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Brief Communication

Neurotrophins BDNF and NT4/5 accelerate dental pulp stem cell migration

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ABSTRACT

Neurotrophic factors play important roles in neuron survival, growth and differentiation. In the present research, the expression of multiple neurotrophins and their effects on cell migration were studied in the dental pulp stem cells (DPSCs). Human DPSCs from five patients were cultured. Expression of neurotrophins and their receptors were evaluated by PCR, immunofluorescent staining and ELISA. Scratch assay was performed in the presence or absence of neurotrophic factors. Level of phosphorylated-ERK was evaluated with Western blotting. Neurotrophins were expressed at various levels in the DPSCs. Treatment of 100 ng/ml BDNF or NT4/5 accelerated wound healing in scratch assay and elevated the expression of phosphorylated–ERK. The work indicated that neurotrophins promoted human DPSCs migration *in vitro*.

Human Dental Pulp Stem Cells (DPSCs) were first isolated from extracted third molars [1]. They are mesenchymal cells that express multiple stem cell markers, and possess pluripotency and regeneration activity [2]. DPSCs were reported to have higher proliferation rate than bone marrow derived stem cells, and has been proposed as a promising candidate for tissue regeneration [3].

It has been reported that DPSCs can promote neuronal tissue regeneration. DPSCs could be induced to differentiate into neuron-like cells as verified by the expression of neuronal markers *in vitro* and *in vivo* [4]. The conditioned medium derived from cultured DPSCs contained multiple neurotrophic factors and induced neurite growth [5]. The mRNA of neurotrophic factors nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), glial cell line derived neurotrophic factor (GDNF), neurotrophin 3 (NT3) and NT4/5 were expressed in a temporal–spatial pattern during tooth development in mice and human [6].

Neurotrophic factors are growth factors that can nourish neurons and promote neuron survival and regeneration. The most well studied neurotrophic factors are neurotrophin (NT) family members, which include the nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), NT3 and NT4/5 [7–9]. All NTs bind with membrane receptor p75 at low affinity, whereas NGF and NT3 bind with tropomyosin receptor kinase TrkA and TrkC with high affinity respectively. BDNF and NT4/5 has high affinity with TrkB [10]. Glial cell line derived neurotrophic factor

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(GDNF) family and neuropoietic cytokines are also identified as neurotrophic factors that are able to support neuron survival, proliferation, maturation, damage repair [11,12].

Previous work from our group showed that GDNF family ligands (GFLs) and their receptors were expressed by the adult human DPSCs and exogenous GDNF promoted migration of DPSCs *in vitro* [13]. In the current study, we further evaluated the expression of NT family neurotrophic factors and found that BDNF, NT4/5 and their receptor TrkB were highly expressed in the DPSCs and promoted DPSCs migration.

Materials and methods

Cell culture

The human DPSCs were gift from Dr. Songtao Shi (University of Pennsylvania). DPSCs were isolated and characterized as previously described by Dr. Shi's lab [1]. Samples were de-identified prior to being received for this study and did not meet the federal definition of human subject research. IRB approval was exempted from the Office of Research and Graduate Studies, University of the Pacific. DPSCs were cultured in DMEM (Gibco) supplemented with 10% FBS and antibiotics. Passage 4–8 cells were used in the study.

PCR

PCR was performed as previously described [13]. Primers sequences could be found in Table 1.

Immunofluorescent staining

Experiments were performed as previously described [13]. Briefly, DPSCs were cultured on gelatin coated coverslips, fixed and labeled overnight at 4 °C with anti-NGF, anti-BDNF, anti-NT3, anti-NT4/5, anti-TrkA, anti-TrkB, anti-TrkC, anti-p75, anti-integrin α5 and anti-integrin β1 antibodies (Abcam), followed with fluorescent secondary. Images were acquired using a Leica TCS SPE confocal microscope.

ELISA

Serum free medium were collected from DPSCs culture and concentrated using Amicon ultra centrifugal filter of 10 kDa (Millipore). ELISA was done following manufacturer's instruction (Abcam).

Scratch assay

Scratch assay were done as previously reported [14]. Briefly, DPSCs were serum starved overnight after reaching confluence. Scratches are created on confluent DPSCs. Images are captured at the beginning and different time intervals. Quantification was done with ImageJ.

Transwell migration assay

Experiments were performed as previously described [13]. Briefly, 5×10^4 DPSCs were plated in the transwell inserts in the upper chamber. Serum free medium containing 100 ng/ml of BDNF or NT4/5 were added in the lower chamber. After 18-h incubation, cells in the upper chamber were removed and cells migrated across the membrane were fixed and stained. Pictures were taken under Leica DMil camera. Quantification was done with ImageJ, which is an open source image processing program developed at the National Institutes of Health.

Western blotting

DPSCs lysates were collected with RIPA buffer (Sigma) and probed with integrin α5 (1:1000), integrin β1 (1:1000) and laminin (1:1000) (Abcam).
DPSCs were serum starved overnight, followed by incubation with recombinant BDNF or NT4/5 at 100 ng/ml (R&D Systems). Cell lysate was collected at different time point with RIPA buffer. Blots were probed with phospho-Erk (1:1000) (Cell Signaling Technology) and tubulin (1:5000) (Abcam).

Statistics

Data were expressed as SEM. Statistical ANOVA and Student's t tests (2-tailed) were used to compare the data. $P \leq 0.05$ was considered to be significant.

Results

Expression of neurotrophins and cell adhesion molecules in human DPSCs

NT family members NGF, BDNF, NT3 and NT4/5 could be amplified through PCR in human DPSCs. NT receptors except for TrkA were also amplified through PCR (Fig. 1A). BDNF was

Table 1 The list of primer pairs for PCR.

Gene	5′ primer sequence	3′ primer sequence
β- actin	AGAGCTACGAGCTGCCTGAC	AGCACTGTGTTGGCGTACAG
Ngf	GGCAGACCCGCAACATTACT	CACCACCGACCTCGAAGTC
Bdnf	GGCTTGACATCATTGGCTGAC	CATTGGGCCGAACTTTCTGGT
Nt3	CCGTGGCATCCAAGGTAACAA	GCAGTTCGGTGTCCATTGC
Nt4/5	CTGTGTGCGATGCAGTCAGT	TGCAGCGGGTTTCAAAGAAGT
TrkA	AACCTCACCATCGTGAAGAGT	TGAAGGAGAGATTCAGGCGAC
TrkB	ACCCGAAACAAACTGACGAGT	AGCATGTAAATGGATTGCCCA
TrkC	ACGAGAGGGTGACAATGCTG	CCAGTGACTATCCAGTCCACA
P75	CCTACGGCTACTACCAGGATG	CACACGGTGTTCTGCTTGT

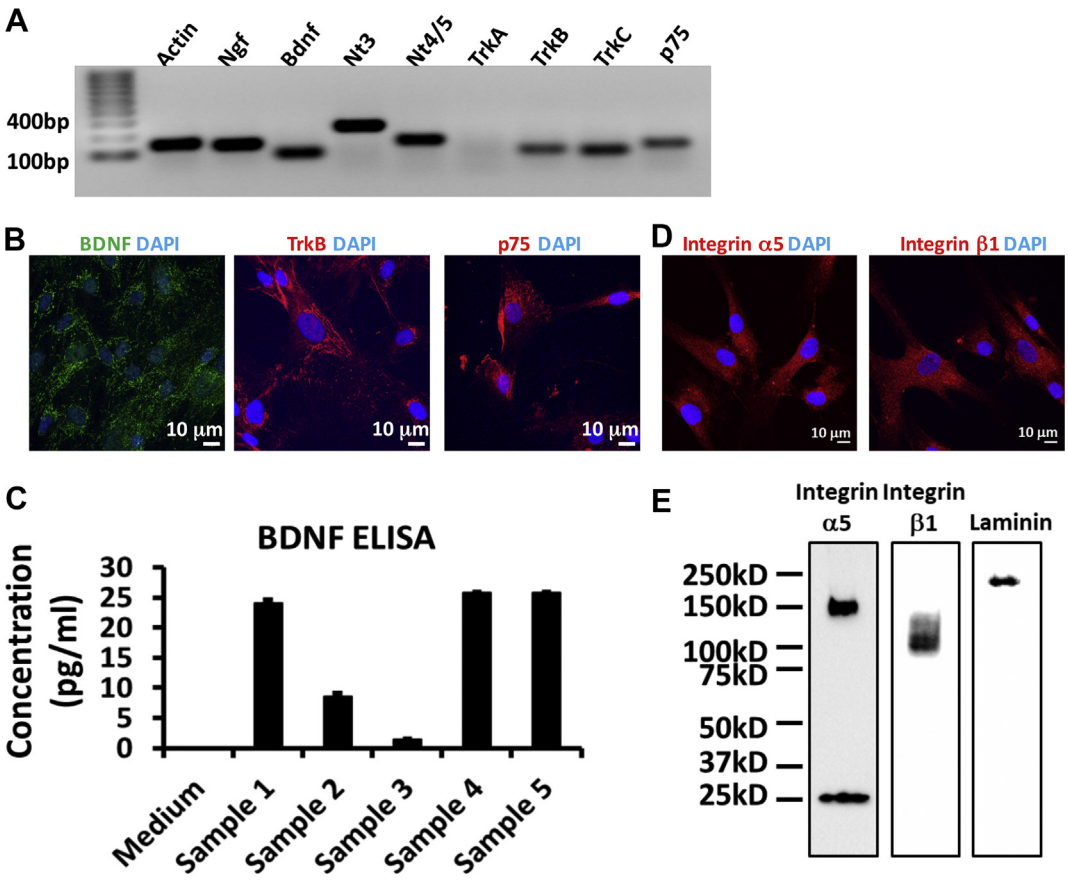

Fig. 1 **Expression of neurotrophic factors and cell adhesion molecules in human DPSCs. (A)** Expression of the neurotrophic factors Ngf, Bdnf, Nt3, Nt4/5, their receptors TrkB, TrkC, and the co–-receptor p75 in the human DPSCs. 10% samples were loaded for internal control actin. **(B)** Cellular localization of BDNF, its receptor TrkB, and co–-receptor p75 in DPSCs. **(C)** ELISA of the secreted BDNF from DPSCs in the culture medium (n = 5). **(D)** immunofluorescent staining and **(E)** Western blotting of integrin a5, integrin b1 and laminin in the human DPSCs.

found as puncta in the cytoplasm of the DPSCs. Signal of membrane receptors TrkB, which has high affinity with BDNF and NT4/5, was prominent in the cell membrane and the perinuclear region in DPSCs. Signal of the low affinity NTs receptor p75 was observed at the cell membrane and the cytosol (Fig. 1B). Secretion of BDNF was confirmed in all five human DPSCs samples through ELISA. The expression level ranged between 1.46 and 25.9 pg/ml in concentrated serum free culture medium of DPSCs (Fig. 1C).

Despite detection of NGF through PCR in DPSCs, we were unable to detect intracellular NGF expression through immunofluorescent staining, nor secreted NGF in the culture medium through ELISA. Expression of NT3, NT4/5, TrkA and TrkC was not detected through immunofluorescent staining either.

The integrin family proteins, and laminin are important cell adhesion protein. Previous report showed blocking Integrin β1 with antibody reduced cell adhesion on laminin [15]. Immunofluorescent staining (Fig. 1D) and Western blotting (Fig. 1E) showed that integrin α5, integrin β1, and laminin all expressed in the human DPSCs.

BDNneurotrophins promoted humanF and NT4/5 promoted migration of DPSCs through MAPK pathway in vitro

Recombinant BDNF and NT4/5 significantly accelerated the DPSCs migration to close the wound 48 h post scratch in serum free medium (Fig. 2A). Quantification of the width of the scratch showed that compared to the control, recombinant BDNF or NT4/5 increased the cell migration to close the wound. The effect of BDNF and NT4/5 was comparable to that of GDNF, which was previously reported to promote DPSC migration (Fig. 2B). Cell proliferation assay showed that neither BDNF nor NT4 induced rapid cell proliferation at the dose of 100 ng/ml during 4 days of observation (Supplementary Fig. 1).

Transwell assay further confirm that compared to control, 100 ng/ml BDNF and NT4/5 significantly accelerated the DPSCs migration across the membrane after 18 h of incubation (Fig. 2C and D).

Because TrkB-ERK pathway was reported to mediate BDNF induced migration in human endothelial cells [16], we evaluated the ERK phosphorylation post BDNF treatment. ERK

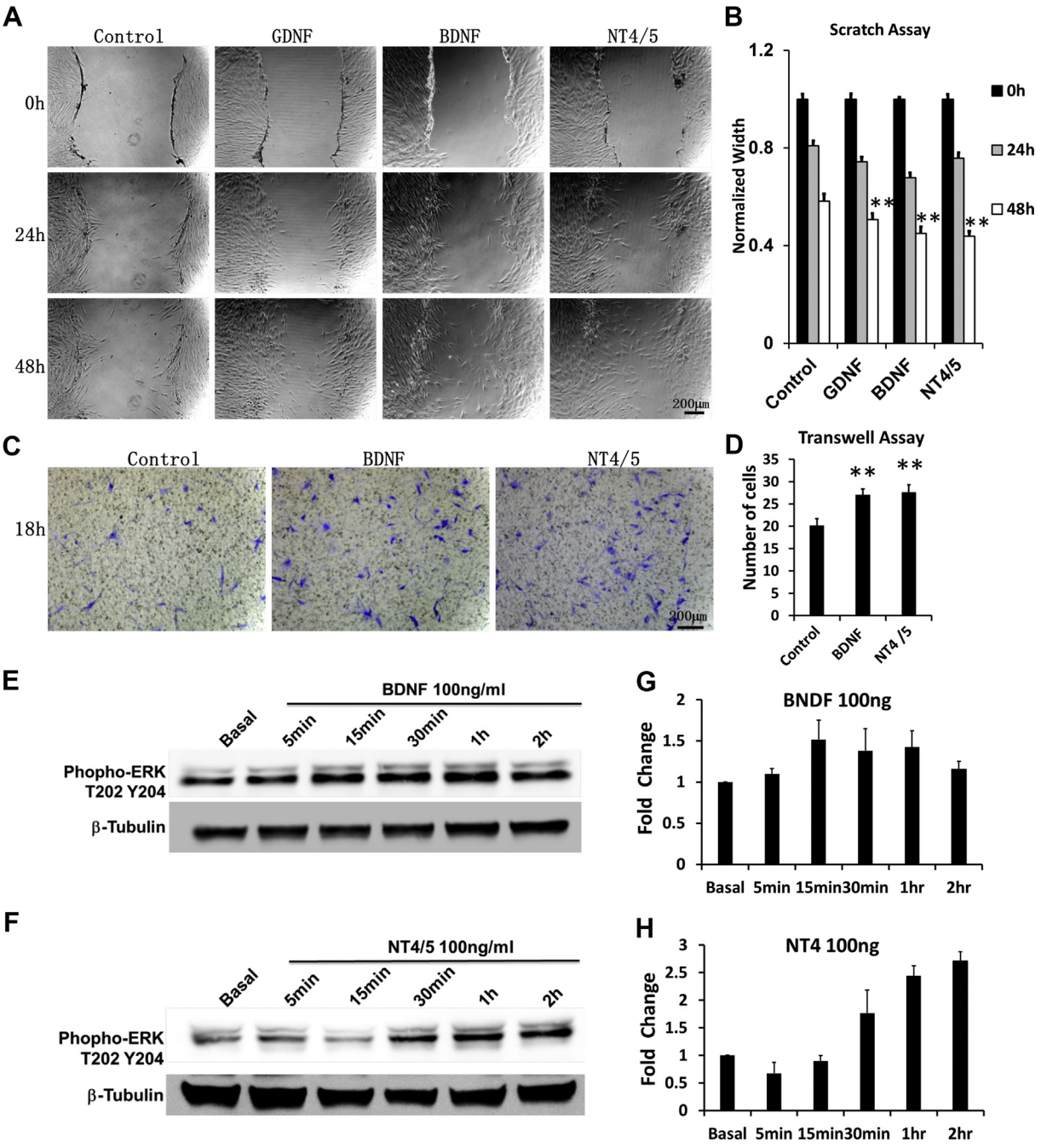

Fig. 2 **Migration of DPSCs through MAPK pathway after BDNF and NT4/5 treatment *in vitro*.** **(A)** Scratches were created on serum starved confluent DPSC monolayers. GDNF, BDNF or NT4/5 at a concentration of 100 ng/ml of were added to the medium after the scratch is created. Images were taken at the time of scratch and then 24 h and 48 h after scratching. **(B)** The width of each scratch was measured at multiple locations and normalized to time zero hour.Experiments were repeated three times. $^{**}p < 0.01$ compared to control at the same time point. **(C)** Transwell migration assay showed DPSCs migration to the lower chamber. BDNF or NT4/5 at a concentration of 100 ng/ml was added in the serum–- free medium in the lower chamber. Cells were stained with crystal violet (blue). **(D)** The number of cells migrated to the lower chamber was quantified at multiple views (n = 15). $^{**}p < 0.01$ compared to control. **(E)** BDNF or **(F)** NT4/5 at a concentration of 100 ng/ml were added to serum starved DPSCs. Phosphorylation of ERK was measured at different time points. **(G-H)** Densitomeric analysis of phospho–-ERK at different time points post treatment of 100 ng/ml BDNF or NT4/5. Data were normalized to β–-tubulin signal (n = 3).

phosphorylation increased 15 min to 1 h post BDNF treatment (Fig. 2E). Similar elevation was observed 30 min to 2 h post NT4/5 treatment, which also activates the TrkB pathway (Fig. 2F). Experiments were repeated three times. Although the result was not statistically significant, it suggested that BDNF induced phosphorylation of ERK appeared at an earlier time than NT4/5 (Fig. 2G and H).

Discussion

Dental pulp cell migration is regulated by the extracellular matrix protein and many chemoattractants that can be found in the dental pulp, such as TGF-β1, FGF, EGF [17]. It was reported that solubilized human dentine extracellular matrix released neurotrophic factors NGF and GDNF along with other hormones such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), insulin-like growth factor (IGF). The extracts from dentine extracellular matrix induced dental pulp cell growth and migration [18].

In addition, DPSCs also express and secrete neurotrophic factors. Co–culture of DPSCs with trigeminal neurons induced neurite outgrowth, while co-culture of fibroblasts with trigeminal neurons did not induce neurite outgrowth [19]. While anti-NGF and anti-BDNF antibodies abolished the neuroprotection effects of human DPSCs on mouse dopaminergic neurons *in vitro* [20].

Previously, we found GDNF family ligands and their receptors were highly expressed in adult human DPSCs. The results indicated neurotrophic factor GDNF promoted the migration of DPSCs though activation of the PI3/AKT and MEK/ERK pathways [13]. Our current investigation revealed that neurophins and their receptors were expressed in the cultured adult human DPSCs. BDNF could be secreted into the culture medium at among DPSC from five different patients. Despite the detection of NGF cDNA in DPSCs, we were unable to detect the expression of its dominant receptor TrkA. Secretion of NGF was not detected either. Therefore, we focused on the effect of BDNF and NT4/5, both of which mainly bind to TrkB receptor. BDNF and NT4/5 promoted DPSCs migration. The effect was comparable to same dose of GDNF (100 ng/ml). Both BDNF and NT4/5 increased phosphorylation of ERK, a common downstream factor mediating cell migration.

The study showed that neurotrophin family members BDNF and NT4/5 accelerated DPSCs migration *in vitro*. It would be interesting to further evaluate if expression of different neurotrophic factors in DPSCs fluctuates in response to various stimulations, and the differential effect of neurotrophic factors on DPSCs. These work would further reveal the function and mechanism of the neurotrophic factors in DPSCs regeneration, and potentially reveal them as future novel drug candidates in vital pulp therapy in the dental clinic.

Conflicts of interest

Authors have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bj.2020.03.010.

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