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# Wnt3a expression during the differentiation of adipose-derived stem cells into cholinergic neurons★

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## Abstract

The present study analyzed changes in Wnt3a expression during differentiation of adipose-derived stem cells into cholinergic neurons. Immunocytochemistry and immunofluorescence revealed significantly increased nestin, neuron-specific enolase, microtubule-associated protein 2, and choline acetyltransferase expression in adipose-derived stem cells isolated from Sprague-Dawley rats and cultured *in vitro* in neural-induced medium. These expressions increased with prolonged induction time. Real-time reverse transcription-PCR and western blot assay results demonstrated significantly increased choline acetyltransferase and Wnt3a protein and mRNA expressions, respectively, in adipose-derived stem cells following induction. Choline acetyltransferase expression positively correlated with Wnt3a protein and mRNA expressions. These results demonstrated that neural-induced medium induced differentiation of adipose-derived stem cells into cholinergic neuronal-like cells, with subsequent increased Wnt3a expression.

## Key Words

adipose-derived stem cells; cholinergic neurons; Wnt3a; induction; differentiation; neural stem cells; neural regeneration

## Research Highlights

Neural-induced medium increased differentiation of adipose-derived stem cells into cholinergic neuronal-like cells, with subsequent increased Wnt3a expression.

## Abbreviations

NIM, neural-induced medium; NSE, neuron-specific enolase; MAP2, microtubule-associated protein 2

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## INTRODUCTION

The Wnt family consists of secretory cellular signal transduction proteins that are involved in the regulation of early embryonic development *via* the signal transduction pathway. In addition, the Wnt family plays an important role in cell differentiation, proliferation, and growth[1-3]. Wnt3a has been shown to promote differentiation of neural stem cells into neurons and astrocytes[2]. Muroyama *et al* [4] showed that the addition of Wnt3a to neural stem cell culture medium promotes neural stem cell differentiation into neurons. However, differentiation is suppressed when the Wnt signal pathway is blocked, suggesting that Wnt3a plays an important role in neural cell differentiation. A previous study confirmed that adipose-derived stem cells differentiate into neural cells capable of synthesizing the neurotransmitter acetylcholine[5]. These results suggest that Wnt3a participates in adipose-derived stem cell differentiation into cholinergic neurons.

The present study analyzed changes in Wnt3a expression during adipose-derived stem cell differentiation into cholinergic neurons. In addition, the correlation between Wnt3a and choline acetyltransferase expression was determined, and the effects of Wnt3a on adipose-derived stem cell differentiation into cholinergic neurons were examined.

## RESULTS

### Morphology of isolated and cultured rat adipose-derived stem cells (Figure 1)

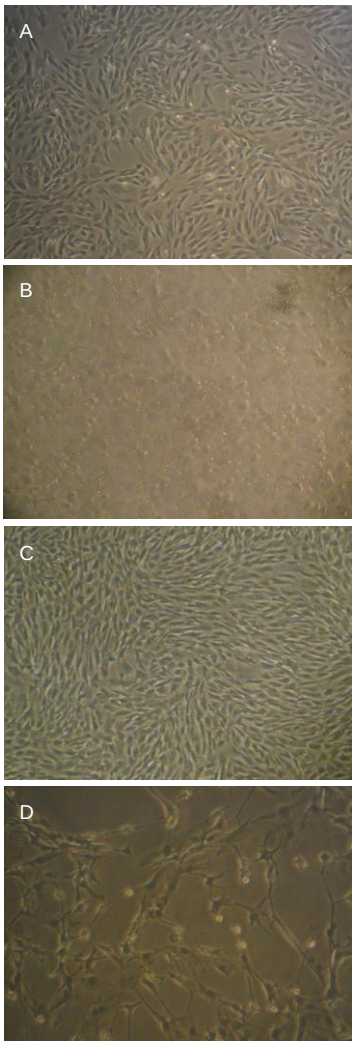


Figure 1 Morphology of isolated and cultured rat adipose-derived stem cells (inverted phase-contrast microscope, × 40).

(A) Cells grow in a swirled pattern at 7 days after first medium replacement.

(B) Cells are suspended with increased refraction.

(C) Sub-cultured cells are spindle- and swirl-shaped.

(D) Cells following induction in neural-induced medium exhibit neuronal-like morphological changes.

Under an inverted phase-contrast microscope, the primary adipose-derived stem cells appeared rounded and in suspension. The culture was mixed with some impure cells and lipid droplets. After 24 hours, a large number of cells were adhered to the wall and exhibited a uniform spindle shape. After 7–10 days, 80% of the cells were confluent and arranged to form whirlpool-like structures (Figure 1A).

Sub-cultured cells were rounded and in suspension (Figure 1B), and the adherent cells grew rapidly. Following passage, the cells were purified and exhibited a spindle shape and a swirled pattern (Figure 1C).

Following induction with neural-induced medium (NIM), the cytoplasm contracted and the nucleus formed the center and extended towards two sides. The cell bodies became globular with the presence of processes. Stereopsis and refraction increased (Figure 1D).

### Nestin, neuron-specific enolase (NSE), microtubule-associated protein 2 (MAP2), and choline acetyltransferase expression in adipose-derived stem cells following NIM induction

Immunocytochemistry revealed low expression of nestin, NSE, MAP2, and choline acetyltransferase in non-induced adipose-derived stem cells. Nestin, NSE, MAP2, and choline acetyltransferase expression significantly increased following NIM induction (Figure 2), and expressions continued to increase with prolonged induction time ($P < 0.01$; Table 1).

Immunofluorescence revealed positive expression of nestin, NSE, MAP2, and choline acetyltransferase in cells, confirming the immunocytochemical results (Figure 3).

### Choline acetyltransferase and Wnt3a mRNA expression in adipose-derived stem cells following NIM induction

Real-time reverse-transcription (RT)-PCR results showed significantly increased choline acetyltransferase and Wnt3a mRNA expression following NIM induction, which increased with prolonged induction time ($P < 0.01$; Table 2).

### Choline acetyltransferase and Wnt3a protein expression in adipose-derived stem cells following NIM induction

Western blot assay results showed that choline acetyltransferase and Wnt3a protein expression significantly increased following NIM induction, which increased with prolonged induction time ($P < 0.01$; Figure 4, Table 3).

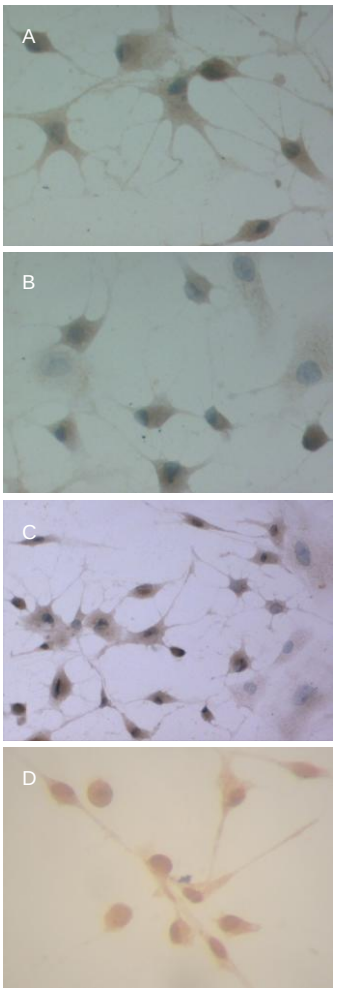


Figure 2 Nestin, neuron-specific enolase (NSE), microtubule-associated protein 2 (MAP2), and choline acetyltransferase (ChAT) expression at 16 hours following induction in neural-induced medium (immunocytochemistry, × 100).

(A), (B), and (C), respectively, represent positive nestin, NSE, and MAP2 expression. Yellow particles are visible in the cytoplasm, with blue nuclei. (D) Positive ChAT expression; brown particles are visible in the cytoplasm and nuclei.

Table 1 Nestin, neuron-specific enolase (NSE), microtubule-associated protein 2 (MAP2), and choline acetyltransferase (ChAT) expression at various time points following neural-induced medium induction (number of positive cells/100 cells)

| Induction time (hour) | Nestin | NSE | MAP2 | ChAT |
|---|---|---|---|---|
| 8 | 28.6±1.62 | 32.3±3.10 | 21.1±2.74 | 13.6±3.32 |
| 16 | 39.1±2.02[a] | 39.1±3.65[a] | 33.4±2.50[a] | 22.6±2.94[a] |
| 24 | 52.2±2.79[ab] | 43.4±3.61[ab] | 46.4±3.38[ab] | 32.2±2.75[ab] |
| 32 | 60.9±1.81[abc] | 50.3±3.16[abc] | 60.3±3.35[abc] | 36.7±2.87[abc] |

Data are expressed as mean ± SD from 10 samples at each time point. [a]$P < 0.01$, *vs*. 8-hour induction group; [b]$P < 0.01$, *vs*. 16-hour induction group; [c]$P < 0.01$, *vs*. 24-hour induction group (analysis of variance).

### Correlation between Wnt3a and choline acetyltransferase expression in adipose-derived stem cells following NIM induction

Spearman's rank correlation revealed that Wnt3a mRNA and protein expressions positively correlated with choline acetyltransferase expressions, respectively ($r_{s(PCR)}$ = 0.994, $r_{s(western\ blot)}$ = 0.968, $P < 0.05$; supplementary Figure 1 online).

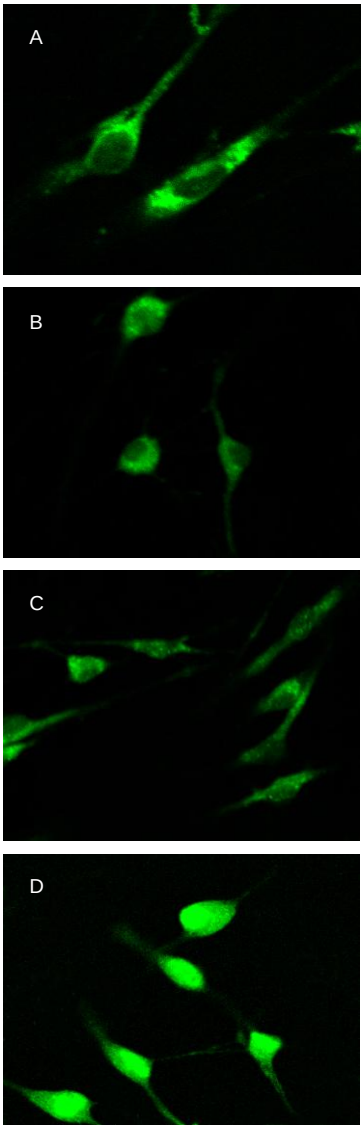


Figure 3 Nestin, neuron-specific enolase (NSE), microtubule-associated protein 2 (MAP2), and choline acetyltransferase (ChAT) expression at 16 hours following induction in neural-induced medium (immunofluorescence staining, × 400).

(A–C) Positive nestin, NSE, and MAP2 expression. Green fluorescence particles are visible in the cytoplasm, and the nuclei are not stained.

(D) Positive ChAT expression; green fluorescence particles (labeled by fluorescein isothiocyanate) are observed in the cytoplasm and nuclei.

Table 2 Relative expression of choline acetyltransferase (ChAT) and Wnt3a mRNA at various time points following induction in neural-induced medium (ratio to control)

| Group | ChAT | Wnt3a |
|---|---|---|
| Control | 1.000±0.000 | 1.000±0.000 |
| 8-hour induction | 1.977±0.034[a] | 1.274±0.068[a] |
| 16-hour induction | 3.412±0.088[ab] | 2.184±0.040[ab] |
| 24-hour induction | 6.854±0.157[abc] | 4.681±0.081[abc] |
| 32-hour induction | 10.907±0.294[abcd] | 6.513±0.056[abcd] |

Data are expressed as mean ± SD from 10 samples at each time point. [a]$P < 0.01$, *vs*. control group; [b]$P < 0.01$, *vs*. 8-hour induction group; [c]$P < 0.01$, *vs*. 16-hour induction group; [d]$P < 0.01$, *vs*. 24-hour induction group (analysis of variance).

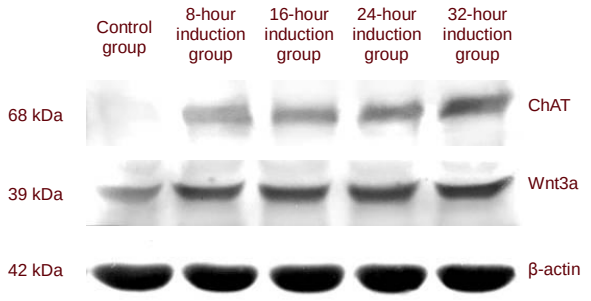


Figure 4 Choline acetyltransferase (ChAT) and Wnt3a protein expression at various time points following induction in neural-induced medium (western blot assay).

Table 3 Choline acetyltransferase (ChAT) and Wnt3a protein expression at various time points following induction in neural-induced medium (absorbance ratio to β-actin)

| Group | Wnt3a | ChAT |
|---|---|---|
| Control | 0.597±0.047 | 0.035±0.015 |
| 8-hour induction | 0.902±0.028[a] | 0.615±0.025[a] |
| 16-hour induction | 1.001±0.011[ab] | 0.649±0.009[ab] |
| 24-hour induction | 1.101±0.016[abc] | 0.689±0.013[abc] |
| 32-hour induction | 1.165±0.012[abcd] | 0.864±0.032[abcd] |

Data are expressed as mean ± SD from 10 samples at each time point. [a]$P < 0.01$, *vs*. control group; [b]$P < 0.01$, *vs*. 8-hour induction group; [c]$P < 0.01$, *vs*. 16-hour induction group; [d]$P < 0.05$, *vs*. 24-hour induction group (analysis of variance).

## DISCUSSION

Adipose-derived stem cells are easily isolated and cultured. The cells are characterized by strong reproductive activity, multiple directional differentiation potential, and lack of immunological rejection following autologous transplantation[6]. Results from the present study revealed positive expression for nestin, NSE, and MAP2 following induction, suggesting that the isolated cells were indeed adipose-derived stem cells and could differentiate into neuron-like cells.

Acetylcholine is an important substance for cholinergic neurons to exert effects, and acetylcholine content indicates function in the cholinergic system. However, acetylcholine becomes degraded by cholinesterase following release. Choline acetyltransferase has been shown to be stable, and choline acetyltransferase content indirectly reflects functions of cholinergic system[7]. The present study utilized immunocytochemistry, immunofluorescence, RT-PCR, and western blot assays to show increased choline acetyltransferase expression in cells following NIM induction. These results suggested that adipose-derived stem cells differentiated into cholinergic neuronal-like cells, as indicated by the production of choline acetyltransferase.

A previous study confirmed that Wnt protein plays an important role in nervous system development, as well as cell growth and differentiation[8]. High expression of β-catenin (a key factor in Wnt signaling pathway) induces a large number of neural stem cells back into the cell cycle, significantly increasing the number of neural stem cells[9]. Zhou *et al* [10] suggested that the Wnt signal transduction pathway plays an important role in early differentiation of stem cells. Wnt3a has been shown to reduce the number of neural stem cell spheres and promote neural stem cell differentiation into neurons and glial cells[8]. RT-PCR and western blot assay results demonstrated increased choline acetyltransferase and Wnt3a protein and mRNA expressions during adipose-derived stem cell differentiation into cholinergic neuronal-like cells. These results suggested that increased choline acetyltransferase expression was associated with Wnt signaling pathway activation.

Results from the present study showed that Wnt3a mRNA and protein expressions positively correlated with choline acetyltransferase expression during adipose-derived stem cell differentiation into cholinergic neuronal-like cells. These results suggested that Wnt3a contributed to adipose-derived stem cell differentiation into cholinergic neuronal-like cells.

The precise mechanisms of Wnt3a in neural cell differentiation remain unclear. The classical Wnt/β-catenin signaling pathway has been shown to be the main pathway for neural stem cell differentiation into neurons[11]. In addition, Wnt3a exerts regulatory effects through the classical Wnt signaling pathway[12], although the precise mechanisms remain poorly understood.

In summary, NIM induced adipose-derived stem cell differentiation into cholinergic neuronal-like cells. During differentiation, Wnt3a expression increased, suggesting that Wnt3a expression induced adipose-derived stem cell differentiation into cholinergic neuronal-like cells.

## MATERIALS AND METHODS

### Design

*In vitro*, comparative, observational study.

### Time and setting

Experiments were performed at the Experimental Center, Hebei United University, China from January 2010 to October 2011.

### Materials

A total of 60 clean, healthy, Sprague-Dawley rats, aged 2–4 weeks, weighing 100–120 g, and of both genders, were purchased from Vital River Laboratories, Beijing, China (certificate No. 0163923). Protocols were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[13].

### Methods

#### *NIM preparation*

Butylated hydroxyanisole was dissolved in 50 mL ethanol. Subsequently, 200 μM butylated hydroxyanisole, 5 mM KCl, 2 mM sodium valproate, 10 μM forskolin, 1 μM hydrocortisone, and 5 mg insulin were added to α-minimum essential medium with a constant volume of 1 L. The solution was then filtered through a Seitz filter and stored at –20°C.

#### *Isolation and culture of adipose-derived stem cells*

Inguinal subcutaneous fat was sterilely obtained from the rats following sacrifice. In accordance with a previously described method[14], subcutaneous fat was washed, cut into pieces, immersed in collagenase solution, supplemented with 250 U/mL type I collagenase and 1% bovine serum albumin, stirred at 37°C for 20–50 minutes, and centrifuged twice. The supernatant was incubated in a cell culture flask, and the solution was replaced for subculture. Cell growth and morphological changes were observed, photographed, and recorded under an inverted phase-contrast microscope (Nikon, Tokyo, Japan).

#### *Adipose-derived stem cell differentiation into neural cells*

Passage 3 adipose-derived stem cells were digested in trypsin (Tianjin Hematology Institute, Tianjin, China). Passage 4 adipose-derived stem cells were incubated in sterile, six-well plates with coverslips. When 70–80% cells were confluent, NIM was added for 8, 16, 24, or 32 hours, respectively. Cell morphology was observed under an inverted phase-contrast microscope. The assessment criteria for cell differentiation were as follows: cells with more than one process, and the length of the process was twice as long as body diameter[15]. Cells treated only with α-minimum essential medium (Gibco, Carlsbad, CA, USA) served as controls.

#### *Nestin, NSE, MAP2, and choline acetyltransferase expression, as determined by immunocytochemistry and immunofluorescence*

Cells were fixed in 4% paraformaldehyde for 30 minutes, and subsequently incubated in PBS containing 0.1% Triton-X 100 for 10 minutes, 3% $H_2O_2$ for 10 minutes, goat serum for 15 minutes, and primary antibodies (Beijing Biosynthesis Biotechnology, Beijing, China) at 4°C overnight: rabbit anti-nestin polyclonal antibody (1:200), rabbit anti-NSE polyclonal antibody (1:100), rabbit anti-rat MAP2 monoclonal antibody (1:100), and rabbit anti-rat choline acetyltransferase monoclonal antibody (1:100). PBS instead of primary antibody served as a negative control. Secondary antibody from the PV6001/PV6002 kit (Zhongshan Golden Bridge Biotechnology, Beijing, China) was added at room temperature for 15 minutes. Subsequently, stainings were visualized by diaminobenzidine (Zhongshan Golden Bridge Biotechnology), and the tissues were counterstained with hematoxylin, mounted, and then photographed under an optical microscope (Olympus, Japan). A PBS wash was performed between each step. The immunofluorescence procedures were identical. However, the secondary antibody was FITC-labeled goat anti-rabbit IgG (1:100; Zhongshan Golden Bridge Biotechnology). Ten non-overlapping fields were randomly quantified using a high-power lens. Positive cells were quantified, and the average number of positive cells was calculated.

#### *Real-time RT-PCR of choline acetyltransferase and Wnt3a mRNA expression*

Total RNA was extracted using Trizol according to Takara instructions. RNA was reverse transcribed into cDNA. PCR primers were synthesized by Shanghai Bioengineering, China. Choline acetyltransferase primer design: upstream 5'-GAG TCA CTA CAA TGC CCA TCC TG-3'; downstream 5'-CCT GGC TCT TCC TGA ACT GC-3'; amplified fragment length: 177 bp. Wnt3a primer design: upstream 5'-TCT GCC ATG AAC CGT CAC AAC AAT-3'; downstream 5'-CCA GCA GGT CTT CAC TTC GCA ACT-3'; amplified fragment length: 304 bp. PCR conditions: 95°C for 15 seconds; 58°C for 15 seconds; 72°C for 45 seconds; for a total of 30 cycles. Results were analyzed using the Comparative Delta-delta Ct method on a Rotor-Gene 3000 real-time PCR instrument (Gene Company Limited, Hong Kong, China).

### *Western blot assay of choline acetyltransferase and Wnt3a protein expression*

The cells were lysed in RIPA buffer, and total cellular protein was extracted[16]. Samples were electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel and transferred onto polyvinylidene fluoride membranes using the semi-dry electro-blotting technique. The membranes were blocked with 5% bovine serum albumin at 37°C for 2 hours and subsequently incubated with rabbit anti-rat choline acetyltransferase, rabbit anti-rat Wnt3a monoclonal antibody, and rabbit anti-rat β-actin (1:200) at 4°C overnight, followed by alkaline phosphatase-labeled goat anti-rabbit IgG (Boster, Wuhan, China) at 37°C for 2 hours. Development was performed using 5-bromo-4-chloro-3-isdolyl phosphate/nitro blue tetrazdiom reagent (Zhongshan Golden Bridge Biotechnology) for several minutes. Double-distilled water was utilized to terminate coloration. The film was scanned using a CANON 9000F scanner (Canon, Tokyo, Japan), and developed bands were analyzed using Image J software (Media Cybernetics, Bethesda, MD, USA). Protein expression levels were calculated as the absorbance ratio of the target band to β-actin.

### *Statistical analysis*

Data were expressed as mean ± SD, and analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA). Intergroup differences were compared using analysis of variance. The relationship of two variables was analyzed utilizing Spearman's rank correlation test. A value of $P < 0.05$ was considered statistically significant.

**Author contributions:** Bin Liu and Chunying Deng participated in study concept and design, as well as manuscript writing. Chunying Deng provided data and ensured data integrity. Chunying Deng and Yuqin Zhang analyzed data. Bin Liu was responsible for manuscript authorization. Chunying Deng and Jinxia Zhang were responsible for statistical analysis.

**Conflicts of interest:** None declared.

**Ethical approval:** This study was approved by the Animal Ethics Committee, Hospital Affiliated to Hebei United University, China.

**Supplementary information:** Supplementary data associated with this article can be found, in the online version, by visiting www.nrronline.org.

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