

Research Article

Efficient One-Step Induction of Human Umbilical Cord-Derived Mesenchymal Stem Cells (UC-MSCs) Produces MSC-Derived Neurospheres (MSC-NS) with Unique Transcriptional Profile and Enhanced Neurogenic and Angiogenic Secretomes

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Cell therapy has emerged as a promising strategy for treating neurological diseases such as stroke, spinal cord injury, and various neurodegenerative diseases, but both embryonic neural stem cells and human induced Pluripotent Stem Cell- (iPSC-) derived neural stem cells have major limitations which restrict their broad use in these diseases. We want to find a one-step induction method to transdifferentiate the more easily accessible Umbilical Cord-Derived Mesenchymal Stem Cells (UC-MSCs) into neural stem/progenitor cells suitable for cell therapy purposes. In this study, UC-MSCs were induced to form neurospheres under a serum-free suspension culture with Epidermal Growth Factor- (EGF-) and basic Fibroblast Growth Factor- (bFGF-) containing medium within 12 hours. These MSC-derived neurospheres can self-renew to form secondary neurospheres and can be readily induced to become neurons and glial cells. Real-time PCR showed significantly upregulated expression of multiple stemness and neurogenic genes after induction. RNA transcriptional profiling study showed that UC-MSC-derived neurospheres had a unique transcriptional profile of their own, with features of both UC-MSCs and neural stem cells. RayBio human growth factor cytokine array analysis showed significantly upregulated expression levels of multiple neurogenic and angiogenic growth factors, skewing toward a neural stem cell phenotype. Thus, we believe that these UC-MSC-derived neurospheres have amenable features of both MSCs and neural stem/progenitor cells and have great potential in future stem cell transplantation clinical trials targeting neurological disorders.

1. Introduction

Mesenchymal stem cells are adult stem cells derived from mesenchymal tissues. Human MSCs can be obtained from various sources such as bone marrow, umbilical cord, cord blood, adipose tissue, even the dental pulp [1–3]. They have great advantages of easy accessibility, easy manipulation, and low HLA typing restriction, com-

bined with their promising features of self-renewal and multipotency, making them the most commonly used adult stem cells in regenerative medicine. MSCs have been widely used in clinical trials for the treatment of diseases including hematological diseases, graft-versus-host diseases, diabetes, end-stage diseases in the liver, kidney, and lung, autoimmune diseases, and various neurological diseases [4–12].

There are several important limitations for current stem cell therapy trials using MSCs for the treatment of neurological diseases. First, for the treatment of neurological diseases, it would be clinically more relevant and efficient if we could use neural stem cells in these trials. But so far, human neural stem cells are extremely difficult to obtain due to ethical restrictions. Even if human neural stem cell can be obtained, the patient will need lifelong immunosuppressive agents [13]. Human umbilical cord-derived mesenchymal stem cells express little HLA antigen and hence can be safely used in a heterologous transplant setting [14], but here comes the second problem. Although MSCs have been shown to be efficiently induced to form neurons under certain induction methods in *ex vivo* experiments [15–19], when these cells are infused *in vivo* when they need *in vivo* microenvironment cues to transdifferentiate, they do not perform so well [20, 21]. It would therefore be ideal to find a cell source which combines the strength of both MSCs and neural stem cells, having both of their desirable features in one cell source.

In the present study, we reported for the first time that UC-MSCs can be efficiently induced to form neurosphere-like cells under standard culture conditions used for neurospheres (DMEM/F12, EGF, bFGF, N2, and B27) within 12 hours. These MSC-derived neurospheres can self-renew to form secondary neurospheres and can be readily induced to form neurons and glial cells. Real-time PCR showed significantly elevated expression of multiple neurogenic genes including *SOX2* and *NESTIN*, as well as moderately increased expression in certain stemness genes such as *OLIG2*, *OCT4*, and *BMI1* after induction. RNA sequencing analysis revealed that these UC-MSC-derived neurospheres have a distinctive transcriptional profile, different from both MSCs and human neural stem cells. Human growth factor analysis on these MSC-derived neurospheres showed that they had greatly enhanced expression in many neurogenic and angiogenic cytokines.

Therefore, these MSC-derived neurospheres represent a new source for neural stem/progenitor cells. They display self-renewal and multipotentialities comparable to neural stem/progenitor cells while still maintaining a low HLA restriction profile of typical MSCs. We believe that these cells have amenable features of both MSCs and neural stem/progenitor cells and will find themselves of tremendous use in future stem cell transplantation clinical trials for various neurological diseases.

2. Methods

2.1. Ethics Statement. All methods used in this study were carried out in accordance with the approved ethical guidelines of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (HUST), Wuhan, Hubei, China. The study protocol was approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology (HUST), Wuhan, Hubei, China. Informed consent was obtained from all subjects prior to the study.

2.2. Isolation and Characterization of UC-MSCs. Human umbilical cords were obtained from mothers (20–30 years old) planning on cesarean sections after obtaining written informed consent. UC-MSCs were isolated and characterized

as previously described [22, 23]. For osteogenic differentiation, UC-MSCs were cultured in DMEM/F12 (Lonza, Walkersville, MD, USA) supplemented with 10% FBS, 0.1 mM dexamethasone, 10 mM β -glycerophosphate, and 50 mM ascorbic acid (all from Sigma-Aldrich) for 3 weeks and then assayed by Alizarin Red (Sigma-Aldrich) staining. For chondrogenic differentiation, UC-MSCs were induced by the chondrocyte differentiation basal medium (Gibco, USA) for 3 weeks and then assayed by 1% Alcian Blue (Gibco) staining to detect the synthesis of proteoglycans by chondrocytes. UC-MSCs were continuously passaged until passage 30. UC-MSCs at P5, P10, P15, and P30 were subjected to karyotyping and neurosphere formation assay.

2.3. Flow Cytometry Analysis. Cultured UC-MSCs were trypsinized (0.25% trypsin-EDTA), washed twice with PBS (pH = 7.4), and suspended in PBS at a concentration of 5×10^6 /ml, and then 1 ml sample was incubated with fluorescein isothiocyanate- (FITC-) conjugated monoclonal rabbit anti-human CD13, CD14, CD44, CD90, CD105, CD34, HLA-DR (BD Biosciences, San Diego, CA, USA), or isotype control for 30 min at 4°C according to the recommendations of the manufacturer. The stained cells were analyzed using a standard Becton-Dickinson FACS Aria instrument and the CellQuest Pro Software (BD Biosciences). MSC-derived neurospheres (Days 1, 3, 5, and 7 postinduction) were dissociated into a single-cell suspension and subjected to the same multicolor flow cytometry analysis.

2.4. Immunohistochemistry Staining. UC-MSCs were washed with PBS and fixed with 4% paraformaldehyde; then, they were incubated for 12 hours overnight at 4°C with the following antibodies: rabbit anti-nestin mAb (Abcam, at final concentrations of 1/250), mouse anti-Tuj1 mAb (Abcam, at final concentrations of 1/300), and mouse anti-GFAP mAb (Abcam, at final concentrations of 1/300), respectively. Primary antibodies were developed with secondary Alexa 488-goat anti-rabbit IgG and Alexa 546-rat anti-mouse IgG, both at final concentrations of 1/500. Secondary antibodies were incubated for one hour at room temperature in the dark. Cell nuclei were counterstained with Dapi. After labeling, the cells were covered with antifade mounting medium. Slides were immediately examined on a three-color immunofluorescence confocal microscope (Nikon Instruments Inc.).

2.5. Generation of Neurospheres from UC-MSCs. UC-MSCs were dissociated with 0.25% trypsin/0.04% EDTA, washed twice with PBS, and plated on T25 plastic tissue culture flasks (Nalge Nunc International, Rochester, NY, USA) at a concentration of $1.5-2 \times 10^5$ cells/cm² in neurosphere culture medium containing DMEM/F12 with 20 ng/ml of both epidermal growth factor (EGF; PeproTech, London, UK) and basic fibroblast growth factor (bFGF; R&D Systems, Inc., Minneapolis, MN) and N2 and B27 supplements (Invitrogen, Carlsbad, CA, USA). The same protocol was also used for the generation of neurosphere from extensively passaged MSCs, namely, passages 10, 20, and 30. At any given timepoint, neurospheres were enumerated under a 100x microscope for 6 random fields. For the apoptosis assay,

UC-MSC-derived neurospheres were digested with 0.25% trypsin/0.04% EDTA for 5 minutes at 37°C and continuous pipetting using 200 μ l pipetting tips to ensure that a single-cell suspension was achieved under a microscope. After spinning down, dissociated neurospheres (10^5 cells) were resuspended in 500 μ l binding buffer. Then, they were incubated for 5 min at room temperature in the presence of 0.5 μ g/ml Annexin V-FITC (R&D Systems, Inc.) and PI in binding buffer as described by the manufacturer. The percentage of apoptosis was determined by FACS.

2.6. Secondary Neurosphere Formation Assay. Primary UC-MSC-derived neurospheres from T25 tissue culture flasks were pooled and digested with 0.25% trypsin/0.04% EDTA for 5 minutes at 37°C and continuous pipetting using 200 μ l pipetting tips to ensure that a single-cell suspension was achieved under a microscope. After that, cells were resuspended in neurobasal medium containing DMEM/F12 with 20 ng/ml of both epidermal growth factor (EGF; R&D Systems, Inc., Minneapolis, MN) and basic fibroblast growth factor (bFGF; R&D Systems, Inc., Minneapolis, MN) and N2 and B27 supplements (Gibco). One thousand MSCs were then distributed into each well of 24-well tissue culture plates (Nalge Nunc International, Rochester, NY, USA), and positive neurosphere formation was scored after 5 days. Secondary neurosphere formation capabilities were measured by a percentage of positive neurosphere formation. For a clonality assay, MSC-derived neurospheres were dissociated into single cells and replated in 96-well plates (Nalge Nunc International, Rochester, NY, USA) at clonal density (100 cells were plated in each well using limiting dilution) with replenishment of EGF and bFGF every 3 days. Positive neurosphere formation was scored after 7 days.

2.7. Multidifferentiation Assay of MSC-Derived Neurospheres. On the third day after MSC-derived neurosphere formation, these neurosphere-like structures were plated (5-10 neurospheres per well) on poly-L-lysine and laminin (both from Sigma-Aldrich) double-coated coverslips on six-well chambers and cultured in DMEM supplemented with 2% fetal bovine serum (HyClone, Logan, Utah, USA). 7-10 days after induction, neurogenic differentiation was assayed by immunofluorescence staining for neural- and glial-specific protein expression as described earlier.

2.8. Quantitative Real-Time PCR and RNA Sequencing. RNA was isolated from UC-MSCs before and after neurosphere induction using the TRIzol Reagent (Invitrogen, Carlsbad, CD, USA). cDNA was transcribed using SuperScript III First-Strand cDNA Synthesis Kit following the manufacturer's instructions (Invitrogen, Carlsbad, CD, USA). Primers used for real-time PCR are summarized in Table 1. A commercially available human iPSC-derived neural stem cell line (NouvNeu hNSC, NC0001, iRegene, China) was used as a positive control. Quantitative real-time PCR was performed with SYBR Green PCR reagents on an ABI Prism 7300 detection system (Applied Biosystems, Foster City, CA). GAPDH was used as an internal control. The normalized fold expression was obtained using the $2^{-\Delta\Delta CT}$ method.

For RNA sequencing, RNA from UC-MSCs before and after neurosphere induction (48 hours after induction) was extracted by using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The same human neural stem cell line was used as a positive control. RNA sequencing was performed by using a NextSeq 550 (Illumina) set for 76 cycles in single end (SE), yielding an average of 4.7×10^7 total clean reads for each sample with an average mapping rate of 97.7%. Sequences were aligned using STAR (version 2.5.3a); association between reads and genes has been performed by feature counts, using GENCODE (version 28) basic annotation as reference. Analysis of count data was performed using the DESeq2 (differential gene expression analysis based on the negative binomial distribution) pipeline (version 1.6.3).

2.9. RayBio Human Growth Factor Cytokine Analysis. UC-MSCs at passage 5 were induced to form neurospheres as described previously in Section 2.5. After neurosphere formation, the supernatant was collected at 1-, 3-, 5-, and 7-day postinduction timepoints, centrifuged at 1000 rpm for 5 minutes, and filtered through a 0.22 μ m syringe filter. 1 ml medium was assayed by RayBio® Biotin Label-Based Human Growth Factor Cytokine Array I (Cat#: QAH-GF-1-1 Human Growth Factor Array, Norcross, GA), respectively, which can detect the expression levels of 40 human growth factors in cell culture supernatants simultaneously. Commercially available human iPSC-derived neural stem cells (NouvNeu hNSC, NC0001, iRegene, China) were used as a positive control. UC-MSCs at passage 5 were grown to subconfluency, and the medium was switched to DMEM/F12 only. UC-MSCs were continued to culture for 48 hours, and the supernatant was collected and also assayed by RayBio® Biotin Label-Based Human Growth Factor Cytokine Array I (QAH-GF-1-1 Human Growth Factor Array, Norcross, GA).

2.10. Statistical Analysis. All values are expressed as mean \pm SD. Comparisons between two groups were analyzed by Student's *t*-test, and comparisons between more than two groups were analyzed by one-way analysis of variance (ANOVA) followed by either a Dunnett or a Tukey post hoc test. *p* values < 0.05 were considered to indicate statistical significance. *p* values < 0.01 were considered statistically very significant. All analyses were performed using GraphPad Prism 8 (San Diego, CA).

3. Results

3.1. Characterization of UC-MSC. Human umbilical cord-derived mesenchymal stem cells (UC-MSC) were isolated and characterized by a multidifferentiation assay as described previously [22]. Representative flow cytometric analysis of MSC-specific cell surface markers is shown in Figure 1.

3.2. UC-MSCs Can Be Efficiently Induced to Form Neurosphere-Like Structures within 12 Hours of Induction. When cultured under serum-free suspension culture using standard neurobasal medium (DMEM/F12, EGF, and bFGF 20 mg/ml, with N2 and B27 supplements), UC-MSCs can be efficiently induced to form neurosphere-like structures within 12 hours. As early as 4-6 hours after converting culture

TABLE 1: Primers used for real-time PCR.

Primer name	Forward primer sequence	Reverse primer sequence
Nestin	CAGCGTTGGAACAGAGGTTGG	TGGCACAGGTGTCTCAAAGGGTAG
Nanog	CAAAGGCCAAACAACCCACTT	TCTGGAACCAGGTCTTCACC
Oct4	GATCCTCGGACCTGGCTAAG	GACTCCTGCTTCACCCTCAG
Sox2	GCCGAGTGAAACTTTGTGCG	GGCAGCGTGTACTTATCCTTCT
Bmi1	CGTGTATTGTTTCGTTACCTGGA	TTCAGTAGTGGTCTGGTCTTG
Olig2	CCAGAGCCCGATGACCTTTTT	CACTGCCTCCTAGCTTGTCC
GFAP	CGAAGCCAACGACTACCG	TCTTCACCACGATGTTCTCTC
Klf4	GGCTGATGGGCAAGTTCCG	TTGGCTTGGGCTCCTCTGG
MAP2	GGGCCTTTCTTTGAAATCTAGTTT	CAAATGTGGCTCTCTGAAGA
PAX6	GCTTCACCATGGCAAATAACC	GGCAGCATGCAGGAGATATGA
β -Tubulin	CTCAGGGGCCTTTGGACATC	CAGGCAGTCGAGTTTTTCAC
hGAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCAT

medium into neurobasal medium, quick clustering of UC-MSCs into sphere-like structures were seen (Figure 2(a)). Within 24 hours of converting culture medium into neurobasal medium, almost all UC-MSCs formed neurosphere-like cells (Figure 2(a)). Apart from the UC-MSC-derived neurosphere-like cells, there were also some loosely formed cell clusters formed at the same time. These cell clusters were easily distinguishable from neurospheres because they were usually much smaller and they did not have the clear and round border of typical neurospheres, as illustrated by blue arrowheads in Figure 2(b).

We next wanted to know more about the neurosphere formation process. We tested several different conditions: (1) complete neurobasal medium which is comprised of DMEM/F12, EGF 20 ng/ml, bFGF 20 ng/ml, and N2 and B27 supplements; (2) EGF and bFGF only: DMEM/F12, EGF 20 ng/ml, and bFGF 20 ng/ml, without N2 and B27 supplements; (3) half regimen: DMEM/F12, EGF 10 ng/ml, and bFGF 10 ng/ml, with N2 and B27 supplements; (4) EGF only regimen: DMEM/F12, EGF 20 ng/ml, and no bFGF, with N2 and B27 supplements; (5) bFGF only regimen: DMEM/F12, bFGF 20 ng/ml, and no EGF, with N2 and B27 supplements; (6) N2 only regimen: DMEM/F12 with N2 supplement only, no EGF or bFGF; and (7) B27 only regimen: DMEM/F12 with B27 supplement only, no EGF or bFGF. We found that standard neurobasal medium induction led to the most prominent neurosphere-like structure production. Interestingly, EGF and bFGF were not indispensable. DMEM/F12 and N2 and B27 supplements were already enough to induce neurosphere formation. But N2 supplement only was not enough to induce neurosphere formation. EGF only regimen induced mainly large- and medium-sized neurospheres, whereas bFGF only regimen induced mainly medium- to small-sized neurospheres. Representative images are shown in Figure 2(b). The statistical results are summarized in Figure 2(c).

3.3. UC-MSC-Derived Neurospheres Can Self-Renew and Can Be Induced to Form Neurons and Glial Cells. To confirm if these MSC-derived neurospheres are indeed neural progenitor cells rather than a culturing artifact, MSC-derived neurospheres were subjected to self-renewal and multipotency

tests. For a self-renewal assay, UC-MSC-derived neurospheres were dissociated into single cells and replated in a T25 tissue culture flask. Secondary neurospheres can be visualized within 24-48 hours, as is shown in Figure 3(a). If plated at clonal density in a 24- or 96-well plate for secondary neurosphere formation, secondary neurospheres can be seen formed within 5-7 days.

For a multipotency assay, neurospheres were plated on six-well plates with poly-L-lysine and laminin double-coated coverslips and cultured in DMEM supplemented with 2% fetal bovine serum. 7 days after induction, differentiated neurospheres were positive for neural- (β -tubulin III, Figure 3(b)) and glial- (GFAP, Figure 3(c)) specific protein expression.

3.4. Real-Time PCR Showed Upregulation of Multiple Stemness and Neurogenic Genes. UC-MSCs already expressed multiple stemness genes (*Oct4*, *Nanog*, *Bmi1*, *Olig2*, etc.) and a number of neurogenic genes such as *Nestin*, *Pax6*, and *Sox2*, as assayed by reverse transcriptase PCR (Figure 4(a)), similar to human neural stem cells (NouvNeu Human Neural Stem Cells, NC0001, iRegene, China). Quantitative real-time PCR further showed the upregulation of multiple stemness and neurogenic genes after induction. The expression levels of pluripotent genes *Olig2*, *Bmi1*, and *Oct4* were modestly increased (5- to 10-fold) from as early as 24 hours post-induction compared to preinduction. The expression of neurogenic genes *Sox2* and *Nestin* were both dramatically increased (40-50 times higher just 48 hours after the induction for *Sox2* and 15 times higher for *Nestin*). Interestingly, the expression of *LIF*, or *Leukemia Inhibitory Factor*, also increased 10- to 60-fold from as early as 24 hours postinduction compared to preinduction (Figure 4(b)).

3.5. RNA Sequencing Revealed a Distinctive Profile of MSC-Derived Neurospheres. We are interested to know the true identity of these MSC-derived neurospheres. To answer this question, we did RNA sequencing analysis and compared the gene expression profiles of UC-MSCs before induction and 48 hours after induction with the profile of commercially available human neural stem cells. RNA sequencing analysis showed that 5,579 genes were upregulated and 2,539 genes

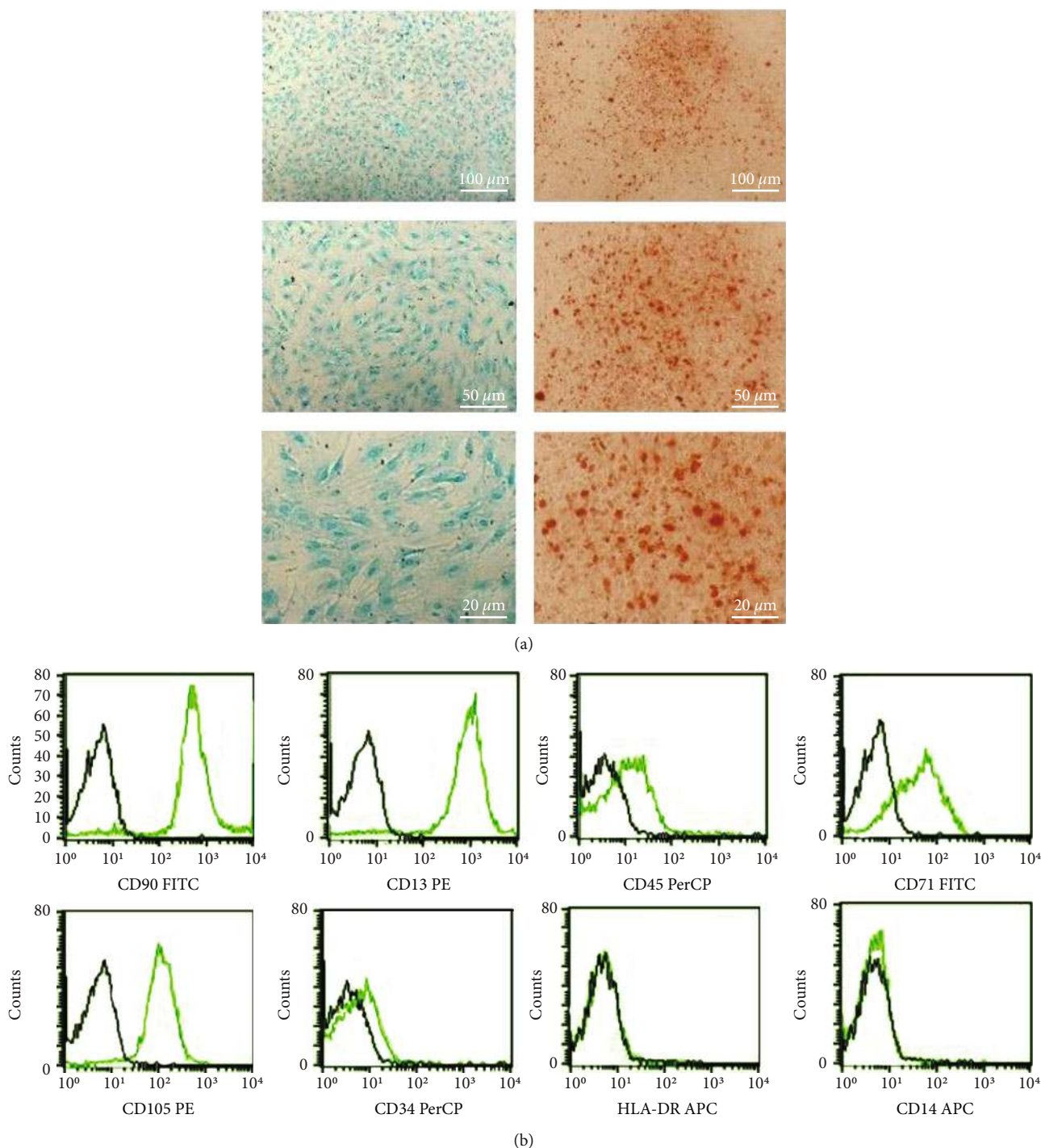


FIGURE 1: Characterization of UC-MSC. (a) Osteogenesis was assessed by Alizarin Red staining, and chondrogenesis was assessed by Alcian Blue staining. (b) Representative cytometric analysis of UC-MSCs at passage 3. Black-lined histograms represent isotype-matched controls. UC-MSCs are positive for CD90, CD13, CD71, and CD105 and negative for CD14, CD34, CD45, and HLA-DR.

were downregulated as early as 48 hours after induction. Gene enrichment analysis indicated that genes involved with the regulation of nervous system development, regulation of neurogenesis, and stem cell population maintenance were significantly enriched after the induction (Figure 4(c)). The overall transcriptional profile of UC-MSC-derived neurospheres distinguishes clearly from UC-MSCs, but it is also different from neural stem cells. These UC-MSC-derived

neurospheres seem to have a unique transcriptional profile of their own, with features of both UC-MSCs and neural stem cells.

3.6. Dynamic Changes in Expression of UC-MSC-Specific Cell Surface Markers after Induction. Multicolor flow cytometry analysis was performed on dissociated neurosphere cells at 1, 3, and 5 days after the induction as well

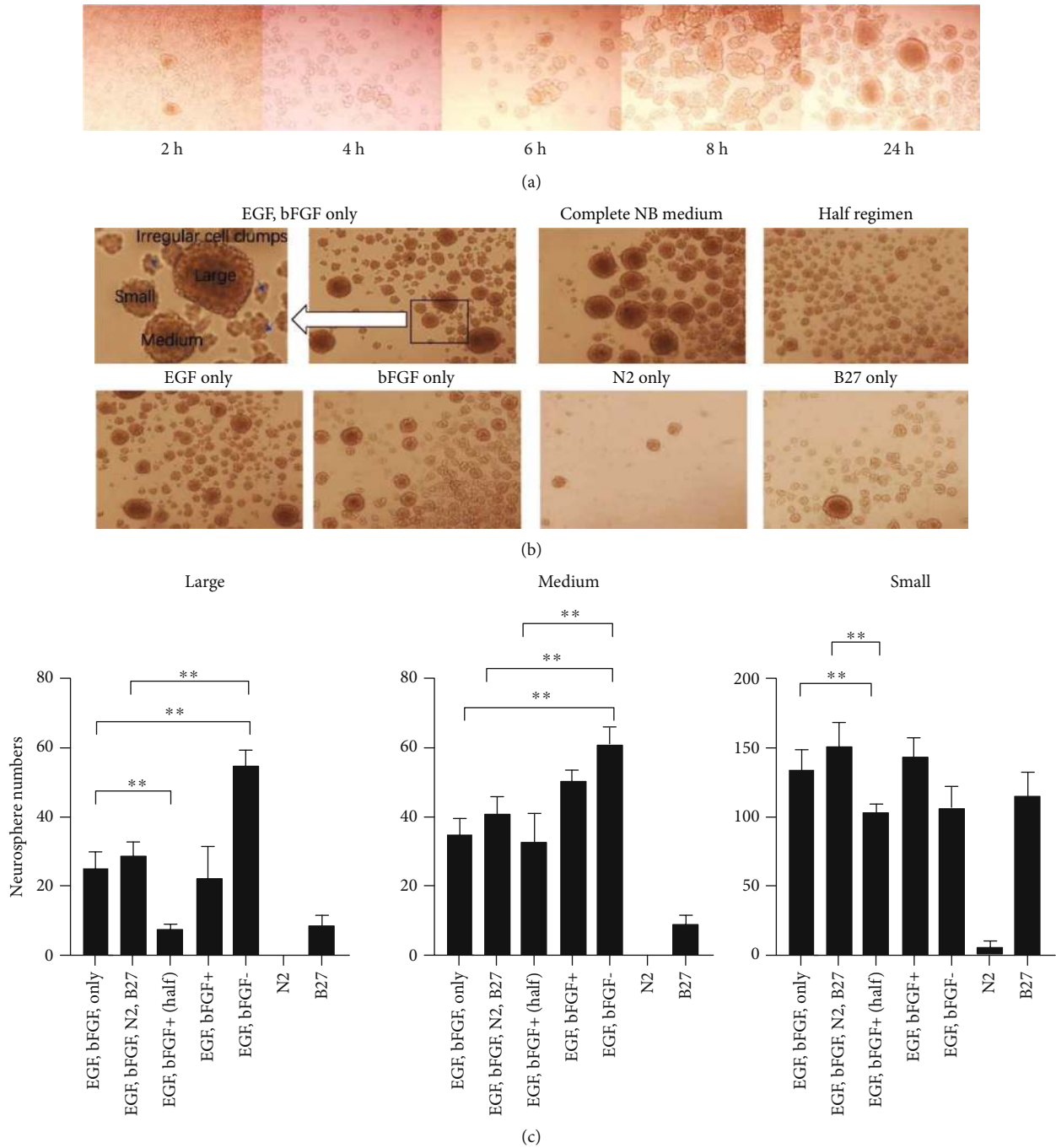


FIGURE 2: UC-MSC-derived neurospheres can be generated using one-step induction. (a) UC-MSC-derived neurosphere formation at different timepoints: 2 hours, 4 hours, 6 hours, 8 hours, and 24 hours after induction. (b) Representative phase contrast images (100x) of UC-MSC-derived neurospheres formed under different regimens, including (1) EGF, bFGF only regimen: EGF and bFGF, both at 20 ng/ml; (2) complete NB (neurobasal) medium regimen (EGF and bFGF, both at 20 ng/ml, with N2 and B27 supplements); (3) half regimen: EGF and bFGF, both at 10 ng/ml, with N2 and B27 supplements; (4) EGF only regimen: EGF 20 ng/ml, no bFGF, with N2 and B27 supplements; (5) bFGF only regimen: bFGF 20 ng/ml, no EGF, with N2 and B27 supplements; (6) N2 only regimen: DMEM/F12 with N2 supplement only, no EGF nor bFGF; and (7) B27 only regimen: DMEM/F12 with B27 supplement only, no EGF nor bFGF. Enlarged image of the black-boxed region denotes the arbitrary classification of large-, medium-, and small-sized neurospheres. Blue arrows denote irregular cell clumps. (c) Statistical analysis of large-, medium-, and small-sized neurospheres produced under different induction regimens. Neurospheres were scored in 6 random fields under the microscope at 100x magnification. $n = 3$. ** $p < 0.01$.

as on UC-MSCs before the induction. Significant downexpression of CD71 and CD105 was seen starting from Day 3 after the induction (Figure 5). CD90 and CD13 first

showed a sharp decrease at Day 3 but regained their expressions at Day 5. The expression of HLA-DR remained negative during this whole process.

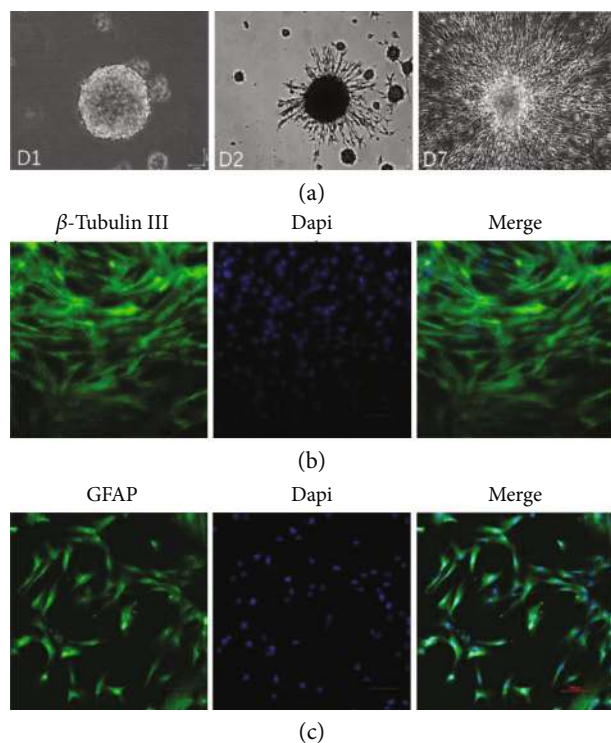


FIGURE 3: Self-renewal and multipotency of UC-MSC-derived neurospheres. (a) Secondary neurosphere formation 24 hours after the dissociation of primary UC-MSC-derived neurospheres, phase contrast image. D2 denotes the differentiation of a UC-MSC-derived secondary neurosphere at day 2 in 2% fetal bovine serum (FBS) differentiation medium on poly-L-lysine and laminin double-coated coverslips. D7 denotes a fully differentiated secondary neurosphere at Day 7 after induction in 2% FBS differentiation medium on poly-L-lysine and laminin double-coated coverslips. (b and c) UC-MSC-derived secondary neurospheres were induced to differentiate into neurons and glial cells using 2% fetal bovine serum induction on poly-L-lysine and laminin double-coated coverslips. Confocal microscopic images of β -tubulin III (b) and GFAP (glial fibrillary acidic protein) immunohistochemistry staining (c) of differentiated UC-MSC-derived secondary neurospheres at day 7 after induction are shown. Dapi represents nucleic staining. Red scale bar = 100 μ m.

3.7. Significantly Upregulated Cytokine Expression Profile after Induction. RayBio human growth factor array analysis was performed to access the secretome of UC-MSCs before and after the induction. The secretome was compared with commercially available human neural stem cells (NouvNeu Human Neural Stem Cells, NC0001, iRegene, China). An overall significant increase in the majority of the human growth factor cytokine expression levels was seen after the induction, among which VEGF was the most prominent with about 300- to 550-fold change beginning as early as 24 hours after the induction. UC-MSCs express very little VEGF, but upon induction, the VEGF level increased to 374.17-, 559.77-, 539.44-, and 535.77-fold on Days 1, 3, 5, and 7, respectively. Among other cytokines which exhibited a significant increase were EGF, bFGF, AR (amphiregulin, a member of the EGF family), insulin, HGF (hepatocyte growth factor), OPG (osteoprotegerin), IGF (insulin-like

growth factor), and GDNF (glial cell line-derived neurotrophic growth factor). In comparison to UC-MSCs before induction, the human growth factor profile is more skewed toward human neural stem cell profile, as is shown in Table 2 and Figure 6(a).

4. Discussion

In this study human UC-MSCs were induced to form neurosphere-like structures under a neurobasal medium culture condition within 12 hours. These findings came as a rather unexpected finding. There are several important features for this simple induction method: (1) The induction is immediate, which takes place usually within several hours. (2) The induction is highly efficient. Usually 80-92% of the MSCs can be induced to form neurospheres. (3) The induction method is simple and well defined. Only standard neurosphere culture medium is used. There is no need to overexpress or downregulate certain genes using retroviral vector intervention, and there is even no need for using a low-attachment plastic cell culture flask for neurosphere formation.

Due to the relative ease of achieving this transformation, the first and foremost question we need to answer is whether this is a culturing artifact during ex vivo culture. We reasoned that if this quick transdifferentiation is only an artifact, we should not see any significant RNA expression profile change during this process. We checked the expression of multiple neurogenic genes and stemness genes. To our surprise, UC-MSCs already expressed a wide variety of neurogenic genes such as *Sox2*, *Nestin*, and *Pax6*. When we compared the gene expression levels before induction to those at days 1, 3, 5, and 7 postinduction by real-time PCR, we found that the expression levels of pluripotent genes *Olig2* and *Bmi1* were modestly increased (5- to 10-fold) from as early as 24 hours postinduction compared to preinduction, whereas the expression of *Sox2*, a well-known neurogenic transcriptional factor, was dramatically increased (45 times higher just 48 hours after the induction). The same is true for *Nestin*. The expression level of another gene, *LIF*, or *Leukemia Inhibitory Factor*, also increased 10- to 60-fold from as early as 24 hours postinduction compared to preinduction.

To get a complete picture of the transcriptional profile change during this induction, we did RNA sequencing. We compared the RNA expression pattern of UC-MSCs before induction to UC-MSC-derived neurospheres at 48 hours postinduction. RNA sequencing analysis showed that 5,579 genes were upregulated and 2,539 genes were downregulated. Gene enrichment analysis indicated that genes involved with the regulation of nervous system development, regulation of neurogenesis, and stem cell population maintenance were significantly enriched after the induction. Thus, these MSC-derived neurospheres are not culturing artifacts and this is a true transdifferentiation process. We propose that UC-MSCs are naturally proneurogenic. They already express many stemness genes, and they also express the most important transcription factors (*Nestin*, *Sox2*, *Pax6*, etc.) which are readily switched on upon induction. On the other hand, UC-MSCs are heterogenous in terms of stemness gene and

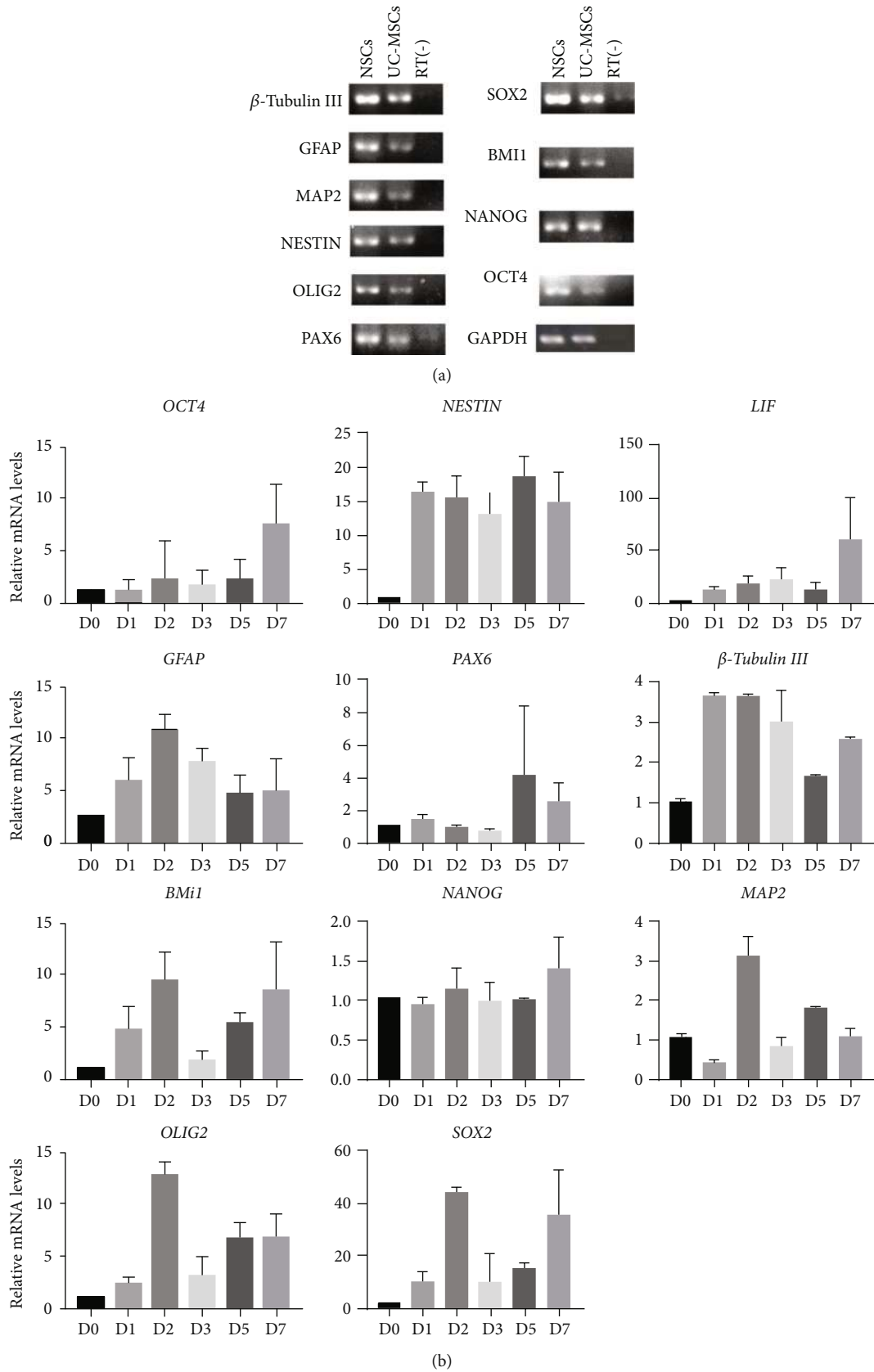


FIGURE 4: Continued.

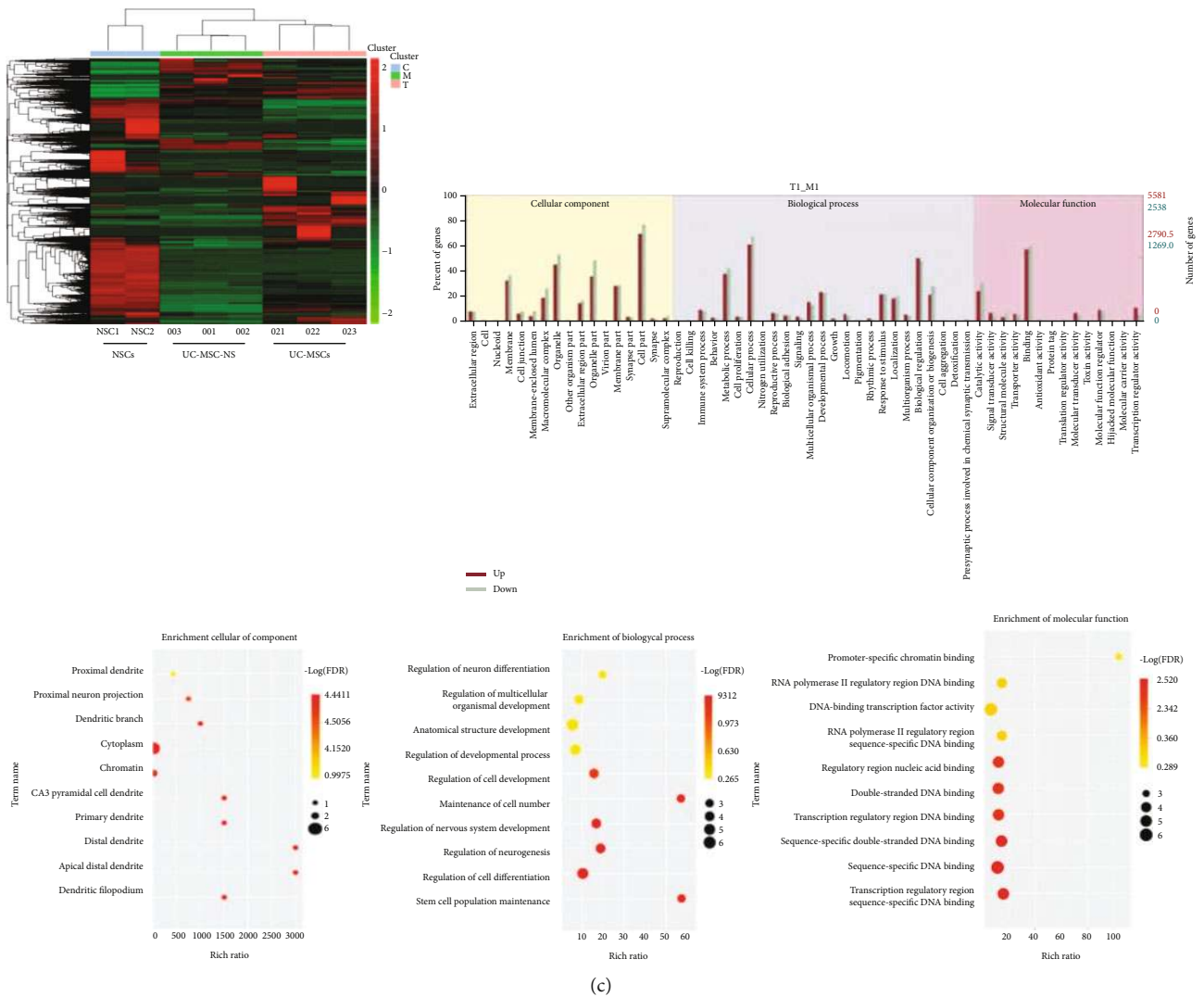


FIGURE 4: Transcriptional profiling analysis after neurobasal medium induction in UC-MSCs. (a) Results of reverse transcriptase PCR. Human NSCs (NouvNeu hNSC, NC0001) were used as a positive control. RT(-): no reverse transcriptase negative control. (b) Quantitative real-time PCR shows that neurosphere medium induction leads to significant overexpression of stemness and neurogenic genes as early as 24 hours after the induction. D0, UC-MSCs before induction; D1, 2, 3, 5, and 7 represent the RNA expression levels at Days 1, 2, 3, 5, and 7 after the induction. The expression of genes was normalized to that of GAPDH. Triplicate PCR amplifications were performed for each sample, and the results are represented as mean values \pm SD of three triplicate samples. (c) RNA sequencing analysis of UC-MSCs before and 48 hours after neurosphere formation. Heatmap represents supervised clustering analysis of transcriptional profiles which sharply distinguishes UC-MSCs (D001, D002, and D003) from UC-MSC-derived neurospheres (D21, D22, and D23) and human neural stem cells (NSC1 and NSC2). GO annotation map shows that genes involved with the regulation of nervous system development, regulation of neurogenesis, and stem cell population maintenance were significantly enriched after the induction.

neurogenic gene expression. The neurobasal medium we used for induction (mainly EGF and bFGF) likely initiated further a selection process so that those cells with higher levels of stemness gene and/or neurogenic gene expression are preferably expanded and gain dominance during the induction, whereas those cells with a relatively lower level of expression quickly go through apoptosis, as is illustrated in Figures 6(b) and 6(c). The serum-free suspension culture seems to facilitate this selection as well. This theory can explain the highly efficient transdifferentiation process, as we demonstrated in this study. However, the overall transcriptional profile of UC-MSC-derived neurospheres seems

to have features of both UC-MSCs and neural stem cells. We believe that these UC-MSC-derived neurospheres represent a unique entity by themselves. These cells can be dissociated into single cells. They can form secondary neurospheres, and they can be passaged and further propagated for at least 3 passages, just like human neural stem cells.

Some people might argue with our findings and suggest another possibility, which is the existence of a rare bipotent or totipotent stem cell subpopulation within MSCs which is predisposed to form neural stem cells [24]. However, our findings argue against this possibility. First of all, the success rate for UC-MSC-derived neurosphere formation is between

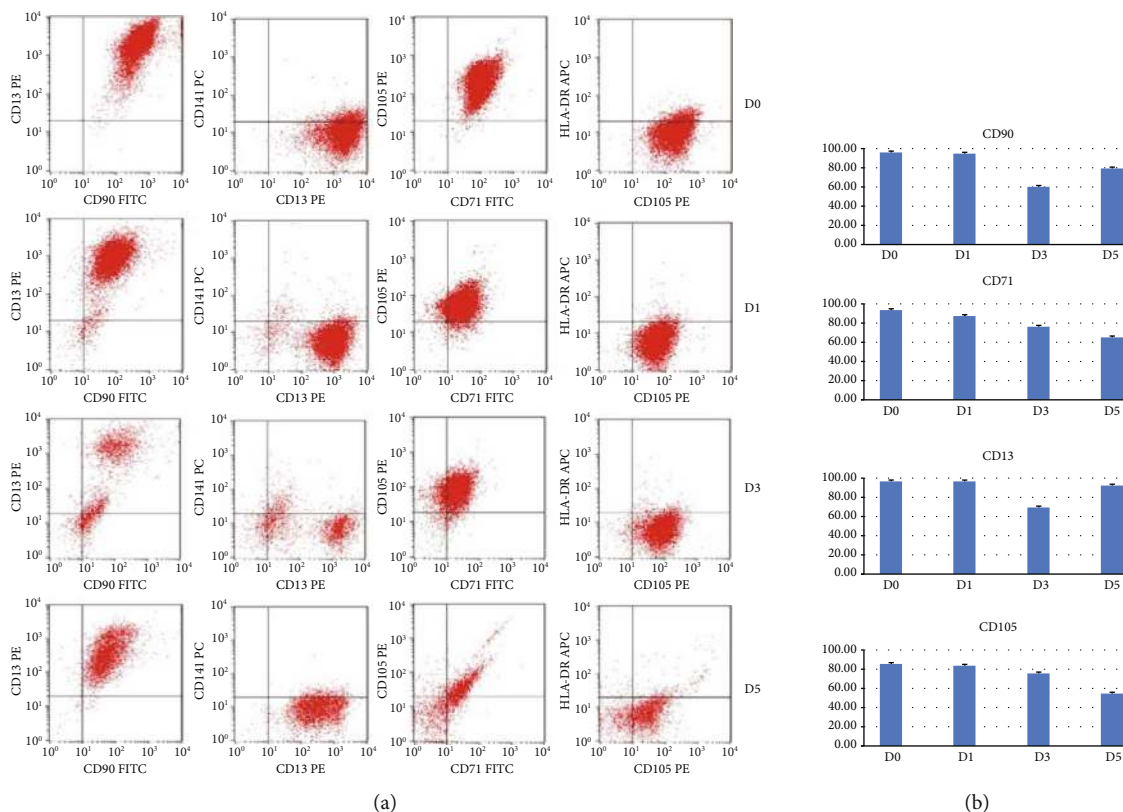


FIGURE 5: Cell surface expression of selected markers before and after UC-MSC-derived neurosphere induction. (a) Representative flow cytometry analysis of UC-MSCs before induction on Day 0, and after induction (flow cytometry analysis was done on dissociated neurosphere cells after induction) on Days 1, 3, and 5, respectively. (b) Dynamic change in UC-MSC-specific cell surface marker expressions after induction on Days 1, 3, and 5 in comparison with Day 0 before induction.

80 and 92%, which is too high for assuming that only a small subpopulation of MSCs has neurogenic capability while others have none. Second, the induction takes place within 12 hours. Although they do need three to four days to grow and further mature, the neurospheres were formed within a maximum of 24 hours after induction. After this induction, UC-MSCs which failed to become neural progenitor cells go through apoptosis. This growth kinetics is certainly not in accordance with the above theory.

We wanted to know more about the neurosphere formation process. We tested several different conditions. We found that complete neurobasal medium induction which is comprised of DMEM/F12, EGF, bFGF (both at 20 ng/ml), and N2 and B27 supplements led to the most prominent neurosphere production. A half regimen led to a less prominent neurosphere production. EGF only regimen and bFGF only regimen both led to a significant number of neurosphere formation, but bFGF only regimen induced mainly medium- to small-sized neurospheres, whereas EGF only regimen led to larger neurospheres. Contrary to our previous assumption, EGF and bFGF are not indispensable. DMEM/F12 and N2 and B27 are already enough to induce neurosphere formation. N2 supplement only is not enough to induce neurosphere formation, whereas B27 only regimen is. As we all know, both B27 and N2 are chemically well defined and have been used extensively for neurosphere for-

mation and the growth of postmitotic neurons in primary cultures from both the peripheral nervous system (PNS) and the central nervous system (CNS). N2 mainly contains insulin, human transferrin (iron-saturated), sodium selenite, putrescine, and progesterone, whereas B27 supplement contains a much more complicated list of vitamins, hormones, proteins, etc. It's interesting that N2 alone cannot induce neurosphere formation. We suspect that some ingredients within the B27 supplement might play an important role in this conversion.

Many researchers, including us, have shown that MSCs, especially UC-MSCs, can be efficiently induced to form neurons under certain conditions. But so far, only a few papers have addressed the question of the direct conversion of MSCs to neural stem/progenitor cells [24–27]. For example, Feng et al. reported the generation of highly purified neural stem cells from human adipose-derived mesenchymal stem cells by Sox1 activation [25]. This conversion is cumbersome, requiring a three-step induction and the use of lentivirus for shRNA delivery. Fu et al. reported that NSCs can be generated from human BM-derived mesenchymal stem cells (MSCs) [24]. In their study, when cultured in NSC culture conditions, 8% of MSCs were able to generate neurospheres. These MSC-derived neurospheres expressed characteristic NSC antigens, such as nestin and musashi-1, and were capable of self-renewal and multilineage differentiation into

TABLE 2: RayBio® human growth factor array. These readouts are the readouts after subtracting the background. Signal strength higher than 150-200 is considered significant. Expression level fold change larger than 1.5 is considered statistically significant.

Cytokines	UC-MSCs—D0	UC-MSCs—D1	UC-MSCs—D3	UC-MSCs—D5	UC-MSCs—D7
AR	360	942	16,610	3,677	17,070
BDNF	8,102	1,229	1,574	1,189	1,272
bFGF	4,602	41,624	31,735	40,812	34,468
BMP-4	934	1,320	1,179	1,228	1,502
BMP-5	692	760	790	696	844
BMP-7	554	723	777	779	737
b-NGF	580	398	471	418	427
EGF	1,659	33,719	47,861	52,847	54,487
EGF R	2,312	5,724	7,688	8,445	4,320
EG-VEGF	218	270	210	201	200
FGF-4	484	718	647	710	652
FGF-7	944	653	1,499	1,014	881
GDF-15	106,615	78,066	128,010	96,329	117,411
GDNF	1,088	916	9,405	1,627	6,965
GH	465	664	583	725	761
HB-EGF	264	498	486	521	795
HGF	9,735	13,519	97,528	32,492	86,731
IGFBP-1	223	578	478	622	636
IGFBP-2	255	679	1,952	1,161	1,930
IGFBP-3	1,898	422	322	397	433
IGFBP-4	2,085	609	2,156	671	892
IGFBP-6	8,868	5,660	7,395	4,292	2,215
IGF-1	5,067	5,372	5,386	6,033	5,988
Insulin	264	3,063	10,150	9,133	10,625
MCSF R	273	514	502	523	536
NGF R	390	703	724	704	659
NT-3	282	489	497	526	510
NT-4	459	663	551	595	550
OPG	1,569	6,750	28,573	22,742	28,604
PDGF-AA	2,167	2,537	2,628	1,165	1,464
PIGF	119	307	309	281	333
SCF	257	474	448	462	378
SCF R	1,000	707	2,247	728	1,533
TGF- α	0	149	207	180	204
TGF- β 1	2,588	1,455	1,504	1,546	1,638
TGF- β 3	118	345	240	292	248
VEGF	88	32,958	49,306	47,515	47,193
VEGF R2	529	775	677	550	705
VEGF R3	360	480	522	699	571
VEGF-D	178	429	627	664	785

neurons, astrocytes, and oligodendrocytes. To a certain degree, their findings are similar to what we report here in the present study. But the major difference is that we use human UC-MSCs and we show a much more efficient neurosphere formation within a much shorter time. It is very possible that UC-MSCs possess higher neural differentiation capabilities than BM-MSCs. But it remains unclear why the reported studies demonstrated much less efficacy because

we could consistently repeat our results in multiple settings, even when using MSCs passaged up to 30.

Paracrine effects play an important role in mesenchymal stem cell transplantation clinical trials [28–30]. Some studies have shown MSCs, especially UC-MSC, to express preferentially secreted factors related to neuroprotection, neurogenesis, and angiogenesis [31–33]. We are interested to see whether the UC-MSC secretome is further shifted toward

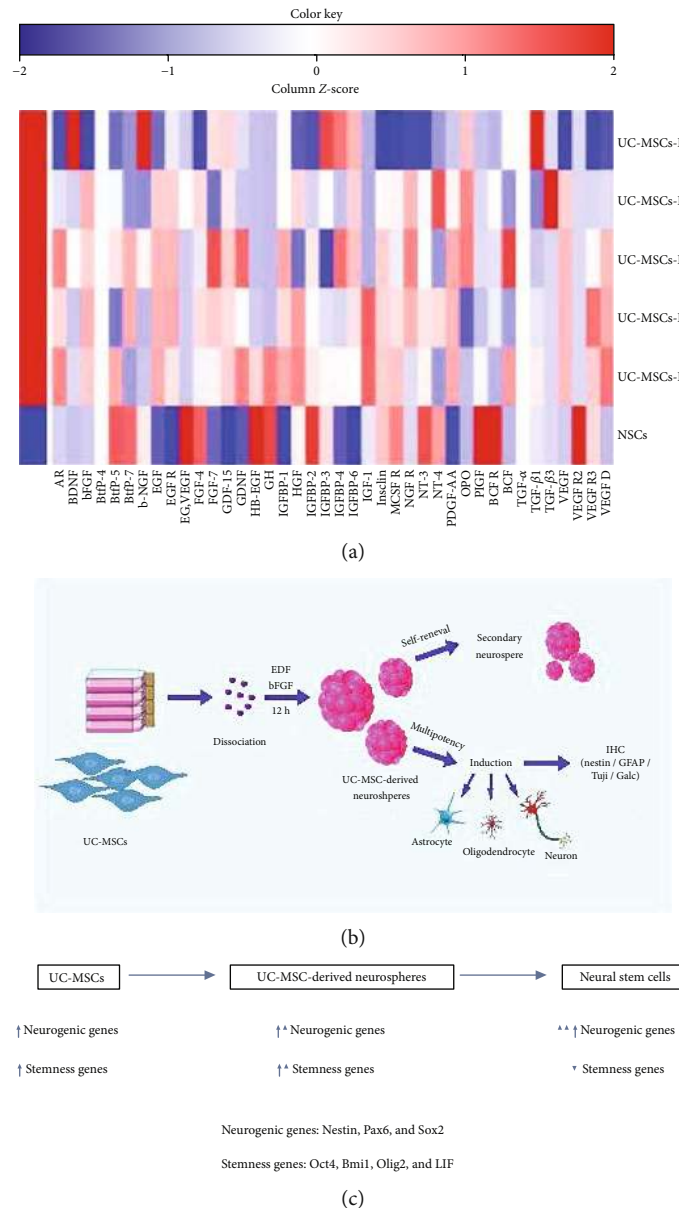


FIGURE 6: (a) Heatmap of the expression levels of human growth factor cytokines before induction (D0) and at D1, 3, 5, and 7 after induction of UC-MSC-derived neurospheres. Human neural stem cells (NouvNeu hNSCs) were used as a positive control. UC-MSCs, UC-MSC-derived neurospheres, and hNSCs have different secretomes, but UC-MSC-derived neurospheres are skewed toward the hNSC secretome. The color scale shown illustrates the relative expression of the indicated cytokines across the samples: red denotes high expression and blue denotes low expression. (b and c) Schematic illustration of UC-MSC-derived neurosphere formation. Serum-free suspension culture with EGF- and bFGF-containing medium led to a significant increase in neurogenic and stemness gene expression (probably neurogenic genes predominate) in UC-MSCs, resulting in neurosphere formation which closely resembles neural progenitor cells with self-renewal and multipotency capacity.

the neural stem cell phenotype after the induction. Using a RayBio cytokine array, we analyzed 40 human growth factors. We compared the profile before the induction and that after induction on Days 1, 3, 5, and 7, respectively. We found an overall significant increase in the expression levels for majority of these cytokines, among which VEGF is the most prominent with about a 300- to 550-fold increase beginning as early as 24 hours after the induction. Among other cytokines which exhibit a significant increase, we found EGF,

bFGF, AR (amphiregulin, a member of the EGF family), insulin, HGF (hepatocyte growth factor), OPG (Osteoprotegerin), IGF (insulin-like growth factor), GDNF (glial cell line-derived neurotrophic growth factor), etc. As we all know, EGF and bFGF have long been shown to play an important role in neural stem cell maintenance and proliferation [34, 35]. The highly increased expression of EGF and bFGF means that these MSC-derived neurospheres can be self-sufficient. On the other hand, AR, insulin, HGF, OPG, and

IGF all have been implicated in neural stem cell maintenance and proliferation [36–41], and thus they can be seen as at least partially neurogenic cytokines. Therefore, our short neural induction protocol not only produced cell phenotypically resembling neurospheres but also led to a quick upregulation and expression of neurogenic and angiogenic cytokines. When we compared this profile with commercially available human neural stem cells, we found that UC-MSC-derived neurospheres not only skewed toward the neural stem cell phenotype but were in many aspects even superior to neural stem cells. MSC-derived neurospheres had much higher expression levels of EGF, bFGF, VEGF, insulin, IGF, HGF, GDNF, AR, etc. in comparison to neural stem cells. The expression levels of BDNF, NGF (nerve growth factor), BMP, insulin, IGF-1, and TGF- β 1 are comparable between the two groups.

Cell therapy has emerged as a promising strategy for treating many neurological diseases such as spinal cord injury, stroke, Parkinson's disease, motor neuron disease, and dementia [42–45]. But finding an ideal cell source has been challenging. The use of embryonic neural stem cells has been restricted due to ethical concerns and availability issues. Another candidate is iPS cells. Although iPS cells were discovered 13 years ago and considerable progress had been made in this field, it does not change the fact that the production of iPS cells is a highly cumbersome and lengthy process. This precludes its use in common stem cell research labs. For the treatment of neurological disorders, iPS cells have to be further induced to neural progenitor cells and that process usually takes another 3–4 weeks [46, 47]. In contrast, here we present an easy way of obtaining neural progenitor cells. The induction is with a well-defined regimen, and therefore, these cells can quickly be produced meeting the GMP standard. The second biggest advantage of using these cells is that we consistently detected no HLA-DR expression in these cells, which means that they can be safely used in a heterogenous transplantation setting. We are now testing the efficacy of MSC-derived neurospheres in animal models.

In summary, we reported for the first time that UC-MSCs can be efficiently induced to form unique MSC-derived neurospheres under a neurobasal medium induction condition within 12 hours. These UC-MSC-derived neurospheres represent a new source of neural progenitor cells. They have the combined features of both MSC and neural stem/progenitor cells. We believe these cells will find themselves of tremendous use in future stem cell transplantation clinical trials for various neurological diseases.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors who have taken part in this study declared that they do not have any conflict of interest to disclose with respect to this manuscript.

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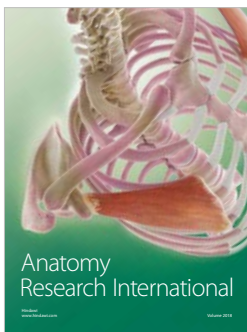
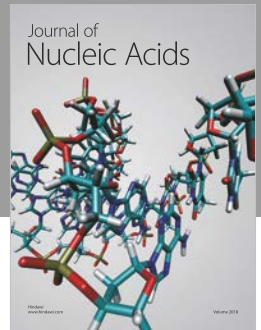
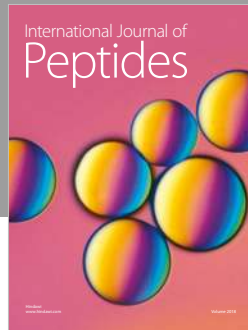
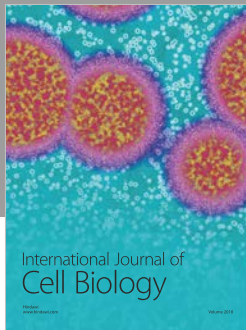
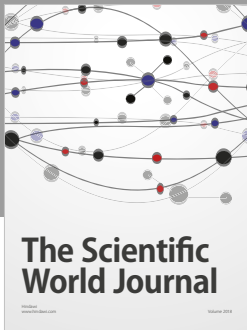
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