ORIGINAL ARTICLE

Effect of Brain-Derived Neurotrophic Factor on the Neurogenesis and Osteogenesis in Bone Engineering

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During bone growth, the lack of a neuralized vascular network in the regenerating area can affect subsequent bone quality. This study aimed to investigate if brain-derived neurotrophic factor (BDNF) could promote neurogenesis and osteogenesis in human bone mesenchymal stem cells (hBMSCs) to improve bone formation during tissue engineering. Initially, a safe and effective BDNF concentration that facilitated hBMSC proliferation *in vitro* was determined. Subsequently, examination of mineralized nodule formation and evaluation of alkaline phosphatase (ALP) activity and *ALP* gene expression revealed that the most effective concentration of BDNF to elicit a response in hBMSCs was 100 ng/mL. In addition, we found out that by binding with TrkB receptor, the downstream Erk1/2 was phosphorylated, which promoted the expression of transcription factors, such as Runx2 and Osterix that are associated with osteoblast differentiation. We also found that by day 7 post-treatment, the neurogenic biomarkers, p75 and s100, were highly expressed in 100 ng/mL BDNF-treated hBMSCs. Finally, the effects of BDNF on osteogenesis and neurogenesis in newly formed tissues were assessed using animal models with a β-tricalcium phosphate scaffold. This revealed that treatment with 100 ng/mL BDNF promoted the osteogenesis and neurogenesis of hBMSCs *in vivo* by increasing expression of the osteogenic marker osteocalcin and various neurogenic biomarkers, including microtubule-associated protein 2, glial fibrillary acidic protein, neural/glial antigen 2, and β-tubulin III. This study has demonstrated that BDNF promotes hBMSC osteogenesis and neurogenesis *in vitro* and *in vivo,* and that BDNF may indirectly promote osteogenesis through increased neurogenesis. This further suggests that encouraging neutralization during bone engineering will lead to effective repairing of bone defects. The study may also provide insight into related fields, such as osseoperception and stress feedback regulation after dental implantation.

Keywords: mesenchymal stem cells, neurogenesis, osteogenesis, tissue regeneration, biomarkers

Introduction

SEVERAL STUDIES HAVE revealed that there is rich innervation and a complex interwoven network of neurons and blood vessels in highly osteogenic areas.[1–7] Further animal and clinical studies have indicated that this innervation plays a vital role in the formation of new bone, primarily through the control of blood flow, regulating bone metabolism, and the secretion of neurotrophic factors.[8–10]

However, during the tissue regeneration process, such as after a bone graft, an efficiently neuralized vascular network can fail to emerge in the center of the regenerating area,

hindering nutrient metabolism. This can have negative effects on the quality of newly generated bone. There have been several studies focused on vascularization in new bone tissue,[11,12] although there has been little research into the neural regeneration that accompanies blood vessel formation in fresh bone tissue.

Of particular importance to bone growth and repair are bone mesenchymal stem cells (BMSCs), adult stem cells that have been shown to be multipotent and able to differentiate into or have a therapeutic value for various cell types, including osteoblasts, chondrocytes, adipocytes, and even nerve cells.[13–16] BMSCs have therefore become a useful cell type

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used for neural tissue engineering.[14–17] However, BMSCs and their neurogenic abilities have not yet been applied in bone tissue engineering due to a limited knowledge of innervation in the newly generated bone. There are also many unanswered questions concerning neurogenesis in neonatal bone tissue, although a number of neurotrophic factors have been detected during new bone formation. These include brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophins 3, 4, and 6 (NT3, NT4/5, and NT6).[18,19]

Among these particular neurotrophins, BNDF is less studied than NGF, yet is also potentially important. It is a multifunctional protein that plays an important role in the survival and differentiation of neurons through the binding of two major receptors, TrkB (NTRK2) and p75NTR.[20–22] BDNF can be detected in many different tissue types (e.g., bone, cartilage, tooth germ, and heart) and cell types (e.g., osteoblasts and vascular endothelial cells) and is not limited to neurons.[23] Several studies have shown that BDNF promotes *in vitro* mineralization in several highly differentiated cells.[24–27] However, the effects of BDNF on the neurogenic and osteogenic abilities of human BMSCs (hBMSCs) remain unclear.

To address this, our study investigated the effects of BDNF on the neurogenesis and osteogenesis of hBMSCs and whether it has a potential role in achieving better bone formation. This was assessed *in vitro* using cultured cells and *in vivo* using β-tricalcium phosphate (β-TCP), a ceramic scaffold widely used in bone tissue engineering due to high biocompatibility, bioactivity, osteoconductivity, and osteoinductivity.[28] We hypothesized that the addition of BDNF as a neurogenic factor, in combination with the osteoinductive β-TCP scaffold, would enhance the multipotency of BMSCs, leading to the generation of better innervated and vascularized bone tissue. This work may lead to better outcomes for patients after significant bone grafts.

Materials and Methods

hBMSC culture

hBMSCs were cultured, and their multipotent differentiation abilities were confirmed using protocols described in our previous research.[12] Briefly, cells were obtained with patient consent from redundant, healthy cancellous bone amputated during orthognathic surgeries. All procedures were approved by the Ethics Committee of the Peking University (Beijing, China) School and Hospital of Stomatology (PKUSSIRB-201520020).

Cell suspensions made from cancellous bone pieces were added to the alpha minimal essential medium (αMEM; GIBCO, Invitrogen, Buffalo, NY) and centrifuged at 168 *g* for 5 min. Subsequent, cell pellets were resuspended and seeded on 35-mm plastic culture dishes in αMEM supplemented with 10% fetal bovine serum (FBS; GIBCO, Invitrogen), penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (250 ng/mL) at 37°C, 95% air humidity, and 5% CO_2. After the hBMSCs had formed a confluent monolayer, the cells were passaged and expanded to obtain enough material. Typically, hBMSCs at passage 4 to 6 were used in the experiments.

Proliferation of hBMSCs treated with BDNF

The proliferation of hBMSCs treated with BDNF was assessed using a CCK-8 assay (Dojindo, Kumamoto-ken,

Japan). hBMSCs were seeded on 96-well plates at a density of 1.0×10^3 cells/well and maintained in a basic culture medium (CM) at 37°C, 95% air humidity, and 5% CO_2 for 24 h. BDNF (Aviscera Bioscience, Santa Clara, CA) was added to the CM at a final concentration of 50 or 100 ng/mL. After 1, 3, 5, and 7 days, the media were replaced with 10% CCK-8 solution and incubated for 4 h at 37°C. The absorbances of the formazan-dissolved solutions were measured using a spectrometer at a wavelength of 450 nm.

Mineralized nodule assays and alkaline phosphatase activities of hBMSCs treated with BDNF

hBMSCs were seeded on 12-well plates at a density of 1.0×10^5 cells/well and maintained in CM at 37°C, 95% air humidity, and 5% CO_2 for 24 h. Four experimental groups were designated as (I) the CM group, (II) osteogenic differentiation medium (OM)-only group (αMEM, 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 250 ng/mL amphotericin B, 50 mg/mL ascorbic acid, 10 mM glycerol-2-phosphate, and 100 nM dexamethasone), (III) a 50 ng/mL BDNF+OM group, and (IV) a 100 ng/mL BDNF+OM group. On day 7, hBMSCs were lysed with RIPA lysis buffer (Solarbio, Beijing, China) and cell lysates were centrifuged at 12,000 *g* for 4 min. The alkaline phosphatase (ALP) activities of the treated hBMSCs were determined using a p-nitrophenyl phosphate (PNPP) kit (Jiancheng Bioengineering Institute, Nanjing, China). On day 14, hBMSCs were fixed with 95% alcohol and stained with alizarin red S to reveal bone nodules (Sigma-Aldrich, St. Louis, MO).

Relative ALP gene expression

hBMSCs were seeded on six-well plates at a density of 2.0×10^5 cells/well and maintained in CM at 37°C, 95% air humidity, and 5% CO_2 for 24 h. Based on the ALP activity and mineralized nodule assays, only a concentration of 100 ng/mL BDNF was used to assess *ALP* gene expression. The three experimental groups assessed were (I) the CM group, (II) the OM-only group, and (III) a 100 ng/mL BDNF+OM group.

On day 3, total RNA was isolated with TRIzol (Invitrogen) and quantified by spectrometry at 260 and 280 nm. cDNA was reverse transcribed using 2 μg RNA template and a M-MLV reverse transcriptase (Thermo Scientific; Life Technologies, Carlsbad, CA) in a total volume of 20 μL. Each real-time polymerase chain reaction (RT-PCR) was performed in duplicate using 1 μL cDNA template in a 20 μL reaction volume with 200 nmol/L of each primer and a FastStart Universal SYBR Green PCR master mix (ROX) (Roche Life Science, Penzberg, Germany). The thermal cycling conditions were an initial step of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 94°C for 15 s and 60°C for 1 min. Reactions were performed using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA).

Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. The sense and antisense primers used to detect *ALP* mRNA were *ACTGGTACT CAGACAACGAGAT* and *ACGTCAATGTCCCTGATGTTAT*, respectively. The sense and antisense primers used to detect *GAPDH* mRNA control gene were *ACAACTTT GGTATCGTGGAAGG* and *GCCATCACGCCACAGTTTC*,

respectively. Relative mRNA expression was assessed using the $\Delta\Delta Ct$ method.[29]

Knockdown of TrkB and the osteogenic mechanism of BDNF

hBMSCs were seeded on six-well plates at a density of 2.0×10^5 cells/well and maintained in CM at 37°C, 95% air humidity, and 5% CO_2 for 24 h. BMSCs were transfected with siRNA-TrkB (sense *GUGAUCCGGUUCCUAAUAUTT* and antisense *AUAUUAGGAACCGGAUCACTT*; Gene Pharma, China) or a negative control using Lipofectamine 2000 Transfection reagent (Thermo Fisher scientific) at a concentration of 100 nM/well. Cells were then treated with 100 ng/mL BDNF and cultured for 48 h and the total RNA was collected using TRIzol (Invitrogen) as described above, and the relative gene expression of *TrkB*, *Runx2*, and *Osterix* was also detected by RT-PCR.

The sequence of the sense and antisense primers used to detect *TrkB* mRNA was *GGGACACCACGAACAGAAGT* and *GACGCAATCACCACCACAG*, respectively. The sequence of the sense and antisense primers for *Runx2* mRNA was *TGGT TACTGTCATGGCGGGTA* and *TCTCAGATCGTTGAACCT TGCTA*. The sequence of the *Osterix* mRNA primers was *CCTCTGCGGGACTCAACAAC* and *AGCCCATTAGTGCTT GTAAAGG*. *GAPDH* was used as a reference gene.

Total protein was extracted and subjected to Western blotting. Transfected cells collected at 0°C in sodium dodecyl sulfate (SDS) lysis buffer (Beyotime Biotechnology, China) were further lysed by supersonic on ice for 2 min discontinuously. Supernatants were collected after centrifuge for 15 min at 4°C, 12,000 g. After being denatured for 5 min at 100 with loading buffer (Huaxingbio, China) and were quantified by the BCA Protein Assay (Pierce), a total of 20 μg protein/lane was used for SDS/polyacrylamide gel electrophoresis (SDS-PAGE).

Each sample was run on a 10% polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (Millipore). The membranes were incubated in blocking buffer (5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20) for 1 h and incubated with the following primary antibodies at 4°C overnight: TrkB (1:1000, #4603; Cell Signaling Technology), Erk1/2 (1:1000, #4695; Cell Signaling Technology), phospho-Erk1/2 (1:1000, #4370; Cell Signaling Technology), Runx2 (1:500, abs116236; Absin, China), Osterix (1:300, abs120764; Absin), and GAPDH (1:1000, TA-08; ZSGB-Bio, China). Based on the host species of primary antibody, HRP-conjugated anti-rabbit or anti-mouse IgG (ZSGB-Bio) was used for 1 h at room temperature, The bands were detected with the chemiluminescence methodology (ECL Plus; Solarbio) by a lumino imaging analyzer (VILBER Fusion FX5, France).

p75 and s100 staining of hBMSCs treated with BDNF

To explore the neurogenic potential of hBMSCs after treatment with BDNF, immunofluorescent staining of the neuronal cell markers p75 and s100 was performed using *in vitro* cells. hBMSCs were seeded on cell slides contained in 24 wells with CM at 37°C, 95% air humidity, and 5% CO_2 for 24 h. Four experimental groups were designated as previously described.

Cells were fixed with 4% paraformaldehyde on day 7 and 14 and the slides incubated with 1:200 anti-p75 NGFR antibody (sc-271708; Santa Cruz Biotechnology, Dallas, TX) and 1:100 anti-S100 beta antibody (ab52642; Abcam, Cambridge, UK) at 4°C overnight. These were washed and incubated in the dark for 2 h at 37°C, respectively, with 488-conjugated AffiniPure goat anti-mouse IgG (H+L) (115-545-003; Jackson Immuno Research, West Grove, PA) for p75 or 594-conjugated AffiniPure donkey anti-rabbit IgG (H+L) (711-585-152; Jackson) for s100, and with 4′,6-diamidino-2-phenylindole to stain the nuclei (DAPI; Life Technologies). Slides were washed with PBS three times for 5 min and then mounted with Fluoromount reagent (F4680; Sigma-Aldrich) and a coverslip added. These were then assessed using an Olympus IX71 microscope (Olympus, Tokyo, Japan).

In vivo experiments

Six-week-old male BALB/c homozygous nude (nu/nu) mice were purchased from Peking University Experimental Animal Center, Beijing, China. Animals were allowed a week to acclimatize to the housing environment before experimentation. hBMSCs were cultured for 7 days in different media, according to the experimental design (experimental groups were (I) β-TCP, (II) β-TCP+CM-treated hBMSCs, (III) β-TCP+OM-treated hBMSCs, and (IV) β-TCP +100 ng/mL BDNF+OM-treated hBMSCs). β-tri-calcium phosphate particles (β-TCP, 50–500 μm; Bicon, Boston, MA) were incubated with each CM at 37°C for 1 h to aid cell adhesion. hBMSCs from group II, III, and VI where then resuspended and seeded onto the β-TCP particles.

During the *in vivo* experiments, mice were anesthetized with sodium pentobarbital and four enclosed subcutaneous transplantation cavities created in the dorsal area using hemostatic forceps. β-TCP complexes from each of the four groups were transplanted into individual subcutaneous cavities ($n = 6$ per group at each time point). After surgery, animals were carefully monitored two times a day and no adverse effects due to the surgery were observed in any individual. Animals were sacrificed at 4 and 8 weeks using CO_2 asphyxiation and samples from each group were harvested and immediately fixed in 4% paraformaldehyde. All animal studies were performed in accordance with procedures approved by the Animal Care and Ethics Committee of Peking University Health Science Center (LA2015158).

Histological study

Specimens were decalcified for 14 days in 10% EDTA (pH 7.4). After decalcification, specimens were dehydrated and embedded in paraffin wax. Next, 5-μm-thick tissue sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome stains. Osteogenesis was evaluated by immunohistochemical staining using 1:200 osteocalcin (OCN) antibody (sc-30044; Santa Cruz). Neurogenesis was evaluated by immunohistochemical staining using 1:200 microtubule-associated protein 2 (MAP2) (sc-20172; Santa Cruz), 1:200 glial fibrillary acidic protein (GFAP) (ab33922; Abcam), 1:500 neural/glial antigen 2 (NG2) (ab139406; Abcam), and 1:1000 β-tubulin III (ab78078; Abcam) antibodies. Three fields from three slides from each sample were used to semiquantitatively assess the optical density of
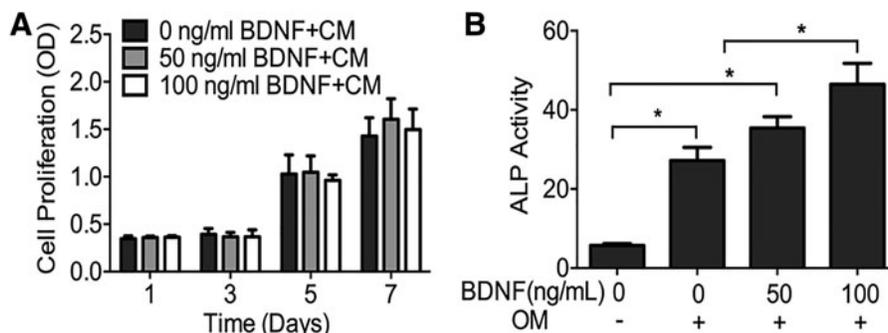
FIG. 1. Effect of BDNF on cell proliferation and ALP activity. **(A)** Cell proliferation of hBMSCs assessed using a CCK-8 assay at day 1, 3, 5, and 7. There were no significant differences between cells treated with 50 and 100 ng/mL BDNF, nor between CM and BDNF, from days 1 to 7 ($p > 0.05$). **(B)** ALP activity at day 7 after stimulation by BDNF. ALP activity was significantly higher in cells treated with 100 ng/mL BDNF compared to the other groups (*$p < 0.05$). ALP, alkaline phosphatase; BDNF, brain-derived neurotrophic factor; CM, culture medium; hBMSCs, human bone mesenchymal stem cells.

positive staining (integrated optical density/tissue area) using Image Pro Plus (Media Cybernetics; Roper Technologies, Lakewood Ranch, FL).

Statistical analysis

Data were analyzed using SPSS software (SPSS; IBM, Armonk, NY) and statistical significance was assessed by one-way analysis of variance and t-tests (nonparametric tests). *Post hoc* testing for multiple comparisons was carried out using a Fisher's least significant difference test. When variance was not homogeneous, a Kruskal–Wallis test was used, followed by a Nemenyi test for multiple comparisons. For all tests, the threshold for statistical significance was $p < 0.05$.

Results

Cell proliferation and osteogenic differentiation of hBMSCs

The assessment of cell proliferation showed that hBMSCs proliferated over time and that BDNF did not show any

FIG. 2. *ALP* gene expression and mineralized nodule formation after stimulation by BDNF. **(A)** Three days after 100 ng/mL BDNF treatment, RT-PCR revealed significantly more *ALP* mRNA expression compared to the other groups (*$p < 0.05$). **(B)** Matrix mineralization nodule assays revealed more nodule formation in the groups treated with 50 and 100 ng/mL BDNF relative to the OM and CM groups by day 14. OM, osteogenic differentiation medium; RT-PCR, real-time polymerase chain reaction. Color images available online at www.liebertpub.com/tea
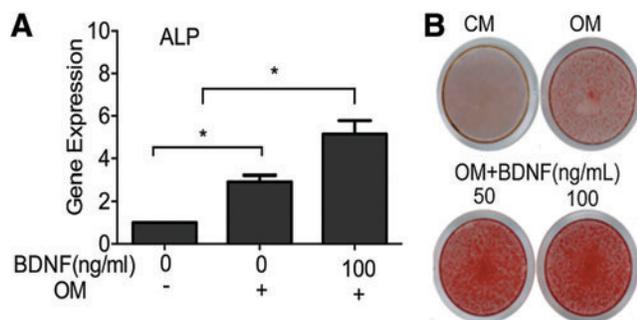
effect on proliferation. There was no significant difference between the control group and the BDNF-treated group, regardless of the concentration of added BDNF (Fig. 1A). However, 100 ng/mL BDNF did significantly promote ALP activity in hBMSCs when compared to cells cultured in OM only ($p = 0.037$). This was slightly higher than the ALP activity observed in the 50 ng/mL BDNF group, although there was no statistical difference. OM was also found to promote osteogenic differentiation by hBMSC compared to the control medium (Fig. 1B). An analysis of gene expression indicated that BDNF significantly increased the expression of *ALP* mRNA compared to cells grown in CM or OM only without BDNF ($p < 0.05$) (Fig. 2A). This was supported by the ALP activity assay. Finally, alizarin red staining demonstrated that BDNF promoted the formation of the mineralized extracellular matrix (ECM) by hBMSCs after 14 days, when compared to cells grown in CM or OM only (Fig. 2B).

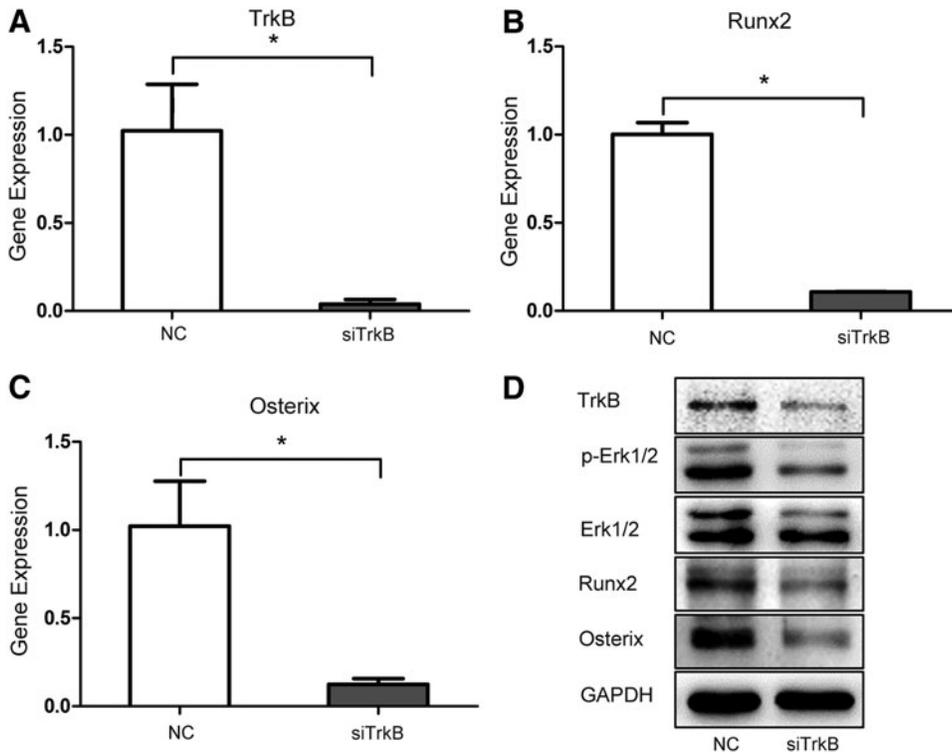#### BDNF promoted BMSC osteogenesis via the Erk/Runx2/Osterix pathway

To clearly determine the signaling pathway responsible for the osteogenesis promoting effect of BDNF, BMSCs were transfected with siRNA-TrkB, treated with 100 ng/mL BDNF, and then cultivated for 48 h to knockdown the BDNF receptor gene. In addition, an siRNA was also used as negative control. RT-PCR results showed that siRNA-TrkB knocked down more than 90% of the original *TrkB* gene. The expression levels of osteogenic transcription factors *Runx2* and *Osterix* were all markedly reduced in siRNA-TrkB knockdown group compared with those in the negative control group (Fig. 3A–C). This result indicated that by binding with TrkB, BDNF played a positive role in osteogenic differentiation of BMSCs. Western blotting assay further confirmed and explored this pathway, and it was observed that the expression level of phospho-Erk1/2 together with Runx2 and Osterix evidently decreased when the *TrkB* gene was knocked down (Fig. 3D).

Neurogenic potential of hBMSCs

Analysis of neurogenesis after BDNF treatment indicated that 100 ng/mL BDNF stimulated hBMSCs to generate more

FIG. 3. Erk/Runx2/Osterix expression after *TrkB* knockdown. Forty-eight hours after transfection of 100 nM siRNA-TrkB, RT-PCR revealed significantly less *TrkB* mRNA expression (**A**), *Runx2* expression (**B**), and *Osterix* expression (**C**) compared to NC group (*$p < 0.05$). (**D**) Western blotting assays showed less expression of TrkB protein, p-Erk1/2, Runx2, and Osterix in the 48-h siRNA-TrkB group.

neurogenic neuron-related proteins compared with cells grown in CM or OM only, or 50 ng/mL BDNF (Fig. 4). By day 7, protein expression of the neuron cell markers p75 and s100 was much higher in the group treated with 100 ng/mL BDNF relative to 50 ng/mL BDNF or cells in OM or CM without BDNF. However, protein expression did not show an increasing trend on day 14 (Fig. 4).

Effect of BDNF on the ectopic bone formation capacity of hBMSCs

As shown in Figure 5, H&E staining revealed no obvious inflammation at 4 and 8 weeks. Four weeks after *in vivo* transplantation, much less newly formed tissue was observed in groups I (β-TCP only) and II (β-TCP+CM only) relative to
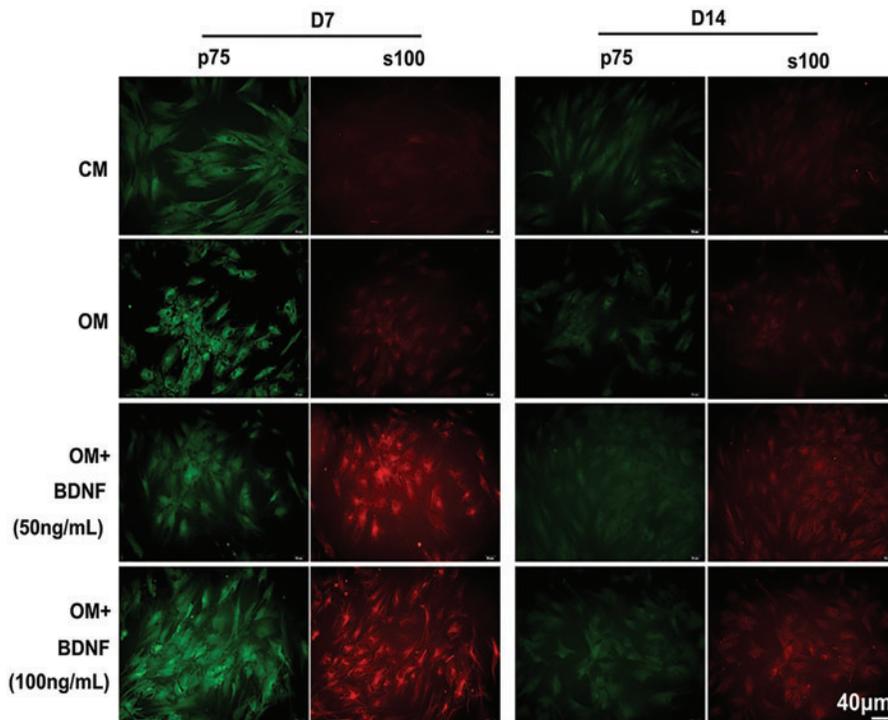
FIG. 4. Neuronal marker expression in hBMSCs stimulated by BDNF. p75 (*green*) and s100 (*red*) expression in hBMSCs at 7 and 14 days after stimulation by BDNF. Immunofluorescent staining revealed that 100 ng/mL BDNF led to more p75 and s100 protein expression relative to CM, OM, and 50 ng/mL BDNF (scale bar represents 40 μm). Color images available online at www.liebertpub.com/tea
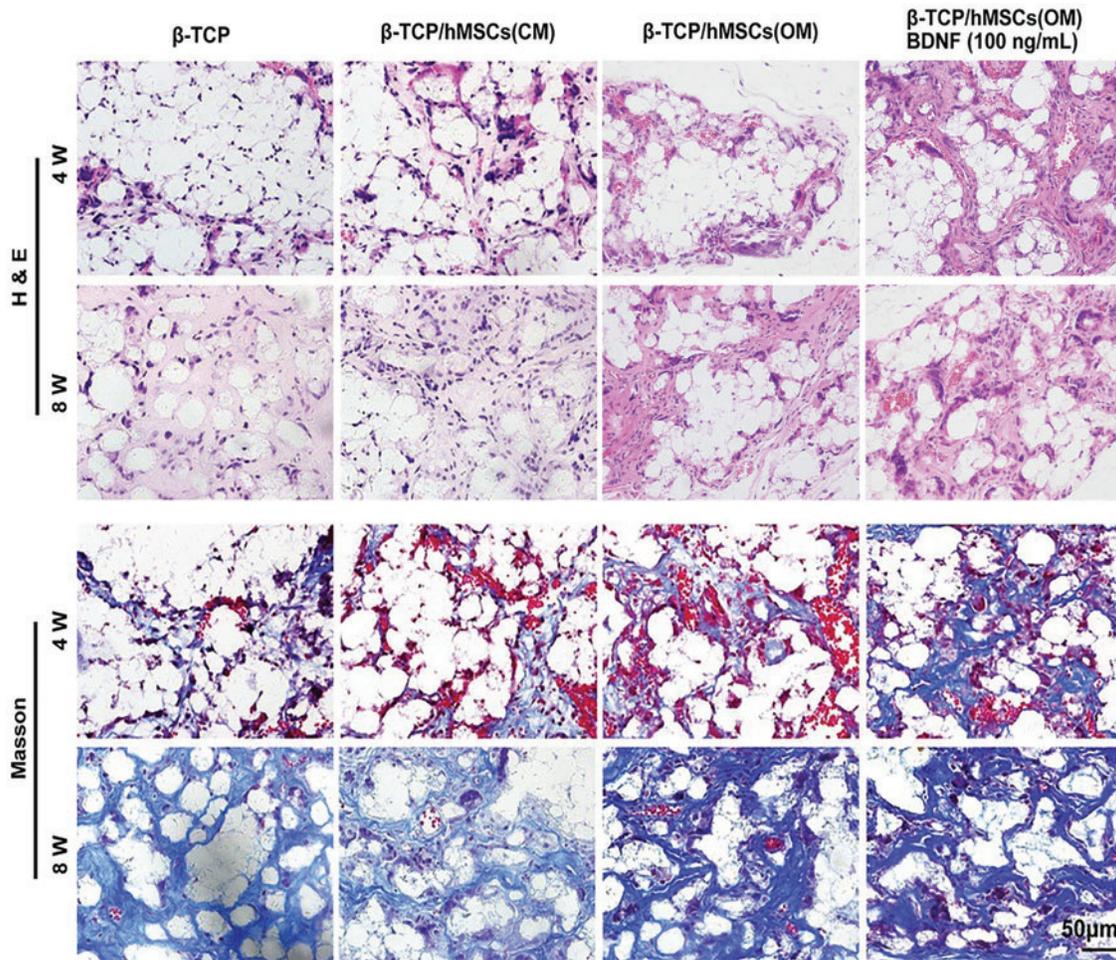
FIG. 5. Immunohistochemical staining of tissue sections. H&E and Masson's trichrome staining of hBMSCs at 4 and 8 weeks after stimulation by BDNF (scale bar represents 50 μm). H&E, hematoxylin and eosin. Color images available online at www.liebertpub.com/tea

groups III (β-TCP+OM) and IV (β-TCP+OM+BDNF). A large amount of eosinophilic bone-like tissue had formed in the ECM around the β-TCP particles, represented by blank areas after decalcification. Scattered sections of small blood vessels were found in groups III and IV (Fig. 5). Eight weeks after *in vivo* transplantation, the amount of newly formed tissue increased in all groups, although in groups III and IV, cells were enclosed by eosinophilic bone-like tissues that were similar in structure to bone lacuna. Masson's trichrome staining revealed a relatively stronger positive staining in group III (β-TCP+OM) relative to group IV (β-TCP+OM+BDNF), both of which were stronger than group I (β-TCP only) and II (β-TCP+CM only). The degree of positive ECM staining in group IV (β-TCP+OM+BDNF) was higher than found in group III (β-TCP+OM), and both groups were higher than group I (β-TCP only) and II (β-TCP+CM only). These results suggest increased proliferation in the BDNF-treated group. At 8 weeks, all groups showed relatively more positive staining than 4 weeks, while the staining in group III and IV was still stronger than the other groups (Fig. 5).
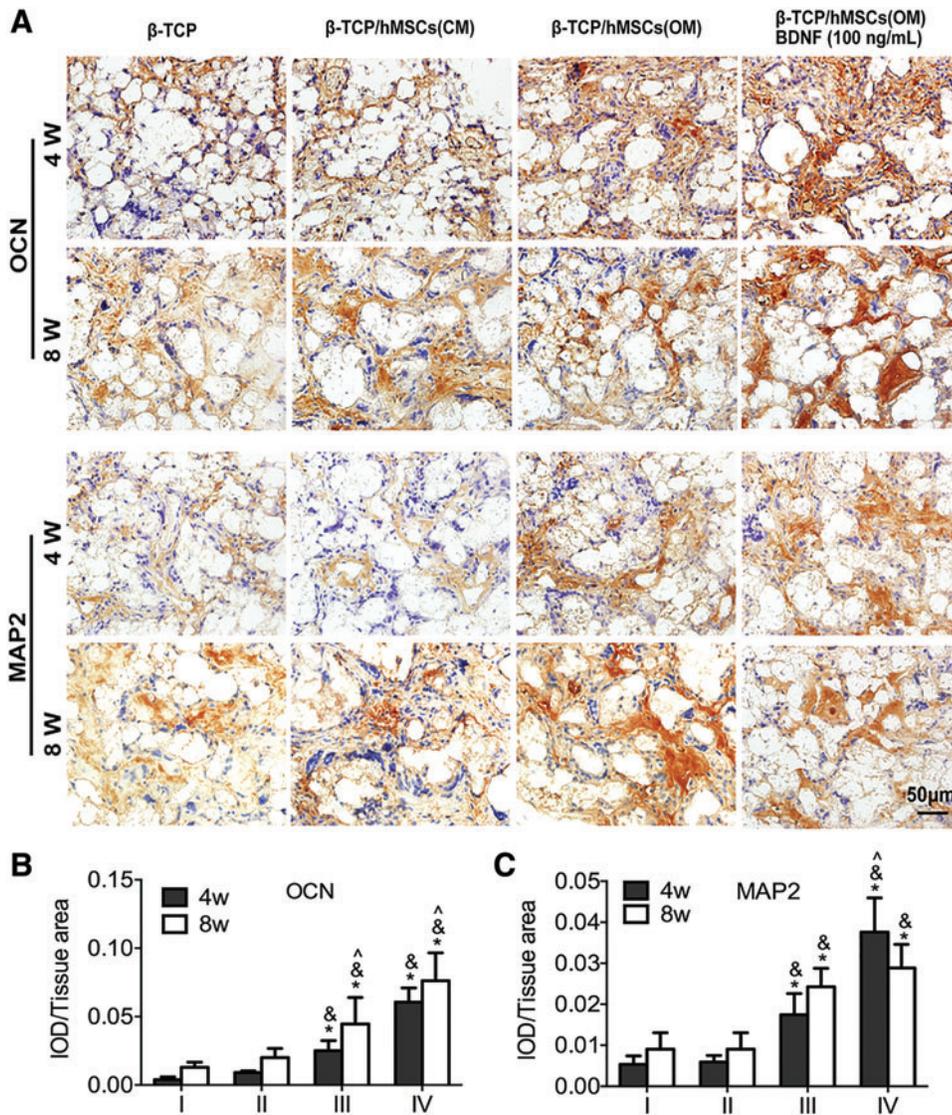
Immunohistochemical staining

To establish if BDNF had an effect on osteogenesis and neurogenesis *in vivo*, we performed immunohistochemical

staining using various osteogenic and neurogenic markers. This showed that the osteogenic marker OCN was more highly expressed at 4 and 8 weeks in group IV (β-TCP+ OM+BDNF) compared with group III (β-TCP+OM), group I (β-TCP only), and group II (β-TCP+CM only). No OCN staining was observed in groups I and II at 4 weeks (Fig. 6A). Quantitative analysis revealed an increasing trend in OCN expression that supported the visual assessment (Fig. 6B).

Immunohistochemical staining showed that groups I and II had little expression of the neuronal marker MAP2, but groups III and IV both expressed significant amounts of the protein (Fig. 6A). Further quantitative analysis examining staining density indicated that treatment with 100 ng/mL BDNF significantly promoted the expression of MAP2 relative to the other groups. At 4 weeks, p-values were <0.001 in all comparisons with groups I, II, and III. At 8 weeks, $p < 0.001$ when comparing the 100 ng/mL BDNF group with groups I and II, and $p = 0.036$ when comparing the 100 ng/ mL BDNF group and group III. There were no statistical differences between 4 and 8 weeks across all groups.

Finally, immunohistochemical staining and semiquantitative analysis of several other neuron markers (GFAP, NG2, and β-tubulin III) showed that both GFAP and β-tubulin III (TUBB) expressions were higher in group IV at 4 weeks,

FIG. 6. Immunohistochemical staining of OCN and MAP2. **(A)** Immunohistochemical staining of OCN and MAP2 at 4 and 8 weeks (scale bar represents 50 μm). **(B)** The average optical density of OCN and **(C)** MAP2 immunohistochemical staining (*$p < 0.05$ compared to Group I, [^&]$p < 0.05$ compared to Group II, and [^]$p < 0.05$ compared to Group III). MAP2, microtubule-associated protein 2; OCN, osteocalcin. Color images available online at www.liebertpub.com/tea

while the expression of NG2 in group IV was higher than other groups I and II (but not group III). At 8 weeks, GFAP, NG2, and β-tubulin III (TUBB) expressions in group IV were all more highly expressed than in other groups (Fig. 7).

Discussion

Previous studies have found that neuropeptides secreted by peptidergic nerves may be involved in regulating osteogenesis. This is hypothesized to be through factors released by nerve fibers that act by binding to receptors found on bone cells. This subsequently regulates bone metabolism and promotes osteogenesis.[19,30] The nervous system also regulates blood flow to an area, indirectly affecting bone metabolism.[31] However, it is still unclear whether there is a direct interaction between neurogenesis and bone formation. In our research, we have identified a safe and effective concentration of the neurotrophic factor BDNF that promotes cell proliferation in hBMSCs. We have used this to study the *in vitro* effects that BDNF had on osteogenesis and neurogenesis of hBMSCs and the mechanisms underlying

these effects. Furthermore, we used a β-TCP scaffold to assess the effects on new bone formation *in vivo*.

Our results demonstrate that neither 50 nor 100 ng/mL BDNF had a negative effect on cell growth. This is consistent with a previous study showing no significant influence on proliferation in human pulp cells by BDNF.[27] We also found that BDNF promoted the ALP activity of hBMSCs in a dose-dependent manner and that a concentration of 100 ng/mL showed an effect on *in vitro* ALP activity, gene expression, and mineralization nodule formation of hBMSCs. This result is supported by numerous studies examining the biological effects of BDNF that have indicated that the most effective concentration of BDNF falls between 50 to 100 ng/mL.[21,22,24,25]

To elucidate a possible mechanism for BDNF, several studies have demonstrated that BDNF can bind the TrkB receptor, activating the ERK1/2 signaling pathway and promoting osteogenesis.[23,27] Prior research has also revealed that activating the ERK signaling pathway in mesenchymal stem cells can lead to osteogenic differentiation.[32] To further examine the role of BDNF in osteogenesis, we utilized
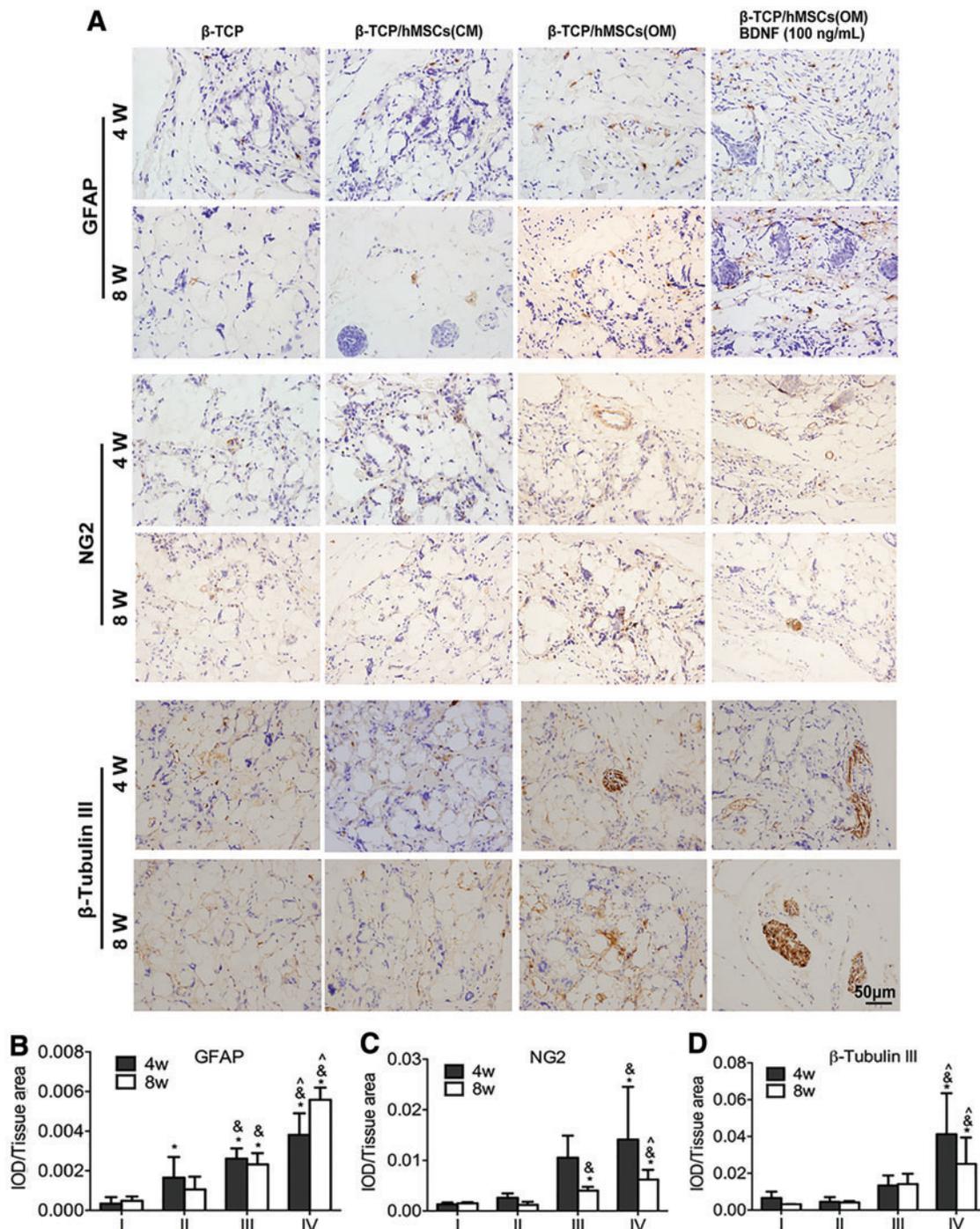
FIG. 7. Immunohistochemical staining of GFAP, NG2, and β-tubulin III. **(A)** Immunohistochemical staining of GFAP, NG2, and β-tubulin III (TUBB) at 4 and 8 weeks (scale bar represents 50 μm). The average optical density of **(B)** GFAP, **(C)** NG2, and **(D)** β-tubulin III (TUBB) immunohistochemical staining ($*p < 0.05$ compared to Group I, $^{\&}p < 0.05$ compared to Group II, and $^{\wedge}p < 0.05$ compared to Group III). GFAP, glial fibrillary acidic protein; NG2, neural/glial antigen. Color images available online at www.liebertpub.com/tea

siRNA-TrkB to knockdown the BDNF receptor gene in BDNF-treated BMSCs, and detected the expression level of p-Erk1/2, Erk1/2, and the downstream transcription factors Runx2 and Osterix. In addition, Western blotting results revealed that after knocking down the *TrkB* gene with the siRNA, phospho-Erk1/2 expression was notably decreased, and RT-PCR and Western blotting assay re-

sults revealed that the expression of Runx2 and Osterix was markedly reduced at both, the gene and protein levels. Hence, our study elucidates a possible mechanism underlying the osteogenesis-promoting effect of BDNF. However, a complete experiment, involving the application of pathway inhibitors, is required to further illustrate the pathway.

During our study, we also used an animal model to assess the possibility of BDNF promoting the neurogenesis and osteogenesis of hBMSCs *in vivo* by directly examining the expression of several osteogenic and neurogenic biomarkers. Our study found that MAP2-, NG2-, GFAP-, and β-tubulin III (TUBB)-positive cells could all be found in newly formed bone-like tissues. Each of these markers are uniquely or strongly associated with neuronal development and neurogenic capacity,[19,25–28,30–35] suggesting that neurogenesis is linked with bone development. Furthermore, after transplanting hBMSCs into the dorsal subcutaneous area of nude mice, our study found that the expression levels of MAP2, GFAP, NG2, and β-tubulin III (TUBB) were all higher in BDNF-treated cells at 4 and 8 weeks. We therefore conclude from these *in vivo* studies and histological staining that both osteogenesis and neurogenesis tend to increase after BDNF treatment.

However, in the *in vitro* and *in vivo* results obtained in this study, we are not able to explain whether BDNF promoted BMSC neurogenesis by directly inducing differentiation into neurons or by indirectly helping BMSCs to secrete neuroprotective factors that could induce nearby cells to differentiate into nerve cells. Referring to previous studies that have reported about the possibility of both the above ways,[36–39] we speculate that these two ways act simultaneously and in a synergistic or complicated manner. This is because apart from our *in vitro* IF assay, in which neuron markers were observed in BDNF-treated cells, indicating that some BMSCs could be induced and differentiated into neuron-like cells, there are studies confirming that BMSCs were also able to secrete neuroprotective factors that help nerve cells grow.[38,39] Therefore, the exact mechanism underlying the effect of BDNF on neurogenesis remains an intricate and challenging question that requires further study.

Previous studies have demonstrated that BDNF can promote vascularization[40–43] and our H&E staining revealed that BDNF showed the tendency to promote the generation of more capillaries within new bone tissue by 8 weeks although there were no significant differences (data not shown). Considering animal ethics and welfare, we used six animals in one group, and therefore, the reason for no significant differences may lie in the small number of samples. In the next step, we will investigate the relationship between neurogenesis and angiogenesis by using BDNF in bone engineering.

In conclusion, our study has shown a clear link between neurogenesis and osteogenesis in hBMSCs and demonstrated that BDNF treatment can enhance both processes, although the exact mechanism remains unclear. This suggests that BDNF may have a future role in improving the outcome for patients requiring bone tissue engineering, such as after bone augmentation in dental implant surgery.

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Disclosure Statement

No competing financial interests exist.

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