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Caveolin-1 regulates neural differentiation of rat bone mesenchymal stem cells into neurons by modulating Notch signaling

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ABSTRACT

Bone marrow mesenchymal stem cells (MSCs) are known to differentiate into neurons *in vitro*. However, the mechanism underlying MSC differentiation remains controversial. A recent analysis has shown that Notch signaling is involved in regulating the differentiation of MSCs. This study examines the potential mechanism of the differentiation of MSCs into neurons, and it considers the role of caveolin-1 in this process. We investigated neuron differentiation and Notch signaling by detecting the expression levels of microtubule-associated protein 2 (MAP-2), Neuron-specific Enolase (NSE), Notch-1, Notch intracellular domain (NICD) and hairy enhancer of split 5 (Hes5). We found that by down-regulating caveolin-1 during induction, MSCs were prone to neural differentiation and expressed high levels of neuronal markers. Meanwhile, the expression levels of Notch-1, NICD and Hes5 decreased. Our results indicate that down-regulation of caveolin-1 promotes the neuronal differentiation of MSCs by modulating the Notch signaling pathway.

1. Introduction

Bone marrow mesenchymal stem cells (MSCs) are non-hematopoietic progenitor cells that have extensive capacities for multilineage differentiation and proliferation (Bianco et al., 2008). Under the appropriate conditions, MSCs derived from the bone marrow of adult animals can differentiate into multiple cell lineages, such as chondrocytes, hepatocytes and myocytes (Pittenger et al., 1999), and recent studies have demonstrated that MSCs have the ability to differentiate into neurons (Bertani et al., 2005). They are interesting cellular sources with potential uses in tissue regeneration, the treatment of degenerative conditions, and immunosuppressive cell therapy. Furthermore, MSCs support both the microenvironment and the regeneration of tissues, and they can provide a target for gene therapy strategies in the treatment of degenerative and autoimmune diseases of the nervous system (Krampera et al., 2005, 2007; Baksh et al., 2004).

Caveolae are plasma membrane invaginations found on the cell surface of differentiated cells; they are abundant in cholesterol and sphingolipids (Parton and Simons, 2007). The caveolin family consists of three proteins, caveolin-1, -2, and -3, with molecular weights of 21–25 kDa (Glenney and Soppet, 1992; Scherer et al., 1996). Caveolin-1 and caveolin-2 are often co-expressed, and they exist in high quantities in endothelial cells, epithelial cells, adipocytes, fibroblasts and smooth muscle cells, while caveolin-3 is mainly abundant in skeletal, smooth and cardiac muscles (Scherer et al., 1997; Song et al., 1996). Furthermore, all three caveolins are expressed in the mammalian brain (Ikezu et al., 1998; Galbiati et al., 1998). They have important physiological functions in regulating endocytosis, transcytosis, and intracellular cholesterol transport (Williams and Lisanti, 2005). They also participate in modulating various associated signaling molecules, such as G-proteins (Chun et al., 1994), H-Ras (Engelman et al., 1997) and protein kinase C (Mineo et al., 1998). Caveolin-1 was the first discovered caveolin isoform, and it has been extensively characterized (Fujimoto et al., 2000). It is considered to be an important marker protein for lipid rafts, which can be expressed in MSCs (Frank et al., 2008; Park et al., 2005), and it is implicated in cell migration, tumorigenesis, neurogenesis and neural stem cell proliferation (Parat et al., 2003; Jasmin et al., 2009).

The Notch signaling pathway is highly conserved across species, and it is involved in the development of the nervous system, regulation and maintenance of various types of stem cells, differentiation, and other biological activities (Artavanis-Tsakonas et al., 1999; Woo et al., 2009). It is also involved in regulating the differentiation of various tissue types through the mediation of intercellular signaling (Woo et al., 2009). The Notch proteins are cell surface transmembrane receptors (Blaumueller et al., 1997). Notch is cleaved when it interacts with the ligands Delta and Jagged, which releases the Notch intracellular domain (NICD) into the

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nucleus so that it can induce the expression of the hairy enhancer of split (Hes) family. In this manner, Notch signaling influences the differences in cell fate observed between cells with equivalent developmental potential, and it participates in the development of the central nervous system (CNS) and peripheral nervous system (PNS) (Artavanis-Tsakonas et al., 1991; Hämmerle and Tejedor, 2007; Gaiano and Fishell, 2002). Notch activity has been extensively studied in the nervous system of worm vulva and Drosophila, and these previous experiments have proven that Notch influences the determination of alternative cell fates in both of these species (Wheeler et al., 2008; Gaiano and Fishell, 2002). Recent studies suggest that the Notch signaling pathway is involved in regulating the neuronal differentiation of MSCs and that caveolin-1 can coordinate and couple with Notch-1 in neural stem cells (Yanjie et al., 2007; Campos et al., 2006). However, the role of caveolin-1 in the differentiation of MSCs into neurons and its effect on Notch signaling are still unknown. In the present study, we conducted a series of experiments to elucidate the role of caveolin-1 in MSC differentiation into neurons. Our results suggest that down-regulated caveolin-1 might improve the efficiency with which MSCs differentiate into neurons by modulating the Notch signaling pathway.

2. Materials and methods

2.1. Cell culture

MSCs were obtained from the femurs and tibias of Wistar rats, aged 6–8 weeks, by flushing the bones with DMEM (Dulbecco's modified Eagle's medium; Invitrogen). The cells were extracted and suspended in a complete medium consisting of DMEM, 10% fetal bovine serum (Invitrogen), and 0.3 mg/ml geneticin.

After 24 h, the non-adherent cells were removed, and the adherent cells were washed with PBS. The complete culture medium was changed every 3–4 days. The cells were harvested with 0.25% trypsin ethylenediamine tetraacetic acid (EDTA) (Invitrogen) when the culture reached 70–80% confluence. The solution was neutralized with DMEM containing 10% fetal bovine serum and then centrifuged to obtain a high-density pellet. The pellet was then resuspended. The cells were replated in complete culture medium and subcultured weekly for 10 passages before being used for the assays.

2.2. Transfection with caveolin-1-siRNA and grouping

MSCs that were cultured for more than 10 passages were plated in 24-well plates (1×10^5 cells/well) with 500 μl complete medium. The cells were then grown in a 37 °C, 5% CO_2 incubator for 2–3 days until the cells reached 50–70% confluence. The MSCs were divided into a non-transfected group, a transfection group (transfected with Rn_Cav1_1 FlexiTube siRNA, Qiagen), and a negative control group (transfected with negative control siRNA, Qiagen). Both the caveolin-1 and control siRNA were modified with Alexa fluor 488. The transfection was carried out according to Qiagen's instructions. Briefly, 1.1 ml of complete medium was added to each well and placed in a 37 °C, 5% CO_2 incubator. Meanwhile, 100 ng of siRNA (Rn_Cav1_1 FlexiTube siRNA or negative control siRNA, Qiagen) was added to 100 μl of DMEM; 2.0 μl HiPerFect transfection reagent (Qiagen) was then added, and the mixture was incubated for 10 min at room temperature. The transfection complexes were distributed into the wells to achieve a final siRNA concentration of 20 nM. The MSCs were cultured for 24–96 h. The transfection efficiency was assessed by fluorescence expression using an inverted fluorescent microscope. The knockdown effect was measured by RT-PCR and Western blotting.

2.3. Neuronal differentiation of MSCs in vitro

In accordance with Woodbury's method (Woodbury et al., 2000), the MSCs were rinsed three times with PBS after 96 h of transfection. To induce neuronal differentiation, the subconfluent cultures were treated with the pre-induction medium, which consisted of DMEM, 10% fetal bovine serum and 1 mM β-mercaptoethanol (β-ME) for 24 h. After the pre-induction, the cells were washed with PBS, and the induction medium was replaced with serum-free medium with 10 mM β-ME for 5 h. The morphology of the MSCs was evaluated before and after the induction using an inverted microscope.

2.4. MTT assay

The 3-(4,5-dimethylthizol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess cell viability. The MSCs were transferred to 96-well plates, treated with MTT (5.0 mg/ml) for 4 h, and then centrifuged. The supernatant was removed, and 200 μl dimethyl sulfoxide was added. Using an enzyme-linked immunosorbent assay, the absorbance (A) at 490 nm was determined. The cell survival (%) for each transfection condition was calculated by comparing it to the control.

2.5. Immunocytochemistry

After washing with PBS, the cells were fixed at 4 °C in 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 for 10 min and then blocked with 10% bovine serum albumin for 1 h. The cells were then incubated with an antibody to one of the following proteins: MAP-2 (rabbit, 1:500, Santa Cruz), NSE (rabbit, 1:500, Santa Cruz), Notch-1 (rabbit, 1:200, Santa Cruz), NICD (rabbit, 1:100, Santa Cruz), Hes5 (rabbit, 1:100, Santa Cruz), or Caveolin-1 (rabbit, 1:200, Santa Cruz) at 4 °C overnight. After being washed with PBS, the cells were incubated with the secondary antibody (anti-Ig-G-Cy3 goat anti-rabbit, 1:500, Santa Cruz) at room temperature for 1 h. The cells were visualized using an inverted fluorescence microscope. The percentage of positive cells in images obtained from similar regions was counted using a double blind, randomized method.

2.6. Western blot analysis

For Western blot analysis, cell lysates (100 μl) were collected from each group. Twenty milligrams of total protein of each lysate was subjected to 8% SDS polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore Corp.). The membranes were blocked in 10% non-fat milk for 2 h and incubated with an antibody to one of the following proteins: MAP-2 (rabbit, 1:1000, Santa Cruz), NSE (rabbit, 1:1000, Santa Cruz), Notch-1 (rabbit, 1:400, Santa Cruz), NICD (rabbit, 1:400, Santa Cruz), Hes5 (rabbit, 1:400, Santa Cruz), Caveolin-1 (rabbit, 1:800, Santa Cruz), or β-actin (1:1000; Santa Cruz) at 4 °C overnight. The membranes were then incubated with horseradish peroxidase-conjugated (HRP) anti-rabbit IgG (1:2000, Santa Cruz) for 2 h at room temperature and visualized by enhanced chemiluminescence (ECL Western blotting detection reagents; Santa Cruz).

2.7. Reverse transcription-PCR

The total RNA was collected with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The primers were designed and obtained from Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. The conditions and cycle numbers for the RT-PCRs were selected according to the instructions provided for the QIAGEN® One-Step RT-PCR kit (Qiagen). The product was used for PCR amplification in a total volume of 50 μl (Buffer 10.0 μl, dNTP Mix 2.0 μl, Enzyme Mix 2.0 μl, 5× Q-Solution 10.0 μl, RNase inhibitor 10 U, RNA 1.0 μg, primer 0.6 μmol/l). The RT-PCR was accomplished in one program (35–40 cycles). Ten microliters of the amplification product with 2.0 μl loading buffer was subjected to electrophoresis on a 2% agarose gel. The bands corresponding to the target and β-actin genes were analyzed with a gel image analysis system (ImageJ 1.42q).

2.8. Statistical analysis

All data are expressed as the means ± SD. To determine whether a difference was significant, variance analysis was performed between groups, and the results of the different groups were compared using Student's t-test. The differences were considered significant if $P < 0.05$. When the multiple comparisons were performed, using a significance level of $P < 0.05/3 = 0.017$ (to maintain an overall significance level of $P < 0.05$).

3. Results

3.1. The effect of transfection on the expression of caveolin-1 in MSCs

We used siRNA to knock down the expression of caveolin-1. After 24 h of transfection, we observed obvious green fluorescence in both the transfection group and negative control group (Fig. 1a and c). The transfection efficiency increased gradually with time (Fig. 1g). There were no significant morphological changes, but there was a small decrease in the number of cells, and we observed that cell bodies slightly contracted into spherical or spindle shapes. Compared to the negative control or non-transfected group, the MTT values of the transfection group were dramatically lower (Fig. 1h). In both Western blotting (Fig. 1e and i) and RT-PCR (Fig. 1f and j), the expression level of caveolin-1 was significantly lower in the transfection group than in the other groups.