

**Report**

# Directed Differentiation of Neural Cells from Human Umbilical Cord Stroma-derived Neural Stem Cells

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**Abstract:** Objective to investigate the directed differentiation of human umbilical cord stromal-derived stem cells into neural cells. Methods: (1) Human umbilical cord stromal cells (HUMSC-NSCs) were induced to differentiate from primary human umbilical cord stromal cells, and two different methods were used to induce differentiation to neural cells, which were observed under electron microscope. (2) The neural cells induced to differentiate by different induction methods were identified by morphological differences, immunohistochemistry, and Western blot. Results: (1) Some of the suspended NSCs started to adhere to the wall 48-72 h after the addition of the inducer, and elongated protrusions could be seen in 5-7 days with the neurosphere as the center, and cells gradually migrated outward from the neurosphere. 10 days later, several cells with different morphologies could be seen. Some of the cells had increased refractive index and the length of the protrusions increased, showing bipolar growth. In some cases, the protrusions were short and dense, centered on the cytosol, and protruding in a discrete manner. Under electron microscopy, the neurospheres consisted of multiple clonal clusters and two different morphologies of cells, shaped like neurons and astrocytes, could be clearly observed. The number of bipolar neurons was significantly increased in the group with the addition of BDNF. (2) Before induction,  $84.5 \pm 1.6\%$  and  $88.62 \pm 1.1\%$  of HUMSC-NSCs expressed stem cell-specific markers: Stro-1 and nestin; neurospheres still expressed  $62.7 \pm 3.9\%$  of HUMSC-NSCs positive for nestin after 5 d of culture in neuronal cell induction medium. After 10 days of induction, the percentage of immunohistochemically positive stained cells was counted. With the BDMF induction protocol,  $38.6 \pm 2.9\%$  and  $8 \pm 1.9\%$  Hoechst33342 positive cells expressed immature ( $\beta$ -tubulin III) and mature (MAP2ab) neuronal cell markers, respectively. Also,  $15.8 \pm 4.5\%$  and  $20.6 \pm 4.6\%$  of Hoechst33342-positive cells expressed GFAP (astrocyte marker) and GalC (oligodendrocyte marker). Comparison using a paired t-test revealed that the percentage of  $\beta$ -tubulin III ( $P < 0.001$ ) and MAP2ab ( $P < 0.05$ ) positive cells was significantly higher in the BDNF-induced group than in the general induction group. Western blot results confirmed the immunohistochemical data. Conclusion: After adding BDNF to induce differentiation in the culture group, the number of bipolar neurons was significantly increased, which could promote the induced differentiation of human umbilical cord-derived neural stem cells into neuronal cells.

**Keywords:** Human Umbilical Cord Stromal Cells, Neuronal Cells, Directed Differentiation

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## 1. Introduction

Neurological recovery is still a difficult problem, mainly because of the difficulty of repairing nerve damage, and more and more studies have shown that exogenous transplanted

neural stem cells can promote the recovery of neurological dysfunction. Previously, we induced the differentiation of neural stem cells using human umbilical cord stromal cells, but whether and how well such human umbilical neural stem cells have the ability to directionally differentiate,

so the present study, based on the previous study, for the ultimate differentiation of HUMSC-NSCs, was improved based on the method introduced by Hermann *et al.* and confirmed that they could directionally differentiate into neural cells by Our own improved method can one increase the differentiation of neuronal cells with stable neuronal cell morphological characteristics, protein expression, in order to provide experimental theoretical support for clinical application in patients with nerve injury repair.

## 2. Materials and Methods

### 2.1. Experimental Cells and Main Reagents

#### 2.1.1. Experimental Cell Source

All procedures of this experiment were adopted by the Ethics Committee of the Army General Hospital, and umbilical cord tissues were taken from the umbilical cords of healthy full-term parous fetuses in the Army General Hospital, all with the authorized consent of the parents. Stable primary human umbilical cord stromal cells induced differentiated neural stem cells (Human umbilical cord stromal cells- neural stem cell HUMSC-NSCs) have been obtained from previous experiments.

#### 2.1.2. Primary Reagents

Rabbit anti-nestin (1:400; Chemicon), mouse anti-beta-tubulin isotype III antibody (1:800; Sigma), mouse anti-MAP2ab (1:600; Chemicon), mouse anti-GFAP (1:600; Chemicon), rabbit anti GalC (1:400; Chemicon), recombinant human brain-derived neurotrophic factor (BDNF, rhBDNF, R&D Systems, Minneapolis, MN, USA), BCA Protein Assay Kit (HyClone, USA), DMEM, and BCA Protein Assay. HyClone, Inc.), DMEM-F12 medium, (HyClone, Inc.), fetal bovine serum (HyClone, Inc.), 0.25% trypsin (HyClone, Inc.), Neurobasal medium (NB medium, Invitrogen), 20 ng/ml EGF (Epidermal growth factor, EGF, Peprotech, UK).

#### 2.1.3. Main Instruments

Hitachi electron scanning electron microscope (Japan), Fluoview FV300 laser confocal microscope (Olympus), biogel imaging system (Genius).

### 2.2. Pre-induction and Differentiation of HUMSCs-NSCs into Neuronal Cells

Induction of differentiation assay group To induce the ultimate differentiation of HUMSC-NSCs, we modified the method based on that described by Hermann *et al.* We digested the cells with 0.125% trypsin/EDTA, grew HUMSC-NSCs in 6-well culture plates precoated with polylysine and laminin, and added NB medium, 0.5  $\mu\text{mol/L}$  RA (Sigma), 1% FBS, 5% horse serum and 1%  $\text{N}_2$  for culture. Induction of differentiated BDNF experimental group To induce the directional differentiation of HUMSC-NSCs to neurons, we added 10 ng/ml recombinant human brain-derived neurotrophic factor (BDNF, rhBDNF, R&D Systems, Minneapolis, MN, USA) and incubated for 7-10 d. After letting, we observed by light and electron microscopy,

respectively.

### 2.3. Immunohistochemical Identification of Cells

The culture medium was removed from the plates, rinsed 3 times with 0.01 mol/LPBS, and fixed with 4% paraformaldehyde for 30 min; primary antibodies diluted in serum diluent were added: rabbit anti-nestin (1:400; Chemicon), mouse anti- $\beta$ -tubulin isotype III antibody (1:800; Sigma), mouse anti -MAP2ab (1:600; Chemicon), mouse anti-GFAP (1:600; Chemicon), rabbit anti-GalC (1:400; Chemicon).

Incubate overnight at 4°C in a wet box, remove primary antibody, rinse 3 times with 0.01 mol/L PBS; add secondary antibodies Alexa Fluor 594-labeled sheep anti-mouse IgG (1:200) and Alexa Fluor 488-labeled sheep anti-rabbit IgG (1:200) dropwise, incubate for 2h at room temperature, remove secondary antibody, rinse 3 times with 0.01 mol/L PBS. Hoechst 33342 (2 $\mu\text{g/ml}$ ) was used to re-stain the nuclei; then the slices were sealed with VECTASHIELD® Mounting Medium and observed by Fluoview FV300 (Olympus) laser confocal microscope. A negative control test was also set up: antibody dilution was used instead of primary antibody, and the result was negative if no color was shown under the microscope.

### 2.4. Western Blot Assay

#### 2.4.1. Sample Protein Determination Experiment and Protein Electrophoresis

The total protein content of the samples was determined using Bio-Rad's BCA Protein Assay kit according to the described in the kit. Prepare 12% isolation gel 10 ml: perfuse the isolation gel, prepare 5% concentrated gel, sample preparation and loading, take 10 ul of the sample after concentration adjustment, add 10 ul of 2 $\times$ Loading Buffer, mix well, boil for 8 min at 95°C, cool rapidly, centrifuge for min (10, 000 rpm) (protein marker was prepared in advance with 2 $\times$ Loading Buffer). Loading Buffer and store at then remove before use). Add 20ul of sample to each well sample in equal amounts. 2.5h of electrophoresis at constant pressure of 120v until the bromophenol blue migrates to the bottom of the gel. The gel, filter paper and nitrocellulose membrane were put into the transfer buffer for 15 min. -Nitrocellulose membrane-gel-filter paper-sponge pad sequence, and drain the air bubbles between the gel, filter paper and fiber membrane. Put the transfer clip into the transfer tank according to the correct polarity direction (the side with nitrocellulose membrane to the positive pole), add the transfer buffer, connect the electrode, turn on the power, and turn on the power at 4°C, 28V for 5h. Turn off the power, remove the transfer film, and mark the positive and negative sides. The transfer membrane was stained in Lixin Red S staining solution for 5-10 min at room temperature, and the membrane was rinsed in deionized water to position the protein molecular weight standard reference, and the position of the standard protein was marked with a pencil. Continue to soak in deionized water for about 10 min to completely

decolorize the transfer membrane for the following immunoassay method.

#### 2.4.2. Immunostaining

The membranes were rinsed 3 times with TBST for 5 min each. The membranes were closed with 10% skim milk powder TBST blocking solution for 2 h at room temperature. The membranes were rinsed 3 times with TBST for 5 min. The primary antibody, rabbit polyclonal antibody to BDNF (1:200), was added and incubated overnight at room temperature with antibody dilution buffer, and the primary antibody for the negative control experiment was replaced with TBST containing 10% skim milk powder. Add HRP-labeled sheep anti-rabbit IgG (1:5000), also with antibody dilution buffer, and incubate at room temperature 2 h. Aspirate the liquid from the nitrocellulose membrane and place the protein side up. Take 3 ml of double-distilled water, add one drop each of liquid A and liquid B from the ECM kit to each ml, mix well and add to the membrane, shake to distribute it evenly on the membrane surface, and react in a dark room for 5 min. remove the test membrane, aspirate the liquid from the membrane, place the membrane in an X-ray box, cover the nitrocellulose membrane with plastic wrap, carefully squeeze out the air bubbles between the test membrane and the plastic wrap, and finally cut an X film of comparable size and place it on the plastic wrap. Finally, cut an X film of comparable size and place it on the plastic film, close the X-ray box, and expose it for 30-60 seconds. Do not let the film and the film slide after contact. Develop in developer for 1 min, fix in fixer for 3 min, and let dry. Use  $\beta$ -Actin as internal reference control: rinse the nitrocellulose membrane with Stripping Buffer for 30min (50°C), rinse the

membrane 3 times with TBST for 10min each, and again as above (primary antibody is  $\beta$ -Actin, 1:400; secondary antibody is HRP-labeled goat anti-mouse IgG (1:5000)). The bands of each protein region of the gel after SDS-PAGE and the positive bands after Western blotting were imaged using the Biogel Imaging System.

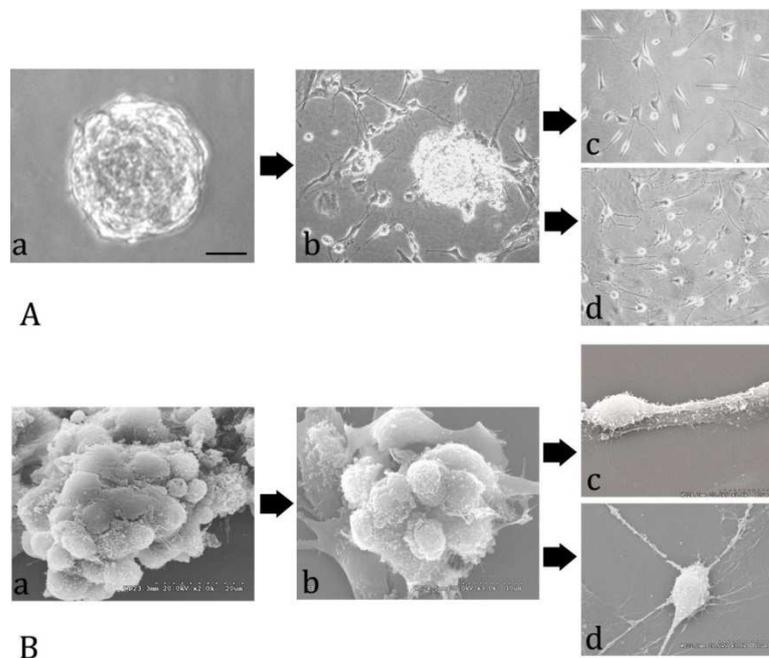
#### 2.5. Statistical Methods

The statistical treatment was performed using SPSS13.0 software. The mean earth standard deviation (.1s\$) was used for normally distributed measures.

### 3. Results

#### 3.1. Morphological Changes of HUMSCs Induced Toward Neuroblasts

Some of the suspended NSCs started to adhere to the wall 48-72 h after the addition of the inducer, and elongated protrusions were visible in 5-7 days with the neurosphere as the center, and cells gradually migrated outward from the neurosphere. Several cells with different morphologies were visible at 10 days. Some of the cells had increased refractive index and the length of the protrusions increased, showing bipolar growth. In some cases, the protrusions were short and dense, centered on the cytosol, and protruding in a discrete manner. Under electron microscopy, the neurospheres consisted of multiple clonal clusters and two different morphologies of cells, shaped like neurons and astrocytes, could be clearly observed. The number of bipolar neurons was significantly increased in the group with the addition of BDNF.

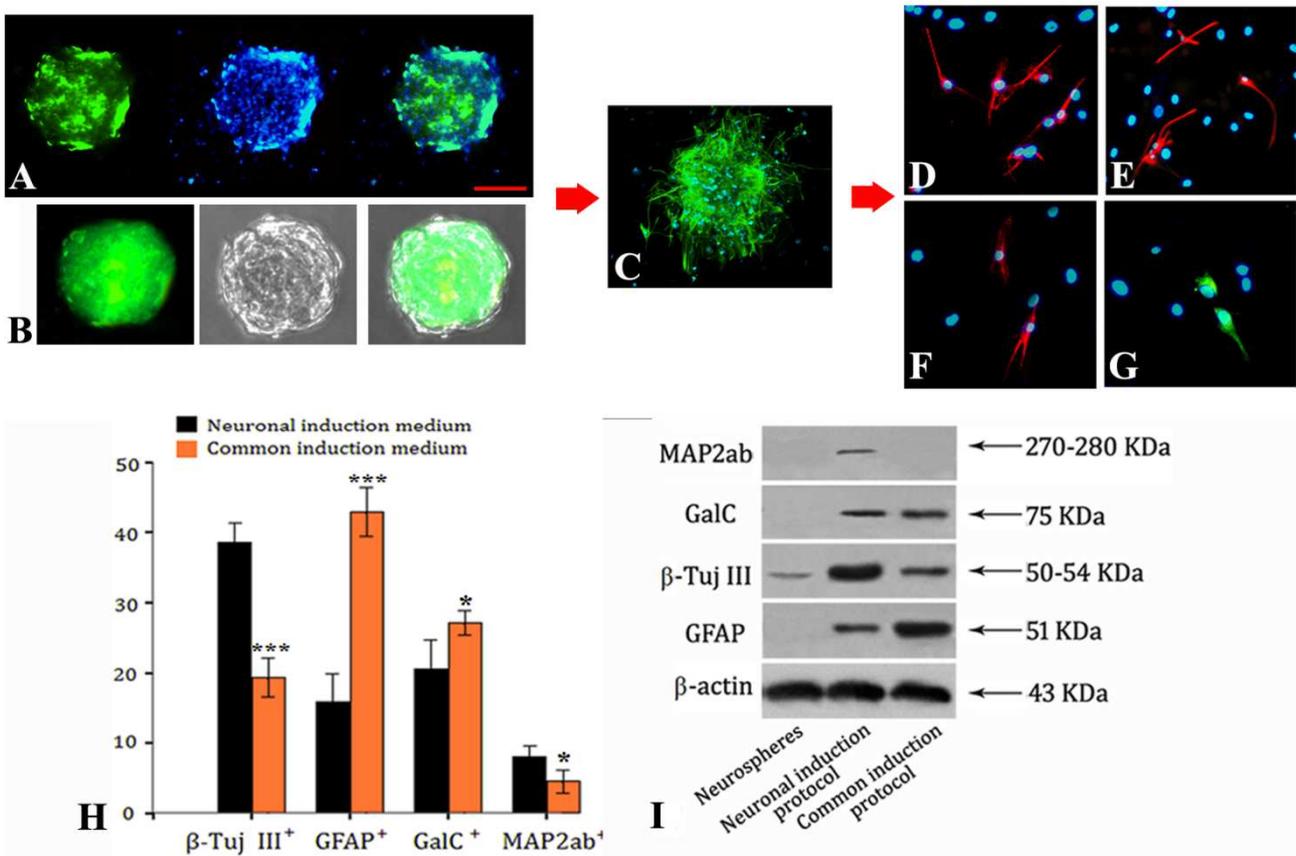


**Figure 1.** The morphological changes of HUMSC-NSCs differentiated towards neural lineage. A-B: the optical microscope (A) and SEM (B) observation, a: free-floating neurospheres; b: 5 days after neurospheres located in the neural induction medium; c-d: two types of cells could be observed, including: neural-like cells (c) and glial-like cells (d) 10 days after neurospheres located in the neural induction medium. Scales bar shown in (A), A (a-d) = 50 $\mu$ m.

**3.2. Immunocellular Fluorescence Chemistry Identification and Western Blot Analysis**

Before induction,  $84.5 \pm 1.6\%$  and  $88.62 \pm 1.1\%$  of HUMSC-NSCs expressed stem cell-specific markers: Stro-1 and nestin; Figure 2A shows Stro-1/Hoechst double-label staining, and Figure 2B shows nestin-positive staining and superimposition of phase contrast micrographs. Neurospheres still expressed  $62.7 \pm 3.9\%$  HUMSC-NSCs positive for nestin after 5 d of culture in neuronal cell induction medium. 3-1-2D-G shows that HUMSC-NSCs differentiated into  $\beta$ -tubulin III, MAP2ab, GFAP and GalC-positive cells. After days of induction, the percentage of immunohistochemically positive stained cells was counted as shown in Figure 2H. the BDMF induction protocol,  $38.6 \pm 2.9\%$  and  $8 \pm 1.9\%$  Hoechst33342 positive cells expressed immature ( $\beta$ -tubulin III

and mature (MAP2ab) neuronal cell markers, respectively.  $15.8 \pm 4.5\%$  and  $20.6 \pm 4.6\%$  of Hoechst33342-positive cells expressed GFAP (astrocyte marker) and GalC marker). In contrast, applying the general induction strategy, found that  $19.2 \pm 3.0\%$  and  $4.4 \pm 1.8\%$  of Hoechst33342-positive cells expressed  $\beta$ -tubulin III and MAP2ab positive staining, respectively, and  $42.8 \pm 3.8\%$  and  $27 \pm 2\%$  of Hoechst33342-positive cells expressed both MAP2ab and GFAP. 2% of Hoechst33342-positive cells expressed GFAP and GalC positive staining. Comparison a paired t-test revealed that the percentage of  $\beta$ -tubulin III ( $P < 0.001$ ) and MAP2ab ( $P < 0.05$ ) positive cells was significantly higher in the BDNF-induced group than in the general induction group. western blot results confirmed the immunohistochemical data.



**Figure 2.** Identification of HUMSC-NSCs and the differentiation of HUMSC-NSCs. (A) Stro-1/ Hoechst33342 double positive indirect immunohistochemistry; (B) Nestin-positive labeling and optic microscope overlay; (C) 5d after differentiation, nestin-positive labeling of HUMSC-NSCs; (D-G) HUMSC-NSCs differentiate into  $\beta$ -tubulin III (D), MAP2ab (E), GFAP (F) and GalC (G) positive cells. Quantification of the percentage of positive cells immunopositive for neuronal markers by using common and neuronal induction protocol. E: Western blot analysis of the protein expressions. Scale bar shown in A, A-G = 50 $\mu$ m.

**4. Discussion**

There are various sources of neural stem cells, including pluripotent stem cells induced by reverse transcription from other cells [1] and neural stem cells directly differentiated cultured; [1] since neural stem cells can differentiate into a variety of cells, how to induce differentiation into the neural

stem cells we need, how to differentiate into the cells that to be repaired after neural injury and how to migrate to the injured neural injury area need further research [2]. The of neural stem cells is also a hot topic of research [3]; cranial nerve injury [4, 5] and neurodegenerative changes are the main cause of disability in the world today [6]; in order to promote nerve recovery, exogenous implantation is the main treatment modality, and many animal experiments have also

confirmed that neural stem cell implantation can promote nerve function recovery [7, 8]; Currently, transplantation can be done by other means such as injury site transplantation, intracerebroventricular transplantation, or intravascular input, but the specific mechanisms regulating migration repair are still not fully understood [9, 10], and some studies have confirmed that it may be related to the environment in which the neural stem cells are located. [11] therefore, we have continued our research in Based on the previous study, we continued to study the function of directed differentiation of human umbilical cord-derived stem cells into neural cells in depth [12, 13], and found that, based on the induction method introduced by Hermann et al. and after continuous the recombinant human brain-derived neurotrophic factor (BDN) was added to this foundation [14, 15]. The number of bipolar neurons was significantly increased in the group with the addition of BDNF, and the differentiation of neuronal was significantly increased, while the protein expression detected by cellular immunohistochemistry and Western blotting was consistent, indicating that the differentiation of neural stem cells could be promoted by our method, which provided the necessary theoretical basis for us to conduct clinical experiments later.

## 5. Conclusion

In conclusion, human umbilical cord-derived neural stem cells can be induced to differentiate directionally into neural cells by adding human brain-derived neurotrophic factor, providing some experimental basis for subsequent animal experiments.

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