Wnt3a induces rat bone marrow mesenchymal stem cells differentiation into neuron-like cells***

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Abstract

BACKGROUND: Wnt signaling pathway is a key regulator of cellular proliferation and differentiation, but its correlation with neural differentiation of bone marrow mesenchymal stem cells (BMSCs) is not very clear.

OBJECTIVE: To find out the molecules of the Wnt family which are involved in differentiation of rat BMSCs into neuron-like cells.

METHODS: The rat BMSCs were separated and cultured *in vitro*. The morphology of the BMSCs was observed. Flow cytometry analysis was performed to detect cell phenotype CD44, CD9, CD34 and CD45. Wnt3a and Wnt5a were respectively combined with basic fibroblast growth factor to induce BMSCs differentiation into neuron-like cells, and then were identified by using immunocytochemistry and RT-PCR.

RESULTS AND CONCLUSION: The BMSCs were long-spindle. CD9 and CD44 were highly expressed, while CD34 and CD45 were lowly expressed. Nestin and neuron specific enolase were positive but glial fibrillary acidic protein were not obviously expressed when they were cultured with Wnt3a. In Wnt5a group, Nestin expression was weakly positive, while neuron specific enolase and glial fibrillary acidic protein were negative. RT-PCR result revealed Nestin expressed both before and after induction in the Wnt3a induced group, neuron-specific enolase exhibited apparent amplified bands 5 days after the induction, and more apparent at 10 days. A weak amplification band of glial fibrillary acidic protein could be seen at 10 days after the induction. In Wnt5a and control groups, BMSCs induced by 10 days weakly expressed Nestin, while neuron-specific enolase and glial fibrillary acidic protein were almost not expressed. It is indicated that Wnt3a molecule can promote the differentiation of BMSCs cultured *in vitro* to neuron-like cells.

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INTRODUCTION

The Wnt signaling pathway is a key regulator of cell proliferation and differentiation[1]. Wnt1 mainly promotes neural stem cell proliferation, also helps to the differentiation of various types of neurons[2]. Wnt3a mainly promotes the differentiation of neural stem cells[3], and Wnt5a (a non-classical Wnt) promotes neuron polarity and axonal growth[4]. Sommer[5] believed that the Wnt signaling pathway have many different roles on neural stem cells during neural tissue development. However, Wnt signaling molecules for promoting the neuron-like differentiation of *in vitro* cultured bone marrow mesenchymal stem cells (BMSCs) has not been reported. This study aims to differentiate the *in vitro* cultured BMSCs into neuron-like cells by using different combinations of cell factors and Wnt signaling molecules, so as to search Wnt signaling molecules that promote differentiation.

MATERIALS AND METHODS

Design

In vitro cell differentiation experiment.

Time and setting

The experiment was performed between May 2008 and December 2009 at Science Experimental Center of Liaoning Medical College.

Materials

Ten SD rats were offered from the Science Experimental Center of Liaoning Medical College (License No. SYXK-Liao-2003-0011), 2 months old, irrespective of genders. Experimental disposals on animals comply with animal ethics requirements in the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of the People's Republic of China[6]. Basic fibroblast growth factor (bFGF), Wnt3a, Wnt5a (Amresco), trypsin-EDTA mixture (Sigma), and L-DMEM (Gibco) were used in this study.

Methods

BMSCs culture

SD rats were anesthetized by intraperitoneal injection, lower limb bone was excised under sterile conditions, medullary canal was washed using D-Hanks, cells were collected on the surface of lymphocyte separation medium (relative density 1.077). Centrifugation at 2 000 r/min for 20 minutes. Cloudy mononuclear cell layer was inoculated in 25-cm^2 culture flask at the density of 1×10^7/L, in an incubator with L-DMEM medium containing 10% fetal bovine serum. The medium was altered 4 days later, and then renewed every 3 days. The microscope observation was performed daily. After cells reached a confluence, they were digested using 2.5 g/L trypsin, passaged by 1:2, and marked for passage 1 (P1).

BMSCs phenotypic identification

The amplified P2 generation of BMSCs were harvested, absorbing culture medium. Then cells were digested and rinsed according to conventional methods, adjusted to a density of 1×108/L cells in each Eppendoff tube, resuspended in 50 μL of PBS solution, followed by addition of rabbit anti-mouse CD34, CD44, CD9, CD45α and CD14 10 μL, negative control tube was supplemented with FITC

labeled rabbit anti-mouse IgG and PE labeled rabbit anti-mouse 10 μL, incubated at 4 ℃ for 30 minutes. After PBS washing, they were analyzed with flow cytometry.

Rat BMSCs differentiation into neuron-like cells

At the density of 1.0×10^7/L, BMSCs of passage 2 were seeded on 35-mm^2 cover slips of culture dish and 6-hole culture plate, 2 mL for each hole. First, pre-induction agent was added for the pre-induction, overnight, removing pre-induction medium and renewing into the induction solution. The morphological changes of cells were observed under inverted microscope, the induction was terminated at 2 weeks after induction, changes in cell morphology were daily recorded. Cell culture medium: DMEM/F12 + 10% fetal bovine serum + 25 mmol/L glutamine + 100 U/mL penicillin + 100 mg/L streptomycin. Pre-induction medium: DMEM/F12 + 20% fetal bovine serum + 100 μg/L bFGF. Pre-induction solution 1: L-DMEM, 10% volume fraction of fetal bovine serum, 100 μg L bFGF, 10 μg/L Wnt3a; pre-induction medium 2: L-DMEM, 10% volume fraction of fetal bovine serum, 100 μg/L bFGF, 10 μg/L Wnt5a; induced fluid 1: L-DMEM, 100 μg/L bFGF, 50 μg/L Wnt3a; induced fluid 2: L-DMEM, 100 μg/L bFGF, 50 μg/L Wnt5a. Wnt molecule single inducer was not added into culture medium of the control group.

Immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR) detection of the induced cells

Immunohistochemistry (two steps): Before induction, 7 days and 10 days after induction, BMSCs were fixed for immunohistochemistry staining. Cells were washed and incubated onto 3% volume fraction of H_2O_2 for 10 minutes, then onto 0.1 g/L Triton PBS for 30 minutes and rinsed. Adding normal goat serum to incubate 30 minutes, then mouse anti-Nestin (1:200), neuron specific enolase (NSE, 1:100), and glial fibrillary acidic protein (GFAP, 1:100) were added, while negative controls were supplemented with PBS instead of primary antibody. 4 ℃ overnight, rinsing and adding the second antibody (HRP labeled rabbit anti-mouse IgG antibody) at room temperature for 60 minutes, PBS rinsing. DAB coloration, hematoxylin counterstained, and mounting were performed.

RT-PCR: Total RNA was extracted to determine the concentration and purity of the extracted total RNA, as well as determine the presence of degradation. Reverse transcription for chain 1 cDNA synthesis, polymerase chain reaction for the amplification of target gene fragments, the products were subjected to agarose gel electrophoresis and stained with EB, image scanning and analysis by using the imaging system. Primers used in experiments are shown in Table 1.

Table 1 Reverse transcription-polymerase chain reaction primer design results

Gene		Primer sequence (5'→3')
GFAP	Forward	AAG TTC TTG GCT ATT ACG ACA
	Reverse	ACA GCA CCT TCA GCA CTC T
Nestin	Forward	CGG AAC ATG CAT GAC TGA GAC
	Reverse	GTC ACG AGG TAC GAC CTC AGA T
NSE	Forward	GGA ACT ATG ACC TCG ACT ACG AC
	Reverse	ACC ATG TCT CCT ACA GTA GCT C
β-actin	Forward	CGT TGA CAT CCG TAA CGA CTC C
	Reverse	ATA GAG CCA CCA TTC CGA CAC AG

NSE: neuron specific enolase; GFAP: glial fibrillary acidic protein

Main outcome measures

By using immunohistochemistry and RT-PCR methods, the influence of Wnt3a and Wnt5a on BMSCs differentiation into neuron-like cells was compared.

Statistical analysis

The third author performed data processing using SPSS 11.0 software package, each set of experimental data were expressed as Mean±SD, the mean value between two experimental groups, between experimental and control groups was compared using analysis of variance and *q* test, a level of $P < 0.05$ was considered significantly different.

RESULTS

Morphological observation of rat BMSCs

After primary culture for 24 hours, some rat BMSCs attached to the wall. Then adherent cells proliferated and increased, cell morphology gradually changed into spindle, with uniform bodies. At 10–14 days, cell colonies were swirling and growing in a radial arrangement, nearly 80%–90% of them reached a confluence. Within 24 hours, the subcultured cells completely adhered, extending as triangle, oval, and gradually re-became long spindle. The proliferation rate was faster than primary cells, passaged cells covered the bottom at 6 days after inoculation.

Rat BMSCs immune phenotype detected by flow cytometry

Flow cytometry showed that 82.1% of the cells expressed CD44, 92.0% of the cells expressed CD9. The majority of these cells was in the undifferentiated state. CD45a expression rate was 3.8%, CD14 3.51%, CD34 4.20% (Figure 1).

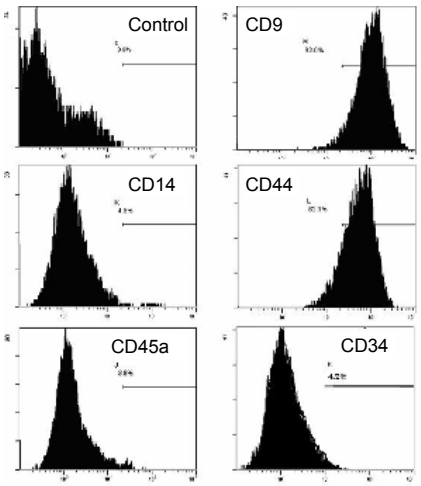
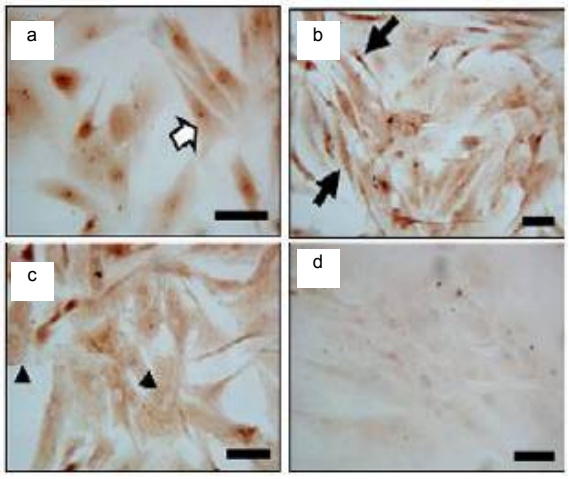


Figure 1 The phenotype of bone mesenchymal stem cells confirmed by flow cytometry

BMSCs differentiation into neural cells by immunohistochemistry

Induced by the Wnt3a for 7 days, the majority of BMSCs were Nestin and NSE positive staining, cytoplasm showed brown yellow granular coloring, while a few cells were GFAP positive staining (Figure 2). In the Wnt5a induced group and control group, at 7 days after the induction of BMSCs, Nestin staining was weakly positive, while NSE and GFAP were negative. There were significant differences

between Wnt3a and Wnt5a groups ($P < 0.05$).



a: Wnt3a group NSE stain (white arrow); b: Wnt3a group 7 d, Nestin stain (black arrow); c: Wnt3a group GFAP stain (▲); d: Wnt5a group 7 d, NSE stain (Bar = 50 μm)

Figure 2 Immunocytochemistry stain of Nestin, neuron specific enolase (NSE), glial fibrillary acidic protein (GFAP) (× 200)

BMSCs differentiation into neural cells by RT-PCR

By agarose gel electrophoresis, all the relevant genes were amplified, results showed the expression of Nestin before and after the induction in the Wnt3a group; distinct amplified bands of NSE can be seen at 5 days after the induction and became more obvious at 10 days; at 10 days after the induction, the weak amplification bands of GFAP appeared (Figure 3). In the Wnt5a group and control group, BMSCs weakly expressed Nestin at 10 days after induction, while no expression of NSE and GFAP was observed (Figure 4).

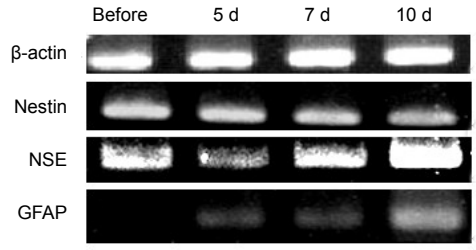


Figure 3 The expression of Nestin, neuron specific enolase (NSE), glial fibrillary acidic protein (GFAP) in Wnt3a group before and after induction

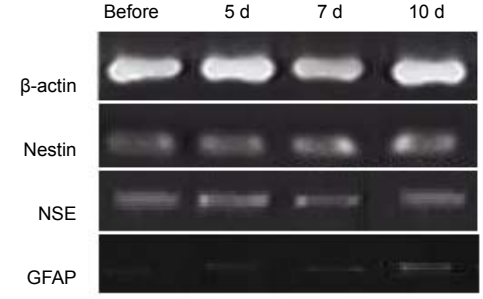


Figure 4 The expression of Nestin, neuron specific enolase (NSE), glial fibrillary acidic protein (GFAP) in Wnt5a group before and after induction

DISCUSSION

Central nervous system degenerative disease or injury may seriously affect the quality of life in patients, and cell transplantation therapy may be one of the most effective treatments. Experiments show that, BMSCs can express neurons and glial cells phenotype-specific proteins under certain conditions, then differentiate into neurons and glial cells[7]. However, the approaches through which the BMSCs transformation into neurons is still unclear. At present, scholars believe that Wnt signaling pathway is a key regulatory link of neural stem cell proliferation and differentiation, it is involved in gene expression regulation, cell adhesion and migration, as well as cell polarization[8]. Immunohistochemical results showed that, Nestin and NSE positive rate was higher in Wnt3a group, RT-PCR results also suggested the expression of nerve-related differentiation markers, indicating a clear improvement of Wnt3a for BMSCs differentiation into neuron-like cells. But how to initialize the mechanism underlying BMSCs differentiate into neuron-like cells remains unclear. Studies have observed that Wnt3a may encourage the expression of β-catenin[9], and Wnt/β-catenin pathway is highly conserved during evolution, the main molecular components and the related regulatory mechanism of this pathway have been basically clarified[10]. In recent years, many studies show that, Wnt signaling pathway plays an important role in neural development, it is involved in neural stem cell proliferation and differentiation process[11], also determines the volume and size of brain tissue[12].

Over-expressed and activated Wnt/β-catenin in the forebrain may lead to an increment of cerebral cortical size, which is due to promoting neural progenitor cell proliferation and inhibiting neural stem cells differentiate into neurons[13]. Gene knockout mice studies have shown that, Wnt signal pathway has an important role in the development of the nervous system in embryonic development[14]. Wnt gene mutations lead to the loss or abnormality of midbrain, hippocampus, spinal cord, neural crest and other nerve tissues. Through Wnt3a expression, spinal cord motor neurons guide sensory neurons to connect with each other right, thus forming neural pathways that can control the muscles. The critical factor for sustained high expression of Wnt signaling pathway, β-catenin, makes a lot of nerve stem cells which should differentiate back to the cell cycle, the number of neural stem cells significantly increased, resulting in the expansion of fetal rat brain cortex surface area[15].

bFGF, as a cytokine, also can promote BMSCs differentiate into neuron-like cells[16], immunohistochemical experiments showed that Nestin expression was weakly positive, indicating a part of BMSCs transformed into neural stem cells. Other studies have indicated that bFGF and other cytokines can jointly promote the transformation of BMSCs into neuron-like cells. bFGF is involved in the regulation of proliferation and differentiation of neural stem cells, and bFGF also plays an important role in the process of neural stem cells differentiate into neurons[17]. bFGF can not only promote neural stem cells mitosis, inhibit neural stem cells differentiation, help to maintain the stem cell state, but also participate in deciding the final differentiation of neural stem cells[18]. bFGF interacted with other cytokines may play a different role. bFGF and Wnt signaling pathway have been

e: Treated CTLs group
C1, D1: death cells; C2, D2: advanced apoptosis; C3, D3: alive cells;

proved to interact in different ways[19]. Through different ways, bFGF can increase the the β-catenin in the *in vitro* cultured neural precursor cells, β-catenin signaling can be also directly or indirectly regulated into neuron gene expression, thus affecting the neural stem cell proliferation and differentiation. In this study, the combination of Wnt3a and bFGF showed that most of the BMSCs could differentiate into neural stem cells, while Wnt5a was not shown to promote differentiation of BMSCs, therefore Wnt3a is believed to promote neural differentiation, which may be related to Wnt3a promotes stem cell proliferation and bFGF promotes neural stem celldifferentiation. In addition, there may be synergies between the two.

REFERENCES

[1] Bejsove A. Wnt passway activation new relations and lacations. Cell. 2005;120(1):11-24.
[2] Royer-Pokora B, Busch M, Beier M, et al. Wilms tumor cells with WT1 mutations have characteristic features of mesenchymal stem cells and express molecular markers of paraxial mesoderm.Hum Mol Genet. 2010;19(9):1651-1668.
[3] Hiraba Lewis SL, Khoo PL, De Young RA, et al. Dkk1 and Wnt3 interact to control head morphogenesis in the mouse. Development. 2008;135(10):1791-1801.
[4] Castelo-Branco G, Wagner J, Rodriguez FJ, et al. Differential regulation of midbrain dopaminergic neuron development by Wnt-1,Wnt-3a, and Wnt-5a. Proc Natl Acad Sci USA. 2003;100(22):12747-12752.
[5] Sommer L. Multiple roles of canonical Wnt signaling in cell cycle progression and cell lineage specification in neural development. Cell Cycle. 2004;3(3):701-703.
[6] The Ministry of Science and Technology of the People's Republic of China. Guidance Suggestions for the Care and Use of Laboratory Animals. 2006-09-30.
[7] Satija NK, Gurudutta GU, Sharma S, et al. Mesenchymal stem cells: molecular targets for tissue engineering. Stem Cells Dev. 2007;16(1):7-23.
[8] Castillo AB, Jacobs CR. Mesenchymal stem cell mechanobiology. Curr Osteoporos Rep. 2010;8(2):98-104.
[9] Lee HY, Kleber M, Hari L, et al. Instructive role of Wnt/beta-catenin in sensory fate specication in neural crest stem cells. Science. 2004; 303(5660):1020-1023.
[10] Daneman R, Agalliu D, Zhou L, et al. Wnt/beta-catenin signaling is required for CNS, but not non-CNS, angiogenesis. Proc Natl Acad Sci U S A. 2009;106(2):641-646.
[11] Ling L, Nurcombe V, Cool SM. Wnt signaling controls the fate of mesenchymal stem cells. Gene. 2009;433(1-2):1-7.
[12] De Ferrari GV, Moon RT. The ups and downs of Wnt signaling in prevalent neurological disorders. Oncogene. 2006;25(57):7545-7553.
[13] yashi Y, Itoh Y, Tabata H, et al. The Wnt/beta-catenin pathway directs neuronal differentiation of cortical neural precursor cells. Development. 2004;131(12):2791-2801.
[14] Clements WK, Ong KG, Traver D. Zebrafish wnt3 is expressed in developing neural tissue. Dev Dyn. 2009;238(7):1788-1795.
[15] Willert K, Brown JD, Danenberg E, et al. Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature. 2003;423(6938):448-452.
[16] Israsena N, Hu M, Fu W, et al. The presence of FGF2 signaling determines whether β-catenin exerts effects on proliferation or neuronal differentiation of neural stem cells. Dev Biol. 2004; 268(1): 220-231.
[17] Holnthoner W, Pillinger M, Groger M, et al. Fibroblast growth factor-2 induces Lef/Tcf-dependent transcription in human endothelial cells. J Biol Chem. 2002;277(48):45847-45853.
[18] Nemeth MJ, Topol L, Anderson SM, et al. Wnt5a inhibits canonical wnt signaling in hematopoietic stem cells and enhances repopulation. PNAS. 2007;104(39):15436-15441.
[19] Shang YC, Wang SH, Xiong F, et al. Wnt3a signaling promotes proliferation, myogenic differentiation, and migration of rat bone marrow mesenchymal stem cells. Acta Pharmacol Sin. 2007;28(11):1761-1774.

Wnt3a 促进大鼠骨髓间充质干细胞向神经元样细胞的分化***

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摘要

背景：Wnt 信号通路是细胞增殖分化的关键调控环节，但与骨髓间充质干细胞神经分化的联系并不十分明确。

目的：寻找促进骨髓间充质干细胞向神经元样细胞分化的 Wnt 信号分子。

方法：首先体外分离培养大鼠骨髓间充质干细胞并传代，行形态学观察，并以流式细胞学方法检测细胞表型 CD44，CD9，CD34 和 CD45。采用碱性成纤维细胞生长因子分别联合 Wnt3a 或 Wnt5a 的方案诱导分化，应用免疫组化和反转录-聚合酶链反应方法比较 Wnt3a 和 Wnt5a 对骨髓间充质干细胞向神经元样细胞分化的影响。

结果与结论：骨髓间充质干细胞为长梭形，CD9，CD44 高表达，CD34，CD45 低表达。Wnt3a 诱导组的巢蛋白和神经元特异烯醇化酶呈阳性，而胶质纤维酸性蛋白无明显表达，诱导后细胞的活力良好。Wnt5a 诱导组巢蛋白呈弱阳性表达，而神经元特异烯醇化酶及胶质纤维酸性蛋白阴性。反转录-聚合酶链反应结果显示，Wnt3a 诱导组巢蛋白在诱导前后均有表达，神经元特异烯醇化酶在诱导后 5 d 可见明显的扩增条带，10 d 后更加明显。胶质纤维酸性蛋白在诱导 10 d 后出现较弱的扩增条带。Wnt5a 组、对照组骨髓间充质干细胞在诱导后 10 d 巢蛋白有微弱表达，神经元特异烯醇化酶和胶质纤维酸性蛋白几乎无表达。提示 Wnt3a 分子能够促进体外培养的骨髓间充质干细胞向神经元样细胞分化。

关键词：骨髓间充质干细胞；Wnt3a；Wnt5a；诱导；神经元样细胞
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来自本文课题的更多信息--

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利益冲突：课题未涉及任何厂家及相关雇主或其他经济组织直接或间接的经济或利益的赞助。

课题的创新点：Wnt 信号通路是较古老的信号机制，但与骨髓间充质干细胞神经分化的联系并不十分明确。课题组在多种 Wnt 信号分子中找到 Wnt3a，证实其具有促进神经转化的作用，为细胞转化的机制奠定了基础。

课题评估的"金标准"：神经转化的标志物一般采用 Nestin、GFAP、NSE 等，另外神经丝蛋白如 TAU 等也是神经分化的标志物。

设计或课题的偏倚与不足：为了证实细胞转化为神经细胞，应采用更多神经标志物如 TAU 蛋白、NF 蛋白等验证，以后的实验应更加系统细致。