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3D microenvironment of collagen hydrogel enhances the release of neurotrophic factors from human umbilical cord blood cells and stimulates the neurite outgrowth of human neural precursor cells

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ABSTRACT

The umbilical cord blood (UCB) cells have been reported to secrete therapeutic signals, including a series of neurotrophic factors. This suggests the cell source provides suitable therapeutic environments for nerve regeneration that ultimately finds a possible cell therapy for nerve tissue. In this study, we observe a collagen hydrogel provides human UCB cells a proper 3D environment that stimulates the release of various neurotrophic factors. When compared to 2D culture, the 3D hydrogel culture significantly enhanced the expression of a series of neurotrophic factors, including neurotrophins, nerve growth factor, brain-derived neurotrophic factor, and ciliary neurotrophic factor as verified by the gene and protein analysis. To confirm the effects of neurotrophic factors secretion, we allowed an indirect interaction of the UCB-environment with human neural precursor cells (hNPCs). Results showed significantly enhanced neurite outgrowth of hNPCs. Collectively, our findings demonstrate that the collagen-based 3D hydrogel provides excellent environment for UCB-derived cells to release neurotrophic factors that will be ultimately useful for the neural repair and regeneration purposes.

1. Introduction

Stem cells play key roles in the regenerative process of damaged and diseased tissues [1–4]. Among the stem cell sources that have been indentified, the umbilical cord blood (UCB) derived stem cells have also gained great attention. UCB cells are easy to harvest and expand for practical applications and have shown relatively low immunogenicity and side effects after transplantation [5,6]. While the major population of UCB stem cells is hematopoietic stem cells, a great fraction of them is mesenchymal stem cells, and many therapeutic efficacies resulted from both cell types [7–11].

One of the major goals of the stem cell therapy is in fact to restore or replace injured tissues through a controlled stem cell transdifferentiation into a cell type of interest. However, recently, a modulatory role of stem cells i.e., immune-modulation of anti-scar formation and nerve protection by their secreted cytokines and neurotrophic factors, has been highlighted [12–14]. UCB cells have also shown to produce several neurotrophic factors, including nerve growth factors (NGFs), brain-derived neurotrophic factors (BDNFs), ciliary neurotrophic factors (CNTFs), neurotrophin-3 (NT3), and -4 (NT4) [15]. Therefore, UCB cells are considered as a possible therapeutic cell source for the treatment of neurotrauma and neurodegenerative diseases [14].

Here we thus hope to make use of the UCB cells for this neural repair and regenerative purposes. In particular, we consider the culture environments of UCB cells would significantly affect their biological functions, particularly their releasing behaviors of neurotrophic factors. We introduce 3-dimensional (3D) hydrogel made of collagen to culture UCB cells. 3D hydrogel environment is considered to mimic the native soft tissue structure, providing stem cells appropriate physical and chemical cues for adherence, spreading, growth and even switch to a lineage differentiation [16]. Collagen, the most abundant extracellular matrix component in human, constitutes a primary structural unit that preserves a gel-like tissue structure, and has been the most-widely studied as the cell culture matrix [17,18]. In fact, stem cell-based therapy has been mainly conducted by transplanting the cells directly to the sites or intravenously. However, this method has been resulted

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in a loss of stem cells at large fractions and a dilution of secreted factors that might come from stem cells, which eventually limit their therapeutic efficacy and clinical applicability [14,19]. In this manner, the collagen hydrogel matrix is considered as a potential UCB cell carrier for neural regeneration.

Here we culture UCB cells within collagen hydrogel matrix, analyze the release of neurotrophic factors and compare the results with those cultured in 2D culture dish. We further demonstrate the biological effects of neurotrophic factors released from UCB cells by co-culturing with neural precursor cells (NPCs) and examining their neurite outgrowth behaviors.

2. Materials and methods

2.1. Isolation and preparation of umbilical cord blood cells

The umbilical cord was obtained with mother's informed consent and approval by Institutional Review Board of Dankook University Hospital at Cheonan, S. Korea. The cord blood was collected after a delivery of the placenta by puncturing the umbilical cord vein with a syringe (>40 ml/sample). Cell numbers, viability, and blood sterility were evaluated before blood storage, which did not exceed 12 h. The mononuclear cell fraction was isolated from the blood on ficoll/hypaque gradient techniques (Pharmacia LKB, Gaithersburg, MD) followed by a washing and resuspension in DMEM/F12 medium (Gibco BRL, Grand Island, NY) at a final concentration of 1×10^6 cells/ml. The cell fractions that proliferated in tight adherence to the plastic culture dish were considered in this study.

2.2. Flow cytometry

For flow cytometric analysis, UCB-derived cells were washed twice with PBS, resuspended and fixed with 4% paraformaldehyde for 15 min, and treated with 0.2% Triton X-100. Blocking was conducted by incubating the cells in 1% BSA at 4 °C overnight, and the cells were probed with either mouse anti-Oct3/4 antibody, mouse anti-Sox2 antibody, anti-SSEA-4 antibody, anti-NGF antibody or rabbit anti-BDNF antibody (Santa Cruz Biotech, Santa Cruz, CA) at 4 °C overnight. The cells were then incubated for 1 h with either Alexa 555-conjugated goat anti-mouse antibodies (Santa Cruz Biotech) for NGF or FITC-conjugated donkey anti-rabbit antibody (Santa Cruz Biotech) for BDNF. After washing in PBS, the flow cytometry was conducted with FACS Caliber flow cytometer (BD Biosciences, San Jose, CA) and analyzed using the Cell Quest-Pro software (BD Biosciences).

2.3. RT-PCR analysis for gene profiling of neurotrophic factors

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed to detect the mRNA levels of neurotrophic factors, including nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), neurotrophin-3 (NT3), neurotrophin-4 (NT4), and basic fibroblast growth factor (bFGF). Total RNA was isolated from each cell pellet by using the RNA isolation kit (RNeasy Mini Kit 74104, Qiagen, Germantown, MD). About 1 μg total RNAs were reverse-transcribed by using the Quantitect RT-PCR Kit (#205311, Qiagen) according to the manufacturer's protocol. Then, the cDNA was subjected to PCR amplification with neurotropic factor-specific primers as listed in Table 1 in the pre-mixed PCR solution (Bioneer, DaeJon, S. Korea) by 35 cycles of an incubation consisting of 95 °C for 30 s, 58 °C for 30 s, followed by 75 °C for 60 s.

2.4. Western blotting for protein profiling of neurotrophic factors

NGF and BDNF protein levels were analyzed by the Western blotting with anti-NGF and anti-BDNF antibody, respectively. About 80 μl of cell lysates was boiled for 5 min with 20 μl of 5× sample buffer. Twenty microliters of boiled protein samples was loaded into each well of a gradient polyacrylamide gel (10%, Bio-Rad, Hercules, CA), and then transblotted to a nitrocellulose membrane. Transblotted membrane was blocked in 5% fat-free milk Tris buffer with 0.5% Tween-20 (TBST) for 1 h at room temperature, and then incubated with primary antibody (mouse anti-NGF antibody or anti-BDNF antibody (Santa Cruz Biotech) that was previously diluted at 1:1000 in the 2% fat-free milk TBST solution overnight at 4 °C. The blotted membrane was washed in 0.5% TBT and incubated with horseradish peroxidase-conjugated anti mouse IgG 1 h, and the immunoreactive bands were detected by using

Table 1
Primer sequences, annealing temperatures, and expected product sizes in PCR reactions.

Target gene	Primer (forward and backward)	Annealing temperature	Product size (bp)
NGF (NM_002506.2)	5-TCACCCCGTGTGCTGTTTAG-3 5-ATTCGCCCCTGTGGAAGATG-3	60	148
BDNF (M61176.1)	5-TTTGGTTGCATGAAGGCTGC-3 5-GCCGAACTTTCTGGTCCTCA-3	60	199
CNTF (AK314118.1)	5-AGGGATGGCTTTCACAGAGC-3 5-CGCAGAGTCCAGGTTGATGT-3	60	163
NT3 (NM_001102654.1)	5-TCTCTTCATGTCGACGTCCC-3 5-TTACCTTGGATGCCACGGAG-3	60	137
NT4 (NM_006179.4)	5-TGACAGGTGCTCCGAGAGAT-3 5-GGGAGAGAAGGTCCCACTCA-3	60	148
RGMa (BC015886.1)	5-GGGAGAGGAGCAGGGTCTTA-3 5-GCACTTTTGGGAGTGAAGCC-3	60	220
RGMb (NM_001012761.2)	5-TGCCCTTGTAATCGGTGTCC-3 5-ACCAGTGTTTCCCCCGTTTT-3	60	242
Netrin-1 (NM_004822.2)	5-CTGAGTGCCTGCTTTACGGA-3 5-TGTCCCTCCCTCCCACATAG-3	60	170
bFGF (NM_004465.1)	5-GTGCGGAGCTACAATCACCT-3 5-ATGCTGTACGGGCAGTTCTC-3	60	131
GAPDH (M33197.1)	5-GAGAAGGCTGGGGCTCATTT-3 5-AGTGATGGCATGGACTGTGG-3	60	231

the Western Bright ECL detection reagent (K-12045, Advansta, Menlo Park, CA).

2.5. Immunocytochemistry for neurotrophic factor expression

Cultured tissues (cell-containing collagen hydrogel) were fixed with 4% paraformaldehyde and washing and smear on the slide glass. Mouse anti-NGF antibody or rabbit anti-BDNF antibody (Santa Cruz Biotech) was applied to the sample slide at 4 °C overnight at a dilution of 1:1000 in PBS with 0.2% BSA and 0.01% Na-azide. Secondary antibodies, FITC-conjugated goat anti-mouse for NGF (Santa Cruz Biotech), or Alexa 555-conjugated donkey anti-rabbit antibody for BDNF (Invitrogen, Grand Island, NY), were diluted 1:500 in the same diluent and applied for 1 h at room temperature.

2.6. 3-Dimensional culture in a collagen-based hydrogel

The preparation of collagen from rat tails and 3-D culture of primary cells in a collagen gel were as previously described [13]. Two hundred microliters of collagen solution were poured into a 50 mm bacteriological petri-dish (Nunc, Roskilde, Denmark), mixed sequentially with 100 microliter of 3× DMEM/F12 (Gibco BRL) and with 1 ml of sorted cell suspension in DMEM 10% FBS (Gibco BRL), and allowed for gelation by gentle agitations. The resulting cell-collagen hydrogel mixture was cultured in DMEM/F12 media at 37 °C in 5% CO_2.

2.7. In vitro functional assays for released neurotrophic factors

Human neural precursor cells (hNPCs) were maintained in DMEM media with the N2 supplement (Gibco BRL). About 1–2 × 10^6/ml of proliferating UCB-derived cells embedded in a collagen hydrogel after 3 days in culture were prepared. For an ex vivo functional assay of secreted neurotrophic factors for the neurite growth and differentiation of hNPCs, UCB-derived cells in a collagen hydrogel were co-cultured with hNPCs on a cover slip placed in the same well such that hNPCs shared the culture media with UCB cells. First, a small cluster of hNPCs was seeded on the matrigel-coated coverslip and incubated at 37 °C in 5% CO_2 overnight. After ensuring that the hNPC cluster was firmly attached to the cover slip, a single 0.5 mm^3 size cube of cell-embedded collagen hydrogel block was cut out and placed in the same well but in about 1 cm distance from the hNPC-loaded cover slip. Enough DMEM/F12 media were added to ensure that the collagen hydrogel block was fully submerged. Following culture at 37 °C in 5% CO_2, a morphological change as well as cellular marker expression was observed by confocal microscopy (Meta 700, Carl Zeiss, Oberkochen, Germany). The expressions of repulsive guidance molecules (RGMa and RGMb), netrin-1, and bFGF were also analyzed by RT-PCR (as shown in Table 1).

2.8. Statistical analysis

Statistical analysis was performed using Student's *t*-test comparing independent two sampling groups, and significance level was considered at $p < 0.05$.

3. Results

3.1. Flow cytometric identification of umbilical cord blood-derived cells

Flow cytometric analysis of UCB-derived cells showed a major cell population expressed stem cell markers Oct3/4, Sox2, and SSEA-4 (Fig. 1).

3.2. Profiling neurotrophic factor expression in umbilical cord blood-derived cells

Next, we performed RT-PCR analysis to examine neurotrophic factor expression in the UCB-derived cells. Fig. 2A showed a typical result of the RT-PCR analysis. There was significant expression of NT4, BDNF, NGF, and CNTF. More quantitatively, the percentage of NGF- and BDNF-positive cell population was estimated by flow cytometry. Our results revealed about 30% of the cell population expressed both NGF and BDNF (Fig. 2B). Fig. 2C showed typical immunofluorescent micrographs of UCB-derived cells after staining for NGF and BDNF. A significant portion of the cells were either NGF-positive cells or BDNF-positive.

3.3. Comparison of the 2-D with the 3-D culture of cells

We compared the 2-D culture of cells with the 3-D culture in terms of neurotropic factor expression. Fig. 3A showed a low magnification microscopic view of cells grown in a regular liquid culture media, which is referred to as 2-D culture, and those in a collagen hydrogel, which is referred to as 3-D culture. Apparently, there seemed to be no morphological difference between the cells in the 2-D and those in the 3-D culture, except the cell density. Overall, all the mRNA levels of BDNF, NGF, CNTF, NT3, NT4, and bFGF were somewhat increased in the 3-D culture compared to the 2-D culture until 12 days in culture. In particular, the expression of NGF and BDNF appeared to be increased the most in the 3-D (Fig. 3B). We also performed the Western blotting analysis to ensure the increase in NGF and BDNF with anti-NGF and anti-BDNF

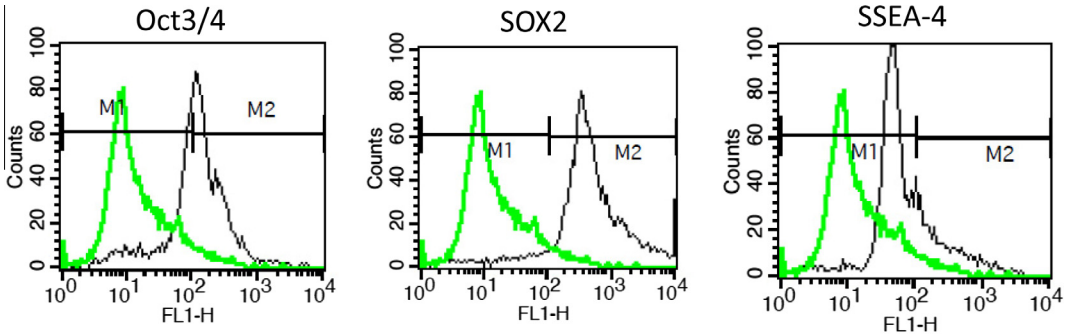

Fig. 1. Stem cell marker expression in umbilical cord blood-derived cells. The mononuclear cell fraction isolated from the umbilical cord blood by ficoll/hypaque gradient techniques was cultured in a plastic culture dish, and the cell population remained attached to the bottom of the dish were subjected to flow cytometry to examine the expression of Oct3/4, Sox2, and SSEA4. The green traces were from the cells after being incubated with secondary antibodies only; the black traces were from the cells after being incubated with primary antibodies and secondary antibodies, sequentially.

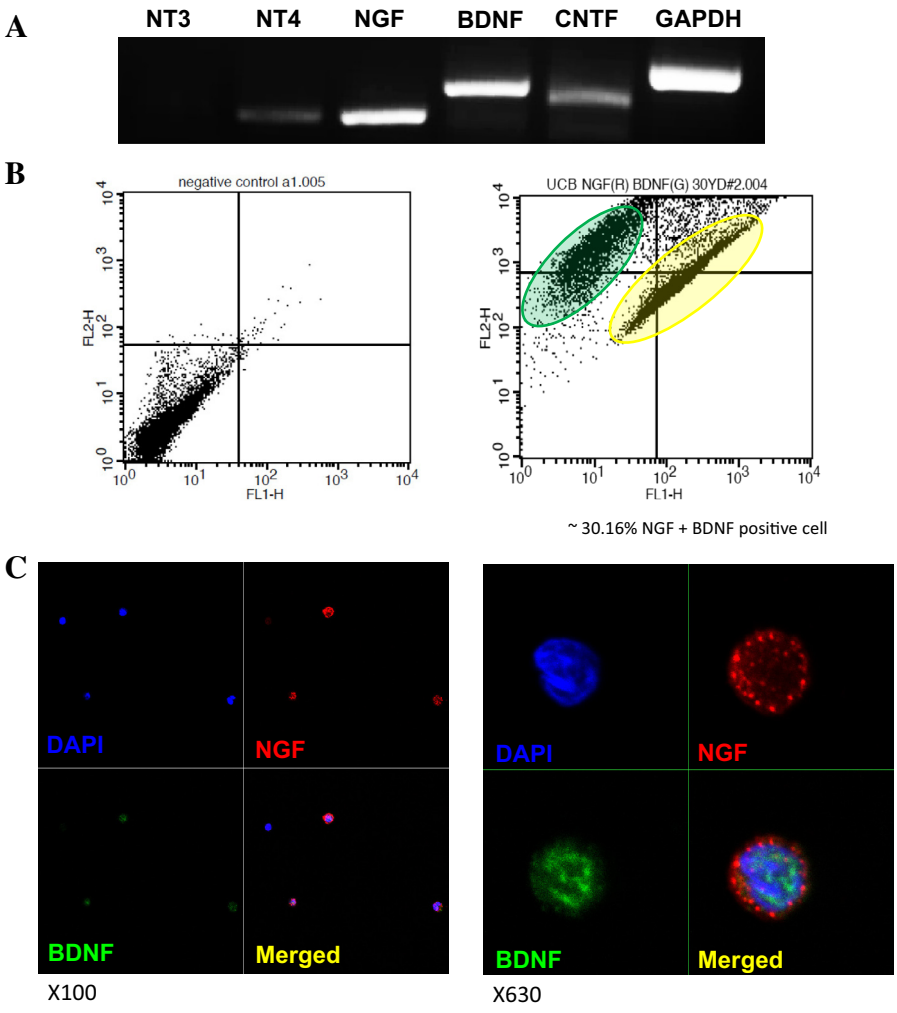

Fig. 2. Profiles of neurotrophic factor expression in umbilical cord blood-derived cells. (A) Total RNAs were isolated from the cell fraction and subjected to the RT-PCR analysis. NT3, neurotrophin-3; NT4, neurotrophin-4; NGF, nerve growth factor; BDNF, brain-derived neurotropic factor; and CNTF, ciliary neurotropic factor. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts were used as an internal control. (B) The NGF and BDNF-positive population was estimated by flow cytometry. The left panel represents the flow cytometic analysis data obtained after incubating the cells with the secondary antibodies only while the right panel represents results obtained after immunostaining with the primary and secondary antibodies. The *x*-axis represents the fluorescence from NGF and the *y*-axis represents that from BDNF. (C) Immunocytochemical staining for NGF and BDNF in cells. The left panel show the fluorescence micrographs at ×100 while the right panel shows the fluorescence micrographs at ×400. DAPI, nucleus staining; Merge, the superimposition of NGF, BDNF and DAPI.

antibodies. We found both NGF and BDNF were expressed in the cells but the intensity of BDNF appeared to be higher in 2-D culture than in 3-D while that of NGF was slightly higher in 3-D with respect to 2-D (Fig. 3C).

3.4. Ex vivo biological effects on human neural precursor cells

We conducted co-culture experiments to examine whether UCB-derived cells in a collagen gel were capable of stimulating neurite outgrowth of human neural precursor cells (hNPCs). Co-culture was carried out by placing UCB-derived cells embedded in a collagen hydrogel in the same culture dish with hNPCs so that two cell populations shared the culture media but no direct contact between. A hNPC cluster grown on a Matrigel-coated cover slip proliferated vigorously by expanding the neurites in all directions (Fig. 4A) without co-culture. However, in the co-culture, the neurite outgrowth was significantly enhanced and directional toward the UCB-derived cells (Fig. 4B). Contrasted to hNPCs cultured alone (upper panel, Fig. 4C), the expanding neurites in the co-culture were positive for βIII tubulin and NeuN (lower panel), suggesting there is a directional neurite-attracting cue generated from UCB-derived cells.

3.5. Directional cue for neurite outgrowth

We performed the RT-PCR analysis to search for directional cues for neurite outgrowth, including repulsive guidance molecule a (RGMa), repulsive guidance molecule b (RGMb), netrin-1, and bFGF in UCB-derived cells. Cells isolated from the collagen gel after 0–12 days in co-culture were subjected to the RT-PCR analysis with specific primers listed in Table 1. Before initiating the co-culture (day 0), cells expressed strong RGMb and weak netrin-1 signal. However, upon initiating the co-culture, the expression level of RGMa and netrin-1 were significantly up-regulated throughout the culture period (Fig. 4D), suggesting the possibility that the collagen gel provides directional cue(s) necessary for the neurite outgrowth.

4. Discussion

In this study, we have shown that various neurotrophic factors, including NGF and BDNF, were expressed from UCB-derived cells. Interestingly, the expression of neurotrophic factors was significantly enhanced by the presence of ambient environment

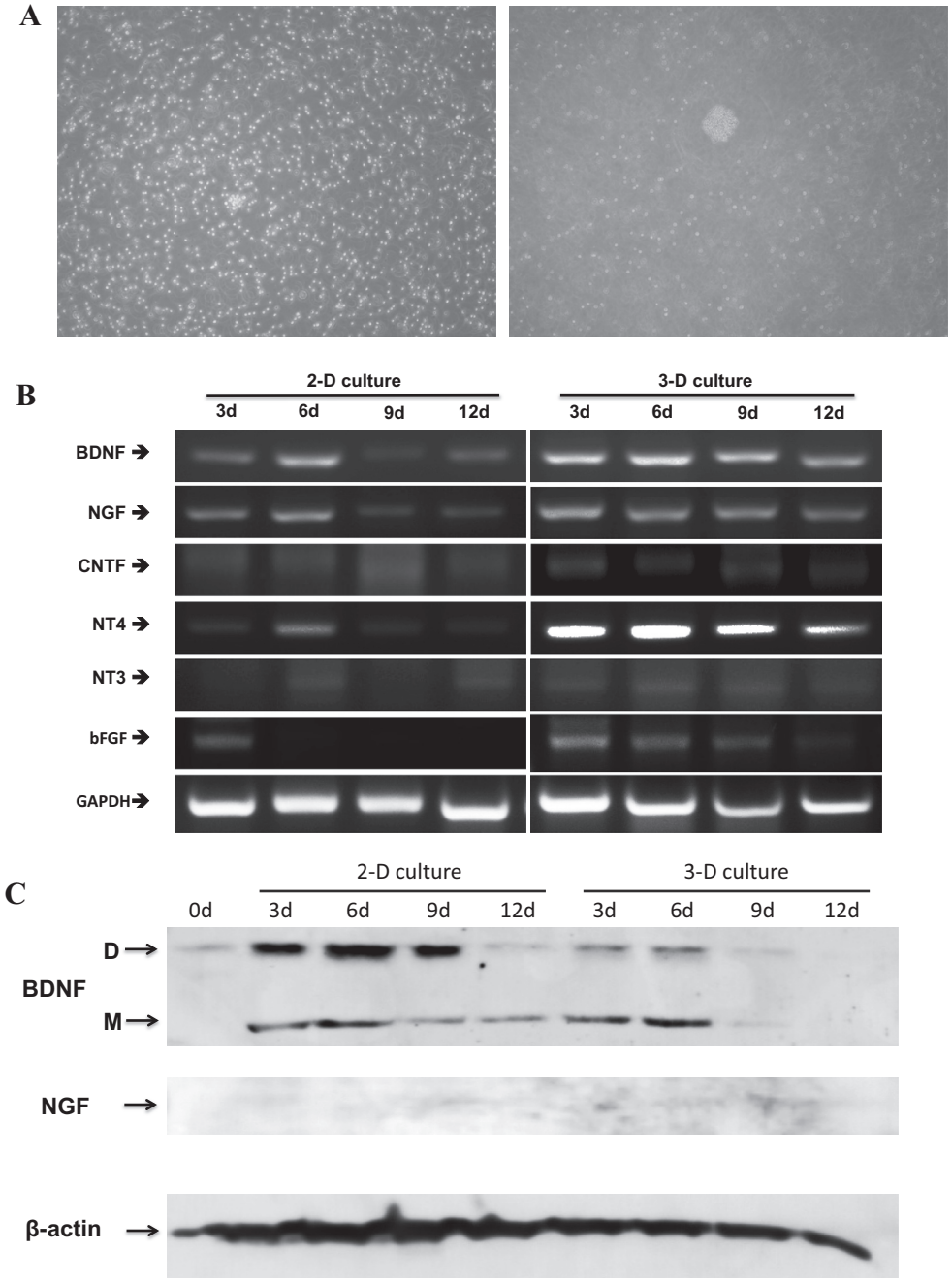

Fig. 3. Comparison of the 2-dimensional culture with the 3-dimensional culture of umbilical cord blood-derived cells. (A) A gross view of cells grown in the 2-D (left panel) and in the 3-D (right panel). A microscopic view of cells was taken after 3 days in culture at ×50. (B) RT-PCR analyses of neurotrophic factor mRNA expression in cells grown in the 2-D or in the 3-D. The cells were grown either in 2-D culture or in 3-D culture for 0–12cells days and subjected to the RT-PCR analysis. NT3, neurotrophin-3; NT4, neurotrophin-4; NGF, nerve growth factor; BDNF, brain-derived neurotropic factor; and CNTF, ciliary neurotropic factor; bFGF, basic fibroblast growth factor. Endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript was used as an internal control. (C) Western blotting analysis of NGF and BDNF expression. The cells were grown either in the 2-D culture or in the 3-D culture for 0–12 days and subjected to the Western blotting analysis. NGF, nerve growth factor (M and D refer to monomer and dimer, respectively); BDNF, brain derived neurotrophic factor; β-actin used as an internal control.

surrounding the cells such as the collagen hydrogel, suggesting that the collagen hydrogel might act as a cell-compatible environment that interacts with the cells thereby stimulating the expression and release of neurotropic factors. Furthermore, an ex vivo study by utilizing the co-culture with hNPCs showed that the neurotropic factors could be delivered to induce neural differentiation of hNPCs.

The 3-D environment was artificially established using a collagen-based hydrogel, which resulted in a significant increase in the expression of neurotrophic factors compared with the conventional 2-D culture. The 3-D culture condition may have provided bio-friendly environment similar to an in vivo extracellular matrix (ECM). Generally, the 3-D culture condition is more likely to allow cells to come in contact with ECM, creating a cell-friendly environment. Thus, the 3-D culture condition may provide a long-term stable cell–ECM interaction that, in turn, may enhance various cellular metabolic activities during the culture period. Therefore, the environment provided by the 3-D collagen hydrogel better mimics native tissue conditions, supplying appropriate physical and chemical cues that might be needed to regulate stem cell behaviors,

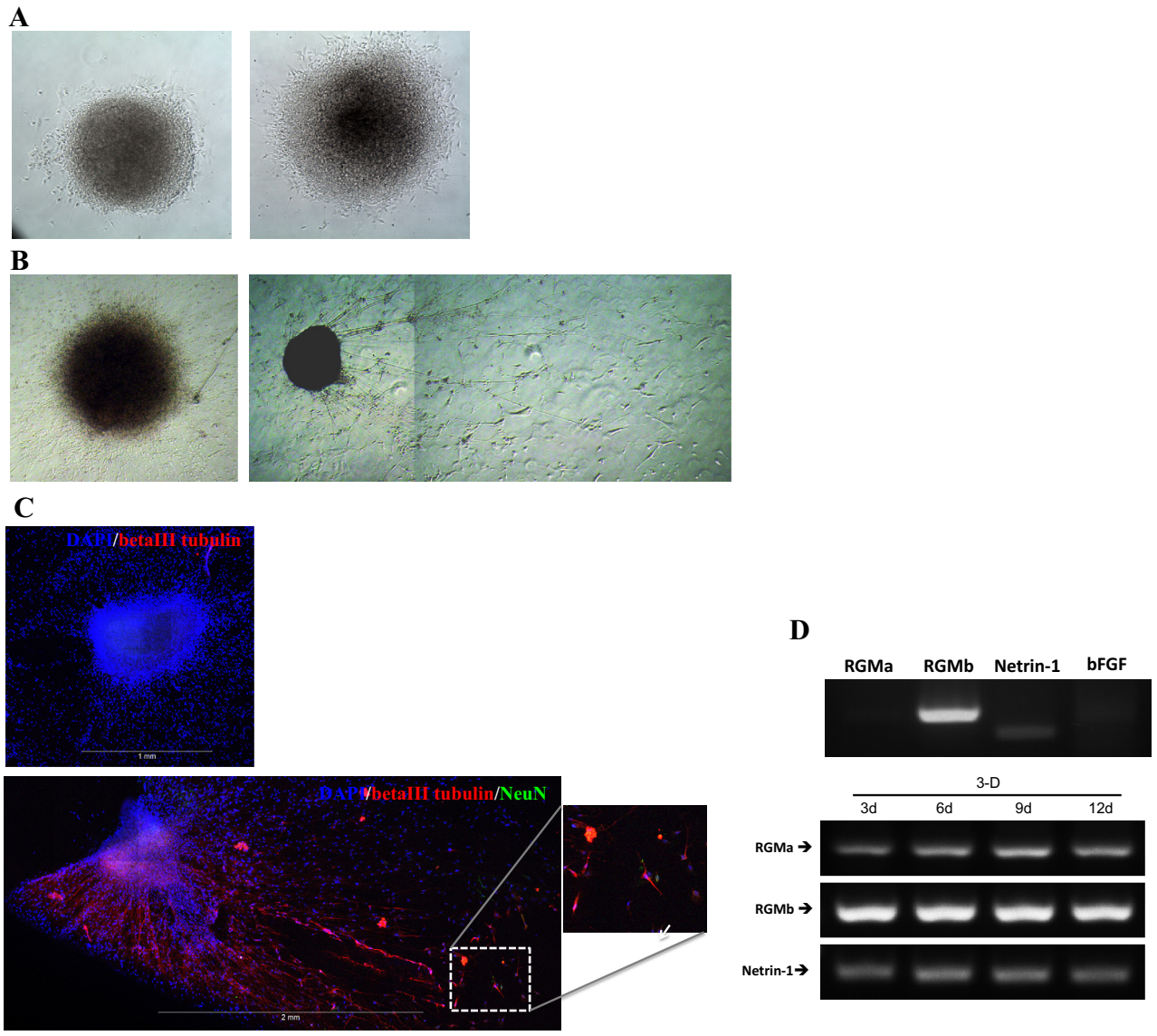

Fig. 4. Effects of neurotrophic factors from UCB-derived cells on the proliferation and differentiation of hNPCs. (A) Phase contrast microscopic images of hNPCs-derived neurospheres grown on the Matrigel-coated cover slip 1 day (left) and 6 days (right) after culture alone. (B) Phase contrast microscopic images of the hNPCs-derived neurospheres grown on the Matrigel-coated cover slip 1 day (left) and 6 days after co-culture with UCB-derived cells in the collagen hydrogel (right). Note a significant directional neurite outgrowth from the neurosphere in the co-culture. (C) Immunocytochemical detection of neural markers βIII tubulin and NeuN in the expanding neurites from hNPCs 6 days after culture alone (top) and after co-culture in the collagen gel (bottom). βIII tubulin in red; NeuN in green; DAPI nucleus staining in blue. (D) RT-PCR analysis of directional guidance cue molecules without co-culture(top) and with co-culture (bottom).

ranging from secretion of tropic factors and other soluble factors to providing mechanical supports. Along with soluble biochemical factors including growth factors, chemokines and hormones, adhesive molecules and mechanical cues such as matrix elasticity, surface topology and roughness have been considered as important determinants of stem cell behaviors [20,21]. We have focused on the expression of neurotrophic factors including NGF, BDNF, GDNF, NT3 and NT4. The mRNA levels, particularly those for NGF and BDNF, were expressed significantly higher when cultured in the collagen hydrogel than in the media.

In fact, neurotrophic factors are a family of proteins that regulate the proliferation, survival, and differentiation of neurons [22]. Some neurotrophic factors, especially BDNF and NGF are already in clinical trial for nerve generation against a dementia like Alzheimer diseases. However, neurotrophic factor therapy suffers from some drawbacks: one is requirement of very long period of treatment, and the other is dilution associated with its delivery to the target.

Therefore, the beneficial effects of collagen hydrogel on UCB-derived cells are considered twofold. One is to stimulate the secretion of neurotropic factors from the cells. The other is to provide a physical reservoir in which the secreted factors can be stored but delivered slowly to other cells nearby. Delivery of neurotropic factors to nearby NPCs was confirmed by an observation that hNPCs displayed neuritis outgrowth upon co-culturing with UCB-derived cells in a collagen hydrogel.

The neurite outgrowth is not a result of a simple expansion and proliferation of neuronal cells but entails the neuronal plasticity that is highly dependent on the microenvironment in vivo [23]. One of them is an involvement of guidance molecules that attract or repulse growing neurites depending on the nerve cell and receptor types. In our study, the expression of three neural guidance

molecules RGMa, RGMb [24], and netrin-1 [25] was greatly induced by the collagen hydrogel. The physical or chemical cues that induce the secretion of neural guidance molecules from UCB-derived cells have not been identified yet, but clearly the increase in those molecules could benefit the damaged yet regenerating neural tissues.

In conclusion, hUCB-derived cells were able to proliferate in the collagen hydrogel and express/secrete various neurotrophic factors and directional chemical cues. The main benefit of using the collagen hydrogel is to stimulate the secretion of neutropic factors andprovide them to nearby neural cells, which collectively would help to regenerate the damaged neural tissues. The present 3-D culture of UCB-derived cells in a collagen hydrogel therefore could provide a novel strategy for the stem cell-based therapy.

Acknowledgments

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