Annals of Stem Cell Research & Therapy

Research Article
Published: 20 Aug, 2018

A Promotional Role of BDNF in Pluripotent Stem Cells Neural Differentiation via Wnt/ β -Catenin and ERK/ MAPK Signaling Pathways

Shuang-Qing Chen[1], Fan Zhang[1], Chen-Lu Liu[1], Ming-Min Tong[1] and Zhong Zhao[2]*

[1]Department of Stem Cells Research, Suzhou Hospital of Nanjing Medical University, China

[2]Department of Neurology, Suzhou Hospital of Nanjing Medical University, China

Abstract

Objective: To investigate the role of Wnt/ β -catenin and ERK/MAPK signaling pathways in Brain-Derived Neurotrophic Factor (BDNF) Induction of Pluripotent Stem Cells (iPSCs) differentiation into Neural Stem Cells (NSCs).

Methods: We induced iPSCs to differentiate into NSCs with BDNF and the expression of β -catenin and p-ERK1/2 in BDNF-induced differentiation of iPSCs using small interference RNA (siRNA)-induced silencing of β -catenin and ERK genes. The differentiation rate of iPSCs was detected by flow cytometry.

Results: We find that Wnt/ β -catenin and ERK/MAPK signaling pathways were activated at the same time by BDNF. However, the expression of p-ERK1/2 was significantly down regulated by siRNA-ERK, whereas the expressions of β -catenin were unaffected by siRNA-ERK. On the contrary, the expressions of β -catenin were significantly down regulated by siRNA- β -catenin, and the expressions of p-ERK1/2 were also partially down regulated by siRNA- β -catenin. It suggested that the Wnt/ β -catenin and ERK/MAPK signaling pathways are not independently involved in the process of BDNF-induced iPSCs differentiation.

Conclusion: BDNF can significantly increase the efficiency of iPSCs differentiating into NSCs by activating the Wnt/ β -catenin and MAPK/ERK signaling pathways, and an interconnected relationship may exist between two signaling pathways.

Keywords: Induced pluripotent stem cells; Neural stem cells; MAPK; Signaling; Differentiation

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Correspondence:

Shuang-Qing Chen, Department of Stem Cells Research, Suzhou Hospital of Nanjing Medical University, No.16, bai-ta-xi Road, Suzhou, Jiangsu Province, 215001, China, Tel: 0086-512- 62364358; Fax: 0086-512-62364358;

E-mail: sznaonao@163.com

Received Date: *10 Jul 2018*

Accepted Date: *16 Aug 2018*

Published Date: *20 Aug 2018*

Citation:

Chen S-Q, Zhang F, Liu C-L, Tong M-M, Zhao Z. A Promotional Role of BDNF in Pluripotent Stem Cells Neural Differentiation via Wnt/ β -Catenin and ERK/MAPK Signaling Pathways. Ann Stem Cell Res Ther. 2018; 2(4): 1022.

Introduction

Pluripotent Stem Cells (iPSCs) have the potential to differentiate into a variety of cells and can differentiate into Neural Stem Cells (NSCs) under appropriate conditions [1-3]. Brain-Derived Neurotrophic Factor (BDNF) can promote the differentiation of iPSCs into early stage NSCs and can further differentiate them into mature neuronal cells [4,5]. However, the exact mechanism of BDNF inducing iPSCs differentiation into NSCs remains elusive. Wnt/ β -catenin and ERK/MAPK are widely present and highly conserved in multicellular animals. β -Catenin and ERK1/2 are key factors of these two signaling pathways, and they may play important roles in cell proliferation and differentiation [6,7]. Whether Wnt/ β -catenin or ERK/MAPK signaling pathways may be involved in the differentiation process of iPSCs into NSCs induced by BDNF is largely unknown. In this study, we examined the expression of β -catenin and p-ERK1/2 in BDNF-induced differentiation of iPSCs using small interference RNA (siRNA)-induced silencing of β -catenin and ERK genes and our results may provide a theoretical basis for exploring efficient and prompt mechanisms to induce iPSC neuronal differentiation.

Materials and Methods

IPSCs culture and identification: iPSCs from C57BL/6 mice (OSKM-1) were purchased from the Chinese academy of sciences. We removed iPSCs from liquid nitrogen and rapidly thawed the stem cells in a 37°C water bath. After centrifugation, cells were re-suspended in 5 ml medium. We then incubated the cells in Mouse Embryonic Fibroblast (MEF)-conditioned medium and placed in an incubator. We examined cells daily and replaced the medium as well. When the cells grew to 80 to 90% confluence, they were removed from medium, rinsed, digested by trypsin, and sub

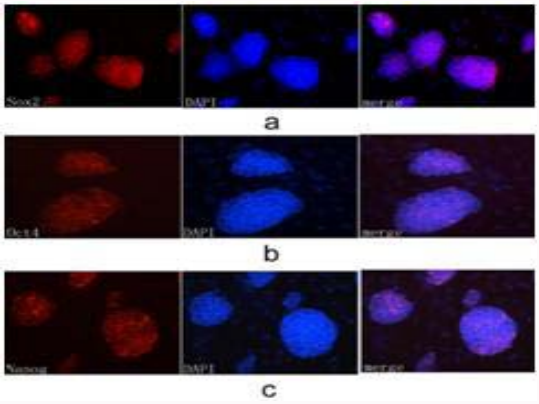

Figure 1: Immunofluorescence staining showed the expression of iPSCs markers. (a) Sox2; (b) Oct4; (c) Nanog.

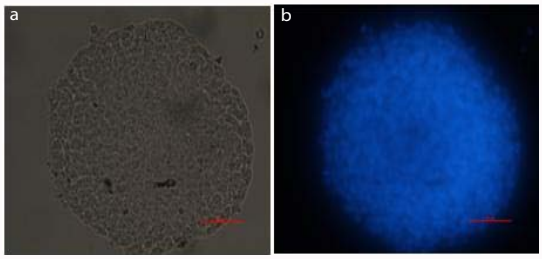

Figure 2: Culture and identification of NSCs. (a) Cultured iPSCs-NSCs were amassed in aggregates as neurospheres. (b) Nestin staining of the neurospheres show strong positive labeling.

cultured at 1:3 ratios. To identify and characterize the cultured cells, immunofluorescence staining was performed. Briefly, the iPSCs in good conditions were cloned, fixed and rinsed, and incubated with Oct4, Sox2, and Nanog primary antibodies (1:100, Abcam) and corresponding fluorescent secondary antibodies (1:400, Abcam). We then detected the expressions of Oct4, Sox2, and Nanog by fluorescence microscopy.

BDNF-induced iPSCs differentiation into NSCs: IPSCs, at a concentration of 5×10^5 cells/ml, were placed in a low-adherent glass culture dish and incubated with iPSCs medium without MEF. We retrieved the cells from culture three to five times a day, and we gently shook the cells to separate the pellets. The embryoid bodies were aspirated, re-seeded in a six-well plate, pre-coated with 0.1% gelatin, and incubated in a differentiation medium containing 10 ng/ml BDNF. We changed the culture medium and examined cells' conditions every other day. To identify and characterize the NSCs, immunofluorescence staining was performed.

RNA interference: To determine the relationship between Wnt/β -catenin and ERK/MAPK signaling pathway in BDNF-induced differentiation of iPSCs, the RNA interference was performed. We diluted 2.5 μl of siRNA stock solution and 3 μl lipo3000 in 50 μl of Opti-MEM to make the transfection medium. We then incubated the cells with the transfection medium at room temperature. IPSCs were digested with trypsin and re-suspended in the medium. We seeded 2×10^6 iPSCs in each well of a six-well plate and cultured the cells in the incubator. We divided the experiment into four groups, including BDNF group, siRNA-ERK/BDNF group, siRNA- β -catenin/BDNF group, and control group.

Flow cytometry: Post-iPSC differentiation, we removed the

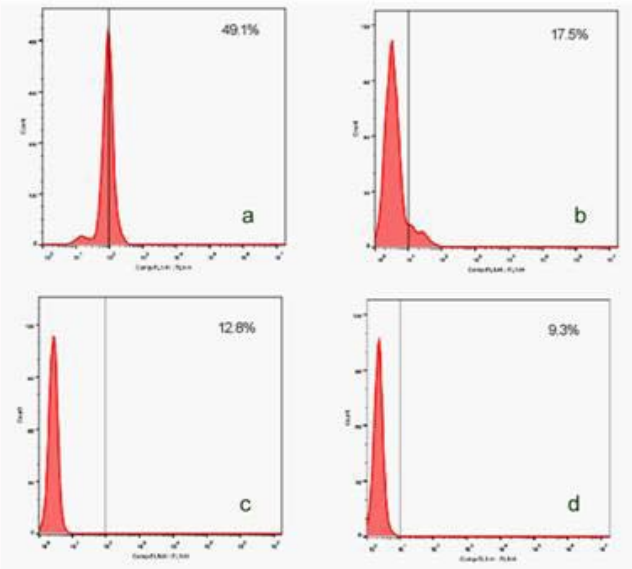

Figure 3: Flow cytometry showed the percentage of Nestin-positive cells. (a) BDNF group; (b) siRNA-ERK/BDNF group; (c) siRNA-β-catenin/BDNF group; (d) control group.

culture medium and immobilized and rinsed the cells. We added a Nestin antibody (1:50, Abcam) at room temperature. The cells were centrifuged and suspended, and then we added donkey antimouse antibody (1:100, Abcam) at room temperature in a dark environment. After suspending the cells in a PBS buffer, cells were re-centrifuged and the expression of Nestin was detected by flow cytometry.

Real-time PCR: Total RNA was extracted by a Trizol reagent after transfection. RNA concentration was detected and cDNA was synthesized by reverse transcription. We performed real-time polymerase chain reaction (RT-PCR) in a 25 μl reaction system. We detected the expression of β -catenin and p-ERK1/2 genes before and after si-RNA transfection, using Glyceraldehyde-Phosphate Dehydrogenase (GAPDH) as the internal control. The reaction conditions were as follows: 95˚C 15 min, 1 cycle; 95˚C 5 s, 60˚C 1.0 min, a total of 40 cycles. The primer sequences are listed in Table 1.

Western blot: We extracted the total protein of each group and used the BCA kit for protein quantification. The proteins were separated by 10% SDS-PAGE gel electrophoresis, and were then transferred onto a Polyvinylidene Fluoride (PVDF) membrane. PVDF membrane was blocked by TBST solution and then incubated with primary antibodies (1:1000, Abcam) and secondary antibodies (1:5000, Abcam) at room temperature. We detected the expression of β -catenin and p-ERK1/2 protein using enhanced chemiluminescence.

Statistical analysis: Statistical data are expressed as mean ± SD (x ± s). All statistical analysis was performed by Statistical Package of Social Science (SPSS 17.0, NY, USA). Inter-group differences were analyzed by one-way ANOVA, and paired comparison was analyzed by LSD. $P<0.05$ was considered statistically significant.

Results

Characterization of cultured iPSCs: The cloned cells were in round or oval shapes, with clear boundaries and refractions. After 3 to 4 days, the clones were increased. After 6 to 7 days, they may be passaged. Immunofluorescence staining showed that iPSCs expressed Nanog, Oct4 and Sox2 markers (Figure 1), indicating that

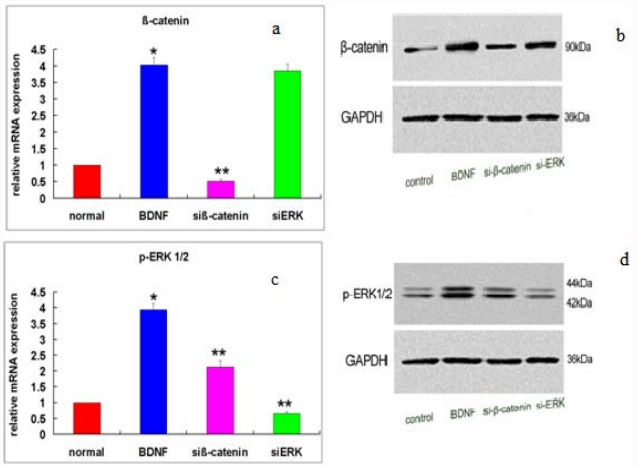

Figure 4: The expressions of key elements of Wnt/ β -catenin and ERK/MAPK signaling pathways. (a) β -catenin mRNA; (b) β -catenin protein; (c) p-ERK1/2 mRNA; (d) p-ERK1/2 protein. *p<0.05 in comparison with the control group, **p<0.05 in comparison with the BDNF group.

neurospheres were composed of undifferentiated iPSCs.

Effect of BDNF on iPSCs differentiation: We could differentiate iPSCs into neurospheres by BDN, and Nestin immunofluorescence of neurospheres was strongly positive (Figure 2), indicating that neurospheres were composed of undifferentiated NSCs. Further, flow cytometry revealed the percentage of Nestin-positive cells in the BDNF group was significantly high than that in the control group (P<0.05) and the percentage of Nestin-positive cells in the siRNA- β -catenin and siRNA-ERK/BDNF group were significantly lower than that in the BDNF group (P<0.05) (Figure 3).

The expression of β -catenin before and after RNA interference: Changes of β -catenin expression in the four groups were measured using RT-PCR (Figure 4a) and Western blot (Figure 4b). Compared with the control group, the expressions of β -catenin were significantly up-regulated in the BDNF group, and the difference was statistically significant (P<0.05). This result suggested that BDNF was able to induce iPSCs differentiation into NSCs by activating Wnt/ β -catenin. However, the expressions of β -catenin in the siRNA- β -catenin/ BDNF group were significantly down regulated compared with the BDNF group (P<0.05), while the levels of β -catenin had no markedly change in the siRNA-ERK/BDNF group. This result indicated that siRNA-ERK could effectively inhibit the activity of the ERK/MAPK signaling pathway, but not the activity of the Wnt/ β -catenin signaling pathway.

The expression of p-ERK1/2 before and after RNA interference: Changes of p-ERK1/2 expression in the four groups were measured using RT-PCR (Figure 4c) and Western blot (Figure 4d). Compared with the control group, the expressions of p-ERK1/2 were significantly up-regulated in the BDNF group, and the difference was statistically significant (P<0.05). This result suggests that BDNF was able to induce iPSCs differentiation into NSCs by activating Wnt/ β -catenin. Further, p-ERK1/2 levels in the siRNA-ERK/BDNF group were significantly down regulated compared with the BDNF group (P<0.05), and the expressions of p-ERK1/2 in the siRNA- β -catenin/BDNF group were partially down regulated (P<0.05). This result indicated that siRNA- β -catenin not only inhibited the activity of Wnt/ β -catenin but also controlled the activity of ERK/MAPK signaling pathways.

Discussion

Stem cell transplantation has been great potential for clinical applications in neurodegenerative diseases, including stroke, Parkinson's Disease (PD), Alzheimer's Disease (AD), and so on. We previously used early embryo-derived NSCs to study the APP/PS1 double transgenic AD mice, and the results showed that the number of neurons in mouse hippocampus was significantly up-regulated by transplantation [8,9]. On the basis of the similarity between iPSCs and ESCs, iPSCs can differentiate into NSCs or various types of neurons with specific functions [10-12]. It shows, however, that the efficiency of naturally differentiated iPSCs is low [13-15]. In this study, we added BDNF to the iPSCs culture, and found that BDNF significantly induced iPSCs differentiation into NSCs. The percentage of Nestin-positive cells in BDNF-treated cells was much higher than that of naturally differentiated cells. This result suggests that BDNF may act as an inducer to promote the differentiation of iPSCs. However, the mechanism of BDNF inducing iPSCs into NSCs remains elusive. Wnt/ β -catenin and ERK/MAPK are the two most important and typical signaling pathways of Wnt and MAPK signaling cascade and they are believed to be related closely to the development, proliferation, and differentiation of nerve cells [6,16,17]. B -catenin is the key intracellular effector of the Wnt/ β -catenin signaling pathway, and the activation of Wnt depends on the level of free β -catenin in the cytoplasm [18]. The ERK/MAPK signaling pathway can be activated by various extracellular factors, initiated by Ras/ Raf/MEK/ERK cascade reactions, and activated by downstream ERK1/2 kinase, as well as other nuclear transcription factors [19,20]. Therefore, the major focus of this study was to investigate whether the Wnt/ β -catenin or the ERK/MAPK signaling pathway was involved in BDNF-induced iPSCs differentiation. During the process of BDNF-induced iPSC differentiation, we used RT-PCR and Western blot to detect significant up-regulation of β -catenin and p-ERK1/2, as compared with the small amount of β -catenin and p-ERK1/2 detected in the natural differentiation group. These results indicate that BDNF indeed can regulate the differentiation of iPSCs by activating Wnt/ β -catenin and ERK/MAPK signaling pathways. Although the Wnt/ β -catenin and ERK/MAPK pathways have not yet been found to target any genes that directly induce iPSC differentiation, this finding confirms our hypothesis that Wnt/ β -catenin and ERK/MAPK may be involved in the process of BDNF-induced iPSC neuronal differentiation.

To further explore whether Wnt/ β -catenin and ERK/MAPK signaling pathway interacted after BDNF induction, we transfected

Table 1: Primer sequences for RT-PCR.

Gene	Forward	Reverse
ERK1	5¹-CATAGCCTGAGTGATGAGGTG-3¹	5¹-CTCCATTCCAGAACGGTCTAC-3¹
ERK2	5¹-CAGGGCAGAGCTCCATATAAC-3¹	5¹-GACATGCTGCAGGAGAAGAA-3¹
β-catenin	5¹-GTTCATCTCAGACCCACCTTTC-3¹	5¹-CTGCTCAGGGAGGAGTAGAG-3¹
GAPDH	5¹-CCAGTATGACTCCACTCACG-3¹	5¹-GACTCCACGACATACTCAGC

iPSCs with siRNA-ERK and siRNA- β -catenin to detect the expression of β -catenin and p-ERK1/2. We found that siRNA-ERK was able to down-regulate the expression of p-ERK1/2 but not β -catenin, suggesting that the ERK/MAPK signaling pathway did not have a significant effect on the Wnt/ β -catenin signaling pathway. However, siRNA- β -catenin not only down regulated the expression of β -catenin but also down regulated the p-ERK1/2 level, suggesting that the inhibition of Wnt/ β -catenin affected the activity of the ERK/MAPK signaling pathway. Our analysis suggests that BDNF not only directly activates the Wnt/ β -catenin and ERK/MAPK signaling pathways, but also indirectly promotes the ERK/MAPK signaling pathway through the activation of Wnt, and coherently regulating associated downstream target genes and promoting the neuronal differentiation of iPSCs. Therefore, the Wnt/ β -catenin and ERK/MAPK signaling pathways are not independently involved in the process of BDNF-induced iPSCs neuronal differentiation.

Conclusion

In conclusion, the results of this study show that BDNF can significantly increase the efficiency of iPSCs differentiating into NSCs by activating the Wnt/ β -catenin and MAPK/ERK signaling pathways. Furthermore, an interconnected relationship may exist between these two signaling pathways. Therefore, further study focusing on the molecular interaction between the Wnt/ β -catenin and ERK/MAPK signaling pathways may elucidate the underlying mechanism of BDNF-induced iPSC neuronal differentiation.

Acknowledgement

This work was supported by the Jiangsu province natural science foundation of China (BK20161231). We also thank stem cell bank of Chinese academy of sciences (Beijing, China) for providing mice iPSCs. We also thank Mr. Zhang for the English revision.

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