The Significance of Notch Signaling Pathway in the Differentiation of Rat Bone Mesenchymal Stem Cells Into Schwann Cells

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Abstract： **Objective**: To investigate the role of Notch signaling pathway in the differentiation of rat bone marrow mesenchymal stem cells (MSCs) into Schwann cells (SCs). **Methods**: BMSCs were isolated from high glucose medium and cultured in fetal bovine serum (FBS) and α-MEM medium. The resulting cells were treated with β-mercaptoethanol (β-ME), all-trans retinoic acid (RA), platelet-derived factor (PDGF-AA), basic fibroblast growth factor (bFGF), Forskolin, Heregulin .After induction of differentiation,cells were divided into three groups: control group, all-induced group and blocker group. Notch pathway blocker DAPT was added in the blocker group upon induction. The expression of Jagg1 ligand, Hes1 target gene, Notch1, Dll1 receptor protein and signal protein (S100, p75, GFAP) were determined by quantitative RT-qPCR. Cell proliferation was measured by CCK-8. Flow cytometry was used to detect the apoptosis of MSCs .**Results**: Compared with the control group, the expression of SCs signal protein (S100, p75, GFAP) was significantly increased in the all-induced group and the blocker group. Compared with theall- induced group, the Jagg1 ligand , Hes1 target gene, Notch1, Dll1 receptor protein was significantly lower; compared with the all- induced of the group, the proliferation effect of the blocker group and the apoptosis rate was significantly lower than the other two groups, the difference was statistically significant, $P < 0.05$) . **Conclusion**: Inhibition of Notch signaling pathway can enhance the differentiation of MSCs into SCs. The possible mechanism is that Notch signaling pathway may promote cell proliferation and promote early apoptosis of MSCs.

Keywords : Notch Pathway ;SCs ;MSCs ; Proliferation ; Apoptosis

MSCs are widely distributed in the human body . Under appropriate circumstances, MSCs can differentiate into chondrocytes, osteoblasts, adipocytes and many other tissue cells, which are the focus of tissue engineering and clinical applications(Aldahmash, Zaher, Alnbaheen, & Kassem, 2012) . Studies have shown that the activation state of Notch signaling pathway plays an important role in cell differentiation during the differentiation of embryonic stem cells and neural stem cells to neural cells (Fior & Henrique, 2005) . However, reports have not been reported that the role of Notch signals in differentiate of mesenchymal stem cells into stromal cells (such as SCs). Notch

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signaling pathway is an evolutionarily conserved signaling pathway. It plays an important role in many cell endings and cell differentiation (Pan & Rubin, 1997) . Four Notch receptors (Notch 1-4) and five ligands (Jagg 1 and 2 and Dll 1, 3 and 4) were found in mammals. The hairy enhancer of split 1 (Hes1) is a basic helical loop repressor and is a downstream target of Notch signaling [3]. Notch signaling results in increased expression of specific genes, including the HES and HEY transcription factor families. As a receptor, Notch binds to ligands, which in turn activate Notch signaling in a typical pathway (Abdallah & Kassem, 2008). This experiment based on the single-cell culture in vitro induction of rat MSCs into SCs, intended to reveal Notch signaling in mesenchymal stem cells differentiate into glial cells (such as SCs), the report is as follows.

1 Materials and methods

1.1 Isolation and culture of rat MSCs MSCs were isolated from rat bone marrow by adherence method according to different growth patterns of bone marrow hematopoietic stem cells and MSCs. Adherent MSCs are spindle-shaped, fusiform and polygonal (Fortier, Nixon, Williams, & Cable, 1998). MSCs were used for the study and MSCs were grown in α-MEM (HyClone, Solarbio, Beijing) supplemented with 10% fetal serum (HyClone, Solarbio, Beijing), incubated at 37 ° C in humidified air with 5% CO^2.

1.2 Induction and Differentiation of MSCs MSCs (80-90% confluency) were dissociated with 0.25% trypsin / EDTA (PAA, Austria) and then plated on 6 cm plastic dishes at a cell concentration of 10^5 / cm^2, MEM, 1 mM β-mercaptoethanol (Sigma-Aldrich, USA). After 24 hours, the medium was removed and the cells were washed three times with PBS (PAA, Austria) and cultured in α-MEM with 10% fetal bovine serum and 35 ng / ml all-trans retinoic acid (Sigma-Aldrich, USA) base. 72 hours later, the cells were washed three times with PBS and cultured in a differentiation culture consisting of α-MEM, 10%

fetal bovine serum, 20 ng / mL basic fibroblast growth factor (bFGF; Peprotech, UK), 5 ng / mL Platelet Derived Growth Factor-AA (PDGF-AA; Peprotech, UK) and 200 ng / mL recombinant human tryptam- β1 (HRG; Peprotech, UK). The medium was additionally supplemented with 50 mg / L streptomycin sulfate (PAA, Austria) and 30 mg / L penicillin (PAA, Austria). Differentiation medium is changed every 3 days. Cells were cultured at 37 ° C in 5% CO2. Cells were incubated for 10 days under these conditions and harvested for further study.

1.3 Cell Grouping Cells were divided into three groups: the control group was MSCs; the all-induced group was MSCs induced; the blocker group, MSCs were induced by DAPT (γ-secretase inhibitor). All groups were cultured in a-MEM supplemented with 10% FBS, 1% penicillin and streptomycin (100 U / ml). When cells proliferate and reach 80% confluence. Subsequently, each group of cells was fixed for immunofluorescence staining.

1.4 Immunofluorescence staining Cells (all-induced cells, blocker cells) cultured on glass slides were fixed in 4% (w / v) paraformaldehyde for 15 minutes at room temperature. After fixation, the cells were treated with 0.1% Triton X-100 for 20 minutes at room temperature and then blocked with 1% BSA for 30 minutes. The primary antibody diluted in 1% BSA in cells was then incubated overnight at 4 ° C. Cells were then washed with PBS and incubated with secondary Alexa Fluor 488 goat anti-rabbit for 1 hour. The following antibodies were used: anti-S100b (1: 100; Bioss, Beijing), anti GFAP (GFAP, 1: 200; Bioss, Beijing), anti p75NGF (1: 200; Bioss, Beijing) Amidino-2-phenylindole dihydrochloride (DAPI, Solarbio, Beijing). Cell staining results were observed using a fluorescence microscope (Olympus, Japan).

1.5 RNA Extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR) Gene Expression Analysis Total RNA was separately

extracted from MSCs and differentiated SCs (day 14), and total RNA was extracted from the cultured cells using Trizol Reagent (Takara, Japan) RNA. To detect mRNA levels of Jagg1, Hes1, Notch1, and Dll1 in MSCs and SCs and protein of S100, GFAP, and p75, quantitative real-time PCR was performed. The sample was added to 1 ml of Trizol reagent and homogenized to extract total RNA. The purified RNA was diluted to 500 ng / ul, and cDNA was synthesized using 1.5 µl of RNA (Takara, Japan) according to the manufacturer's instructions. Real-time PCR (SYBR Green) was performed using the CFX96 TouchTM Real-Time PCR Detection System (Bio-Rad) with the following cycling conditions: 95 ° C for 2 minutes, 95 ° C for 40 seconds, 15 seconds, 60 ° C for 1 min.12.5 µl Taq qPCR Master Mix , Japan) in a final volume of 25 µl containing 2 µl of cDNA per reaction. Gene expression was calculated using the comparative CT (22DDCT) method, and the amount of RNA added to the reverse transcription reaction was normalized using β - actin as a control housekeeping gene. Primers designed by (Sangon, Biotech, Shanghai), and synthesized primer sequence is as follows: for notch1: Forward ACCCACTCTGTCTCCCACAC, Reverse GCTTCCTTGCTACCACAAGC For hes1: Forward GCCAATTTGCCTTTCTCATC, Reverse AGCCACTGGAAGGTGACACT.For jagg1: Forward CAGTGCCTCTGTGAGACCAA, Reverse TGGTATTTGTCAGGCCCAGT For dll1: Forward CCAGCGCTACATGTGTGAGT, Reverse TGGCTCTCCATATGCCTCTC.For Beta Actin: Forward TACAGCTTCACCACCACAGC, Reverse TCTCCAGGGAGGAAGAGGAT.For GFAP: Forward GCCACCAGTAACATGCAAGA Reverse GGCGATAGTCGTTAGCTTCG P75: Forward TACCAGGACGAGGAGACTGG Reverse CTCTGGGCACTCTTCACACA For S100: Forward AGAGGGTGACAAGCACAAGC Reverse CGTCTCCATCACTTTGTCCA

β-actin is used for normalization. Relative gene expression was analyzed using the comparative Ct method (22DDCt). All measurements were done in triplicate. Student's test was performed to compare relative fold changes.

1.6 Cell Counting Kit (CCK-8) Assay Cells were seeded in 96-well plates with 2000 cells per well. Cell proliferation was tested using the CCK-8 kit according to the kit instructions (7sea Pharmatech Co. Ltd, Shanghai). Next, 10 µL of CCK-8 reagent was added to each well. The plate was incubated at 37 ° C for an additional 3 hours. After that, the optical density (OD) at a wavelength of 450 nm was detected with a multi-function microplate reader (Rayto-RT6100). The DMSO concentration in each well did not exceed 0.1% and three replicate wells were established. Repeat the above process three times and collect the average. Based on the OD value of each well, the effect of the Notch signaling pathway on cell proliferation was calculated. The cell viability (%) in each group of different experimental groups was taken as the detection index, and the cell viability was shown by the absorbance measured at 450 nm.

1.7 Apoptosis Assay Apoptosis It was detected using Annexin V-FITC / PI Apoptosis Assay Kit (7sea Pharmatech Co. Ltd., Shanghai). Cells are seeded into 12-well plates and incubated for 24 hours. Next, the cells were harvested and washed once in PBS (4 ° C) and once in 1 × binding buffer. After resuspension in 1 × binding buffer, 5 µl of annexin V was added to 100 µl of the cell suspension, incubated at room temperature for 15 minutes, washed and the cells were resuspended in 1 × binding buffer according to the manufacturer's instructions. After adding 5 µl of PI dye, the percentage of apoptotic cells was analyzed by (BD FACSAria flow cytometry).

1.8 Data and statistical analysis Application SPSS19.0 software for data analysis. Variance analysis of independent samples was used , P <0.05 is considered statistically significant.

2 results

2.1 GFAP, p75, S100 results

The result of immunofluorescence staining showed that the positive expression of mid-green fluorescent markers S100b, p75 and GFAP were seen in the cytoplasm of all-induced and blocker groups (Fig. 1). RT-PCR results showed that the blocker group (B Group) S100, p75, GFAP expression were significantly lower than the all-induced group (group A), the difference was significant, $P < 0.05$, the results shown in Figure 2.1-3. The results show that MSCs have successfully differentiated into SCs, and DAPT can significantly reduce this differentiation process.
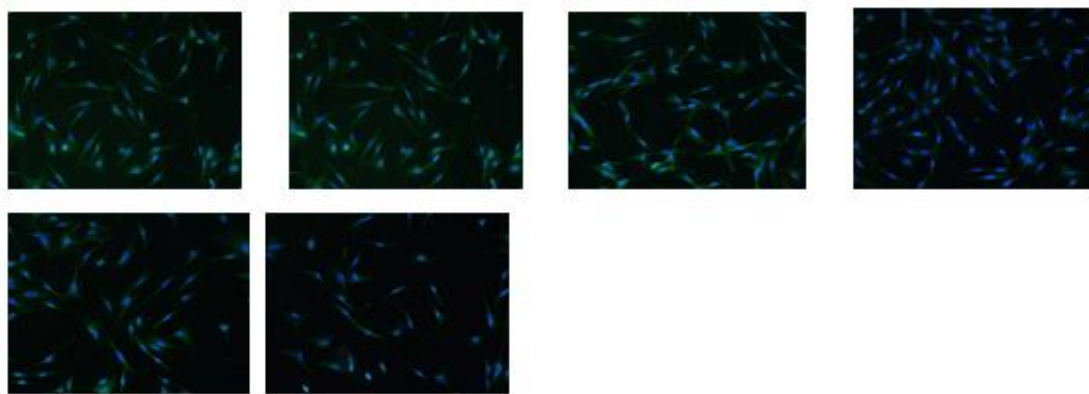
all-induced group S100b   all-induced group p75   all-induced group GFAP   blocker group S100b   blocker group p75   blocker group GFAP
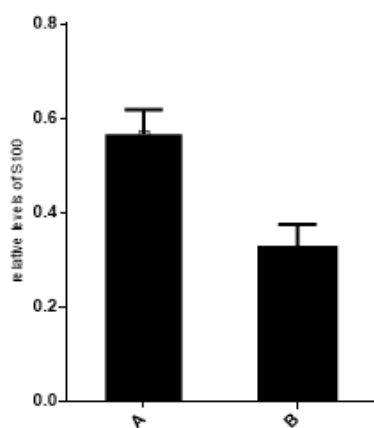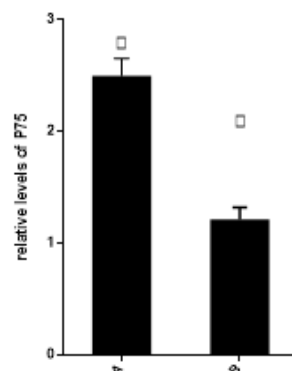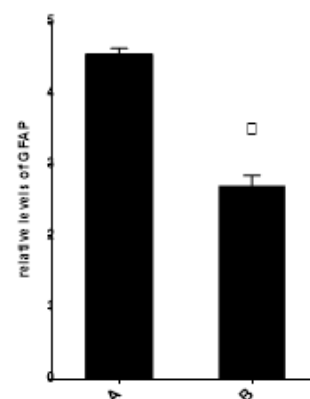

Fig.2.1   Fig.2.2   Fig.2.3

2.2 CCK-8 assay for cell proliferation

The results of CCK8 assay showed that compared with the control group, the cell proliferation ability of the all-induced group was increased. However, the cell proliferation ability was significantly decreased after the addition of the blocking agent (the difference was significant, $P < 0.05$).
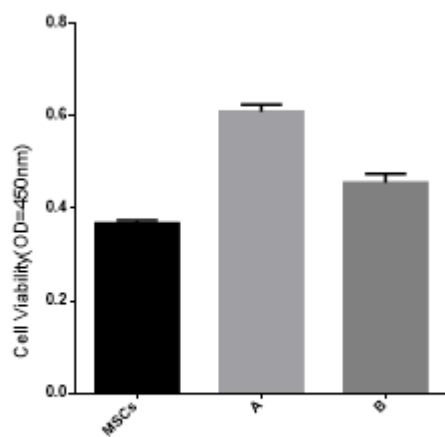
Fig.3

Fig.4

2.3 The rate of apoptosis

The experimental results showed that there was no significant difference in the apoptotic rate between the groups at the early time point, but compared with the control group, the early apoptotic rate of the cells in the all-induced group was decreased. However, the early apoptotic rate was significantly decreased There was significant (P <0.05), the results shown in Figure 4.
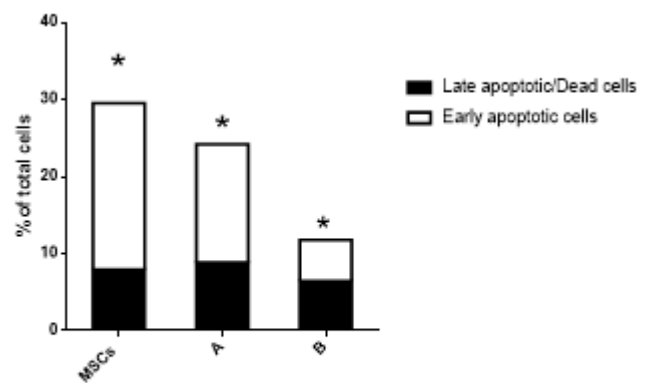
2.4 RT-PCR detection MSCs and SCs Jagg1, Hes1, Notch1, Dll1 mRNA levels

Compared with the control group, the levels of Jagg1, Hes1, Notch1 and D111 in the all-induced group were significantly higher than those in the control group (P <0.05). Compared with the control group, the levels of Jagg1 , Hes1, Notch1, Dll1 mRNA levels were significantly reduced, the difference was significant (P <0.05), the results shown in Figure 5.1-4.
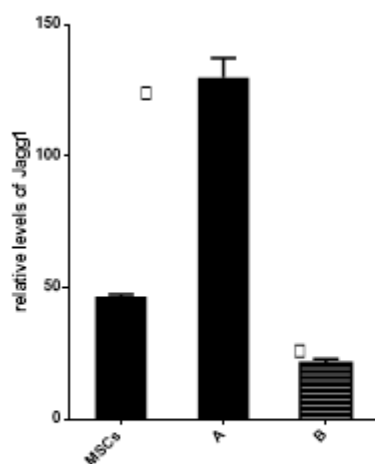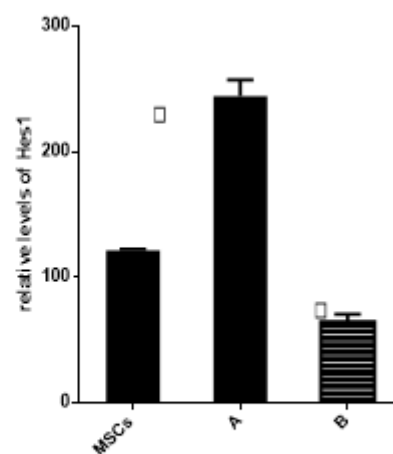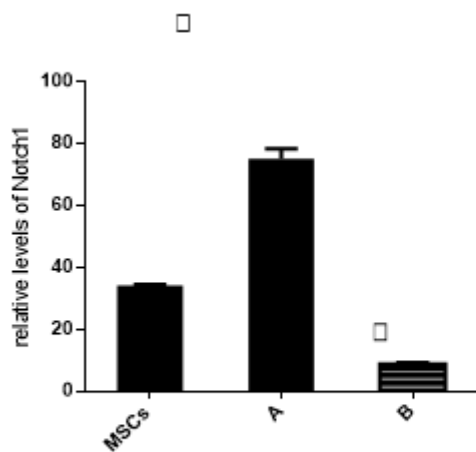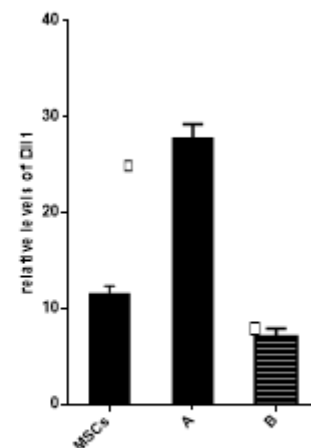

Fig.5.1

Fig.5.2

Fig.5.3

Fig.5. 4

3. Discussion

It is well-known that SCs differentiation plays an important role in the regeneration of nerves, whereas Notch signaling has complex regulation and extensive application in the differentiation of SCs. Because SCS can differentiate and return to an immature state upon axonal loss, it is called dedifferentiated SCs. Removal of myelin debris, rapid and stable proliferation, SCs' differentiation promote axonal regeneration and increase the number of neurotrophic factor synthesis. Neonatal cell degeneration or damage proximal axonal necrosis, cutting off spinal cord cells and axons and the loss of pyramid-shaped nutrient source, called the Waller-type degeneration. Schwann cell proliferation, decomposition, absorption and degradation of axons and myelin sheath phenomenon known as Wallerian Degeneration (Wallerian Degenration). This degenerative process, the beginning of nerve rupture, usually within 8 weeks after the completion of nerve injury. During Werner's denaturation, mature SCs activate, proliferate, demyelinate, and re-express Schwann cell markers of perinatal immature processes.The process is known as SCs differentiation or reversible polarization. These non-myelinated SCs promote axonal regeneration as the best source (Jessen & Mirsky, 2008) . Therefore, Schwann cell differentiation plays an important role

in nerve regeneration. Early regeneration, activation of SCs by Notch signaling effectively promotes neurorestoration, indicating the presence of Notch signaling. It is clinically applicable to the treatment of peripheral nerve defects and the possibility of promoting SCs from SCs precursors and regulates the proliferation of SCs in the pool by controlling the growth and development of precursor size. Notch inhibits myelination, suggesting that myelin may progress forward, such as limiting the Notch signaling of the Krox20 system. Notably, Notch's disease causes demyelination in adults under stress and the results identified in the in vivo signaling pathway induce myelin degradation. The molecular mechanisms of these results may help to understand that Notch system controls SCs differentiation and repair of nerve (Wang et al., 2015).

Based on current research, we analyzed the progress of MSCs differentiating into SCs and whether this differentiation is related to the Notch signaling pathway. In this experiment, we first isolated the rat MSCs by adherent method and used MSCs single-layer cell culture method. After subculturing to the third generation, three-step induction is performed. After induction of differentiation , the expression of SCs specific proteins GFAP, p75 and S100b was detected by immunofluorescence

(Preitschopf et al., 2014).The results showed that they were successfully induced. By blocking the Notch signaling pathway by DAPT, we detected the proliferation and apoptosis rate of SCs in the blocker group. The results showed that Notch signaling pathway could promote the proliferation and early apoptosis of MSCs, but had no effect on the late apoptosis rate.

The study found that the normal development of the nervous system, there is a lateral inhibition mechanism in which neurons around the differentiation of neurons no longer differentiate into neurons and develop into epithelial cells. It was found that Notch signal molecule and Notch signaling increased cell surface expression and downregulated its ligand Delta; on the other hand, Delta downregulated the expression of Notch molecules in cells. With this positive feedback mechanism, the subtle differences in the developmental expression of Notch and its ligands in cells gradually widen early in the developmental process to determine cellular differentiation. It binds to the Notch receptor, which activates the Notch receptor and the soluble Notch intracellular segment is transferred to the nucleus and interacts with the CSL DNA binding protein, eventually regulating the target gene expression (Fuwa et al., 2006).

The results of qPCT showed that the expression of Notch signal Jagg1, Hes1, Notch1 and Dll1 gene was significantly decreased in the blocker group compared with the all-induced group (SCs), indicating that the Notch signaling pathway exists in MSCs and the blocker group Jagg1, Hes1, Notch1 and Dll1 gene expression decreased significantly, suggesting that the blocker group at the Notch signal level based on MSCs block Notch signaling further differentiation. Blockade of the Notch pathway by DAPT has shown to inhibit differentiation (ie, the low level of the Notch pathway contributes to differentiation and then inhibits post-arrest differentiation). More importantly, expression of

Notch signaling proteins (S100, p75, GFAP) in the blocker group was significantly lower than that of the full induction and MSCs groups.

Expression of the Notch signaling pathway effector Jagg1, Hes1, Notch1, D111 is induced in MSCs and remains highly expressed when cultured under conditions conducive to Schwann cell differentiation. Inhibition of the Notch signaling pathway by γ-secretase inhibitors reduces effector expression in differentiated Schwann cells and enhances differentiation of SCs. In this study, we revealed the role of Notch signaling in MSCs activation in vitro. Inhibition of Notch signaling using γ-secretase inhibitor (DAPT) strongly inhibits MSCs activation in vitro. These results demonstrate the key role of Notch signaling in activated MSCs, indicating the differential role of the Notch signaling pathway in differentiation. In conclusion, this study confirms the role of the Notch pathway in the differentiation of MSCs and SCs. It suggests the therapeutic significance of the Notch pathway in MSCs, which may lead to the development of new therapies in nerve cell regeneration, providing a valuable opportunity to restore the health of patients with nerve damage. In this study, we investigated rat MSCs and the mechanisms involved in the Notch signaling pathway in human bone marrow mesenchymal stem cells (MSCs) require further experiments and studies.

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