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RESEARCH ARTICLE

Glycogen synthase kinase 3 (GSK3)-inhibitor SB216763 promotes the conversion of human umbilical cord mesenchymal stem cells into neural precursors in adherent culture

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Abstract The ability to generate neural progenitor cells from human umbilical cord mesenchymal stem cells (hUC-MSCs) has provided an option to treat neurodegenerative diseases. To establish a method for this purpose, we characterized the early neural markers of hUC-MSCs-derived cells under different conditions. We found that neither the elimination of signals for alternative fate nor N2 supplement was sufficient to differentiate hUC-MSCs into neural precursor cells, but the GSK3 inhibitor SB216763 could promote an efficient neural commitment of hUC-MSCs. The results indicated that Wnt/β-catenin might play an important role during the early neural differentiation of hUC-MSCs. Here, we report a method for hUC-MSCs to commit efficiently into a neural fate within a short period of time. This protocol provides an efficient method for hUC-MSCs-based neural regeneration.

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Abbreviations

hUC-MSCs	Human umbilical cord mesenchymal stem cells
GSK3	Glycogen synthase kinase 3
CNS	Central nervous system
NPCs	Neural precursor cells
FACS	Fluorescence-activiated cell sorting
ICC	Immunocytochemistry

Introduction

Stem-cell-based therapies have been used as new strategies to treat neurodegenerative diseases and neural damages, such as Parkinson's disease, Alzheimer's' disease, stroke, and spinal cord injury (SCI) [3, 13, 30, 31]. Some reports have shown that human mesenchymal stromal cells (MSCs) are able to support axonal growth after transplant, enhanced sensorimotor function, and significantly recovered it in vivo [1, 25, 47]. However, there are still some obstacles for stem-cell-based therapies, such as the differences between donors, survival of stem cells after transplantation, and the differentiation ability of stem cell in vivo. Currently, there is no single efficaciously established stem-cell-based strategy, but efforts are being made to achieve one; therefore, certain new strategies focus on combining stem cells with gene-based therapies or small molecules, such as growth factors and chemicals.

Umbilical cord-derived mesenchymal stem cells (UC-MSCs) can develop into neural cells, and have been used to replace damaged tissue in the central nervous system

(CNS) [14, 20, 22]. UC-MSCs have certain advantages for engraftment: (1) they are easily isolated; (2) they are derived from the umbilical cord after birth, so the source of the cells is less controversial; (3) they replicate faster in vitro; (4) the source of the cells is quite young; and (5) they are less immunoreactive after transplantation [8, 35, 48]. Some protocols have been established to direct the differentiation of UC-MSCs into neural cells, involving the use of retinoic acid [29], heparin, basic fibroblast growth factor [20], and epidermal growth factor [6]. UC-MSCs have been found to differentiate into oligodendrocyte precursor cells (OPCs), neural stem-cell-like cells [18], and motor neuron-like cells [20]. However, there are a number of obstacles associated with stem-cell-based therapies, such as the low survival rate of cells after transplantation, the low efficiency of differentiation of the cells, and the uncontrollable differentiation in vivo. Accordingly, the purpose of this study was to find a factor, which can be used both in vitro and in vivo, to promote the differentiation of UC-MSCs into neural progenitor cells. Our previous research showed that the stimulation of the Wnt/β-catenin-signaling pathway could promote the generation of neural precursor cells (NPCs) from embryonic stem cells (ESCs). Other studies have also confirmed that Wnt signaling is essential for the normal development of the cortex and hippocampus [21]. In addition, they found that this signaling pathway plays an important role in the proliferation and maintenance of neural progenitor cells in vitro [24]. Thus, an alternative method which we investigated in this study is based on the ability of SB216763, a small molecule which has been confirmed to activate Wnt-signaling pathway through inhibiting GSK3 and to promote the neural differentiation of UC-MSCs. Specifically, we examined the effect of SB216763 on promoting neural differentiation of UC-MSCs under two conditions. The default condition was routinely used in neural differentiation of ESCs through the default mechanism, which induces neural differentiation by eliminating extra growth factors and signals. This method makes it possible to examine the effect of SB216763 in the absence of other factors. The other condition involved the use of a defined neuroectodermal-induction medium which contains DMEM/F12 and N2. This method provides a simple yet low-cost way to induce the formation of neural progenitor cells. Our ultimate aim was to find out a chemical compound which could direct the neural commitment of UC-MSCs, and to establish a protocol for promoting the differentiation of UC-MSCs into neural precursors which can be used for transplanting in the future. Our results confirmed that SB216763 activates the Wnt-signaling pathway by inhibiting GSK3, to promote the neural differentiation of UC-MSCs. This suggests the potential use of SB216763 to generate neural precursors, which would be

advantageous due to its low cost, high efficiency, and easy manipulation.

Experimental procedures

Isolation and identification of UC-MSCs

The study protocol was subject to approval by the Ethic Committee of the Affiliated Hospital of Guangdong Medical University (Ref. PJ2015014). Fresh human umbilical cords were collected after birth from healthy donors ($n > 6$, age between 20 and 35 years, and gestation period 38–40 weeks), and washed several times with $1\times$ sterile phosphate-buffered saline (PBS; Gibco, Carlsbad, CA, USA) until the cord blood was removed. Subsequently, umbilical arteries and veins were peeled off, and the outer layer of the umbilical cords was removed. The Wharton's jelly was cut into 5 mm^3 pieces and plated on 100 mm petri dish (Corning, USA), after drying for 5 min, the complete medium, which contained Dulbecco minimal essential medium (DMEM) with 10 % FBS and 10 U/ml penicillin, was added to cover the fragments. The petri dishes were kept in an incubator at 37 °C with 5 % CO_2 for days until cells migrated out of the tissues. Then, all tissue fragments were removed from the petri dishes, and the cells were cultured to confluence. For the passaging, cells were detached with 0.25 % Trypsin–EDTA (Gibco), and the trypsin was inactivated by PBS with 10 % FBS. Cells were the centrifuged at 100 g for 5 min, resuspended in complete medium at a density of about 2000–3000 cell/cm^2 and seeded into new plates. The morphology of the cells was examined using an inverted light microscope (EVOS; Life Technologies, Carlsbad, CA, USA). The cells at passage 3 (P3) were collected and processed for flow cytometry (FACScan. BD Biosciences) analysis to assess the expression of monoclonal antibodies (CD31, CD34, CD45, CD73, CD90, and CD105; BD Pharmingen, Franklin Lakes, NJ, USA). SB216763 was used to increase the accumulation of β-catenin in the cytoplasm by inhibiting the activity of GSK-3β. The SB216763 was purchased from Selleck (S1075; Houston, TX, USA).

Neural differentiation of UC-MSCs

For the neural differentiation under default condition, UC-MSCs were cultured in 60 mm petri dishes to 70 % confluence, and the cell monolayer was washed with PBS. Then, instead of complete medium, cells were serum-free DMEM high glucose (Hyclone, Logan, UT, USA) medium [39]. For the direct neural differentiation, UC-MSCs were centrifuged and resuspended at a density of 0.5×10^6 cells/ml in DMEM/F12 (1139; Gibco) plus 1X N2 (Gibco).

Cells were refed fresh medium every 2 days during the neural differentiation of hUC-MSCs.

Immunocytochemistry

Cells were cultured in 24-well plates for 2 days to 60–70 % confluence. The cell monolayer was then washed with PBS before being fixed with 4 % paraformaldehyde (PFA). The fixed cells were permeabilized using PBS/0.3 % triton X-100 for 15 min. The primary antibodies were as follows: monoclonal Anti-POU5F1 (OCT4, 1:1000; SIGMA-ALDRICH, Saint Louis, MO, USA), anti-Nestin (1:50; Beyotime, China), neuronal class III β-tubulin (1:250, Beyotime, China), anti-GFAP (MXB, China), anti-β-catenin (1:50–500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Ki67 (MXB, China), anti-Vimentin (MXB, China), and anti-Cytokeratin (MXB, China). The secondary antibodies were as follows: Alexa Fluor 488 goat-anti mouse IgG2α (1:1000; Life Technologies), Alexa Fluor 488 goat-anti mouse IgG (H + L) (1:1000; Life Technologies), and FITC-conjugated Affini-Pure Goat-Anti-Rabbit IgG (H + L) (1:50–200; Jackson ImmunoResearch, US). The 4,6 diamidino-2-phenylindole (DAPI) was used for nucleus staining. The control cells, which were stained without secondary antibodies, were negative for immunolabeling. A Leica DMI3000B microscope was used to capture the images of cells, and the LAS V4.6 software was used to analyse the results.

RT-PCR analysis

Total RNA was extracted from the cell using the E.Z.N.A TM Total RNA Kit I (OMEGA Bio-Tek., GA, USA). A total of 1 μg RNA was used to synthesize cDNA with oligo primers (TAKARA Bio Inc., Kyoto, Japan), and the cDNA was used directly for the RT-qPCR analysis (SYBR Remix Ex TaqTM II, TAKARA Bio Inc.). All procedures were performed following the instructions of the manufacturer. The specific sequences of genes were used to detect the early neural differentiation and the "stemness" status of cells. The primers were as follows: OCT4-forward: GCAAAGCAGAAACCCTCGTG, OCT4-reverse: GAACCACACTCGGACCACAT; NANOG-forward: CTTCTGCGTCACACCATTGC, NANOG-reverse: CTTCTGCGTCACACCATTGC, NESTIN-forward: GACCCTGAAGGGCAATCACA, NESTIN-reverse: GGCCACATCATCTTCCACCA; PAX6-forward: AGAGAAGACAGGCCAGCAAC. PAX6-reverse: TGGTTGGTAGACACTGGTGC; SOX1-forward: CAACCAGGACCGGGTCAAAC, SOX1-reverse: TCGGACATGACCTTCCACTCG; β-tubulin-forward: GATCGGGGCCAAGTTCTGG, β-tubulin-reverse: GCCTCGTTGTAGTAGACGCT, β-tubulin-reverse: GCCTCGTTGTAGTAGACGCT; β-ACTIN-forward: CCAACTGGGACGACATGGAG, and β-ACTIN-reverse:

AGGGATAGCACAGCCTGGAT. The housekeeping gene β-ACTIN was used as the control in all RT-qPCR analyses. Statistical significance was analyzed by SPSS (SPSS 19.0; IBM Corp., Armonk, NY, USA), and the paired-sample t test was used. A p value of >0.05 was considered as insignificant; a p value of <0.05 was considered as statistically significant; a p value of <0.01 was considered as highly significant; and a p value of <0.001 was considered as very highly significant. All results were collected from at least three independent experiments.

Results

Cytotoxicity and effects of SB216763 on undifferentiated hUC-MSCs

The hUC-MSCs were isolated from umbilical cord stroma, and then examined by the FACS analysis. Similar to other studies, we detected the expression of mesenchymal markers, such as CD90, CD73, and CD105, but neither the endothelial marker CD31 nor hematopoietic stem-cell markers CD45 and CD34 were detected (Supplementary Fig. 1A). The hUC-MSCs isolated were adherent to plastic in culture could differentiate into osteoblasts, adipocytes, and chondroblasts in vitro (Supplementary Fig. 1B).

To determine the concentration of SB216763 with the least effect on the viability, proliferation, and other features of hUC-MSCs, we conducted experiments using a serial dilution of this small molecule. The hUC-MSCs were plated into 96-well plates, and treated with 30, 300 nM, 3, and 30 μM of SB216763 for 2 days. Subsequently, the cell counting kit-8 (CCK8) assay was used to examine the viability of the hUC-MSCs. The results of the CCK8 assay indicate that the population of live cells decreased in the 30 μM, 60, and 120 μM SB216763-treated groups, while the concentration of 3 μM, 300, and 30 nM did not affect the viability or proliferation of hUC-MSCs (Fig. 1a). The examination of the morphology of untreated hUC-MSCs and 3 μM SB216763-treated hUC-MSCs under an inverted microscope revealed no differences between the two groups. This suggested that 3 μM SB216763 did not affect the shape and size of hUC-MSCs (Fig. 1b). In addition, the accumulation of β-catenin was also detected in cytoplasm of SB216763-treated cells (Fig. 1c), suggested that SB216763 could inhibit GSK3 in hUC-MSCs.

To examine the effect of SB216763 on the expression of certain important transcription factors, which related to pluripotency of embryonic stem cells, OCT4, SOX2, SSEA1, and NANOG were examined in this study. The pluripotent marker OCT4 was detected in both untreated and SB216763-treated hUC-MSCs (Fig. 1d). Moreover, the results of the FACS analysis showed that the
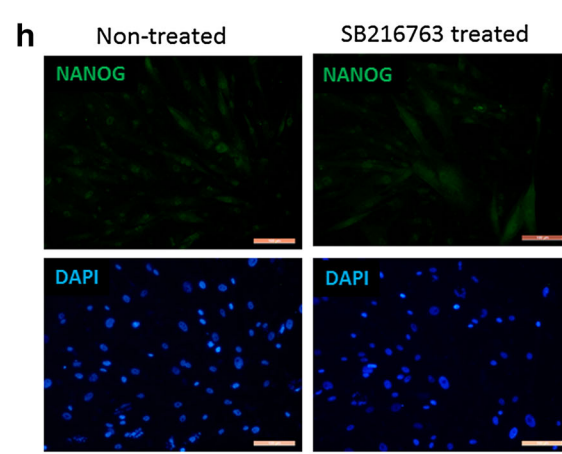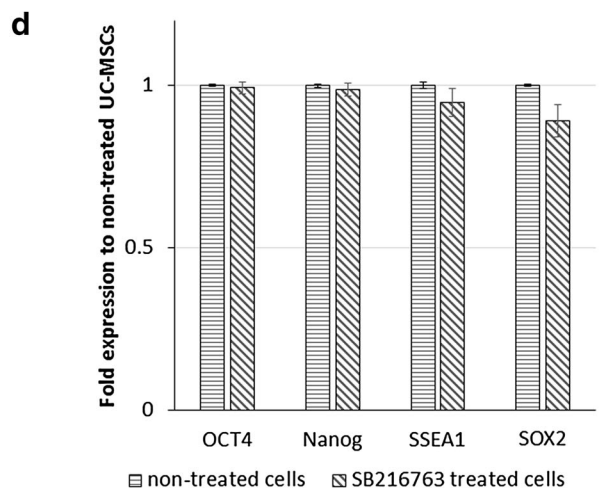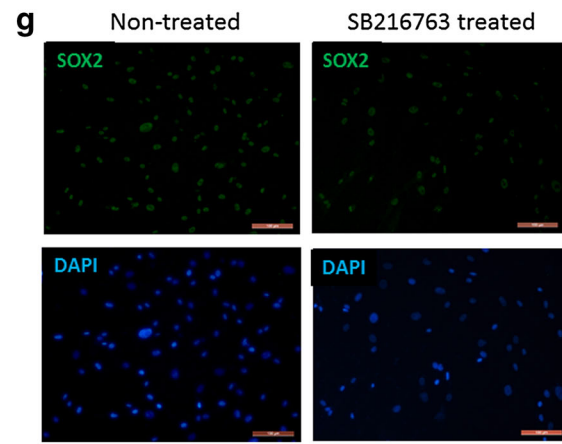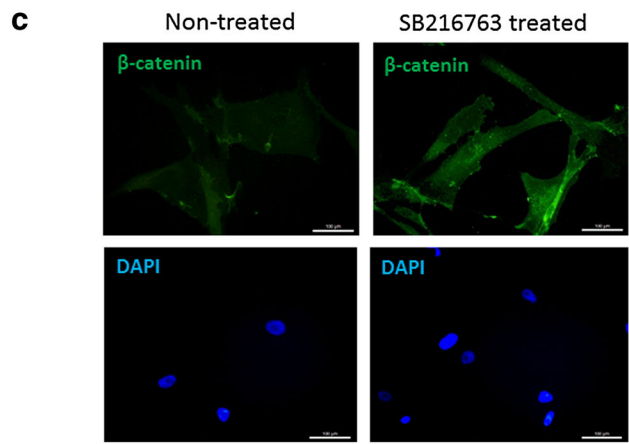
a Cell Viability (%) (Fold increases to Control cells) — bar chart with x-axis: 0nM, 30nM, 300nM, 3uM, 30uM, 60uM, 120uM

b Non-treated（10X） | SB216763 treated（10X） — UC-MSCs

c Non-treated | SB216763 treated — β-catenin / DAPI

d Fold expression to non-treated UC-MSCs — bar chart with x-axis: OCT4, Nanog, SSEA1, SOX2; legend: non-treated cells, SB216763 treated cells

e Non-treated | SB216763 treated — OCT4 / DAPI

f The percentage of OCT4 positive cells — Non-treated UC-MSCs 97.7% | SB216763 treated UC-MSCs 98.2%

g Non-treated | SB216763 treated — SOX2 / DAPI
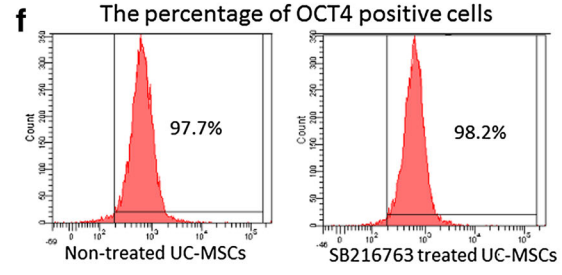
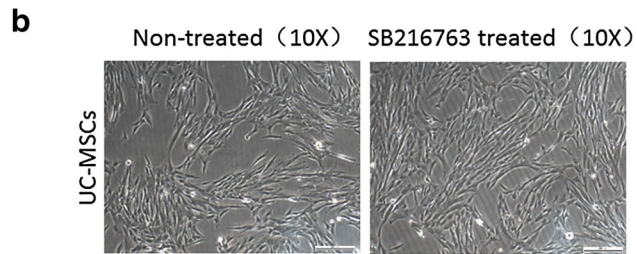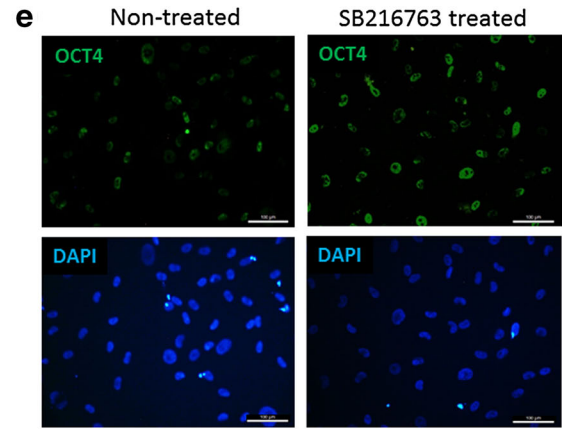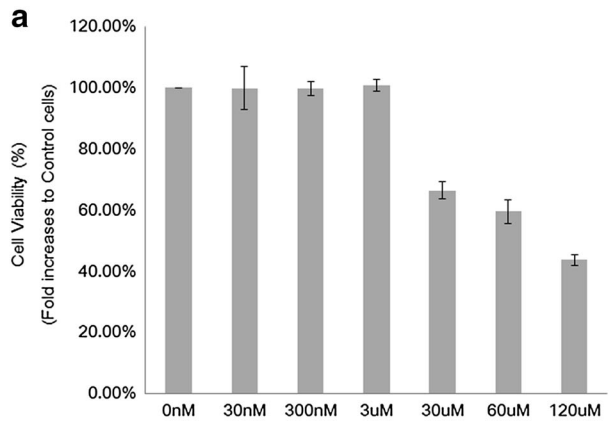**h** Non-treated | SB216763 treated — NANOG / DAPI

◀**Fig. 1** Characterization of the hUC-MSCs and optimal concentration of SB216763 to treat cells. **a** Dosage-dependent cytotoxicity of SB216736 on undifferentiated UC-MSCs was measured using CCK8 assay 48 h after treatment. Values are showed as a percentage of control values (0 nM). Data are presented as mean ± SEM of three separate experiments performed in three independent experiments. **b** Morphology of non-treated hUC-MSCs and SB216763-treated hUC-MSCs under inverted microscope (×10), the images, showed that no obvious morphological changes after treating. **c** β-catenin (anti-β-catenin, *green*) expressed in non-treated hUC-MSCs and SB216763-treated cells. The nuclei were stained with DAPI (*blue*). *Bar* 100 μm. **d** Expression of OCT4 (Anti-POU5F1, *green*) in non-treated and SB216763-treated groups, respectively. The nuclei were stained with DAPI. *Bar* 100 μm. **e** FACS analysis results showed that the percentage of OCT4 positive cells did not have a significant change between two groups. **f** Expression of transcription factor OCT4, NANOG, and SSEA1 in non-treated and SB216763-treated UC-MSCs. There were no significant difference between non-treated and SB216763-treated groups. Data are the average of three assays, and *error bar* represents mean ± SEM. $*p < 0.05$, $**p < 0.01$

percentage of OCT4 positive cells was not significantly different between the untreated group and the SB216763-treated groups (Fig. 1f). These results indicated that 3 μM SB216763 did not affect the characteristics of hUC-MSCs. Therefore, 3 μM SB216763 was adopted as an optimal concentration for treating hUC-MSCs in this research. In addition, to evaluate the gene expression of SB216763-treated hUC-MSCs, qRT-PCR analysis was applied. The analysis of the expression of *OCT4, NANOG, SSEA1,* and *SOX2* revealed that the expression of these three genes was not significantly affected after treatment with SB216763 (Fig. 1d). Moreover, the ICC images showed that the expression of stem-cell markers SOX2 and NANOG was detected in both SB216763-treated and non-treated cells (Fig. 1g, h). These results suggested that SB216763 did not influence the expression of certain important "stemness"-related transcription factor genes.

SB216763 protects hUC-MSCs from serum starvation-induced apoptosis and enhances the neural fate of hUC-MSCs by increasing the expression of neural specific genes

To examine the effect of SB216763 on neural commitment of hUC-MSCs, we adopted two adherent monoculture methods, "default mechanism" and neural differentiation medium. The "default mechanism" is one of the method to trigger the neural differentiation of ESCs in vitro, which has been used to eliminate the effects of some exogenous factors during neural differentiation. To examine the neural commitment of hUC-MSCs in the absence of other factors, we adopt "default mechanism" to check the effect of SB216763 on neural differentiation of hUC-MSCs. Therefore, SB216763 was added into the serum-free

medium at different time points during the culturing, the design of the experiment is shown in Fig. 2a.

To examine the percentage of cells surviving 48 h after differentiation under "default mechanism", the CCK8 assay was used. The CCK8 results showed that the cells in the group constitutively treated (CT) with SB216763 survived twice as long as the untreated group cells (Fig. 2b). This suggested that SB216763 either protected cells from apoptosis induced by serum starvation or stimulated the neural differentiation of hUC-MSCs. However, which effects caused the increased cell population in the constitutively treated group was not clear. Therefore, we cultured the non-treated hUC-MSCs under "default condition" for 1 day, and then treated the cells with SB216763. The CCK8 result showed that the cell population in the group subjected to treatment at day 1 (ET) was as large as that in the untreated group (Fig. 2b). This indicated that the SB216763 might play a major role in preventing the death of hUC-MSCs at the early stage under the default mechanism.

In addition, the expression of some neuronal specific markers increased after treating the hUC-MSCs with 3 μM SB216763 under "default condition". For instance, 2 days after treatment, the expression of *NESTIN* was found to be significantly increased, and the expression of *class III β-tubulin* was very significantly increased. In addition, the expression of *SOX1* and *PAX6* was increased in the SB216763-treated cells, but the increase was not statistically significant when compared with the untreated group (Fig. 2c). These RT-qPCR results suggested that although treatment with SB216763 did not impair the "stemness" of hUC-MSCs, it enhanced the neural differentiation potential of hUC-MSCs by increasing the expression of neural specific genes.

The expression of NESTIN was monitored by immunocytochemistry (ICC), 2 days after neural differentiation of hUC-MSCs under "default condition" (Fig. 2c). The results indicate that there was no obvious NESTIN expression in the untreated group, but it was clearly detected in the group treated constitutively with SB216763 (Fig. 2d). Moreover, ICC result showed that the Ki-67, which is a nuclear protein associated with cellular proliferation, was detected in SB216763 constitutive-treated groups (Fig. 2e). These ICC analysis results showed that SB216763 itself was able to stimulate and accelerate the neural differentiation of hUC-MSCs into neural precursor cells as well as protect proliferation of the cells under serum starvation. However, this method could not produce a large number of neural progenitor cells. Moreover, its low efficiency makes the method less appealing to researchers who want to produce more hUC-MSCs derived progenitor cells. To overcome this problem, we developed a new protocol that involves the
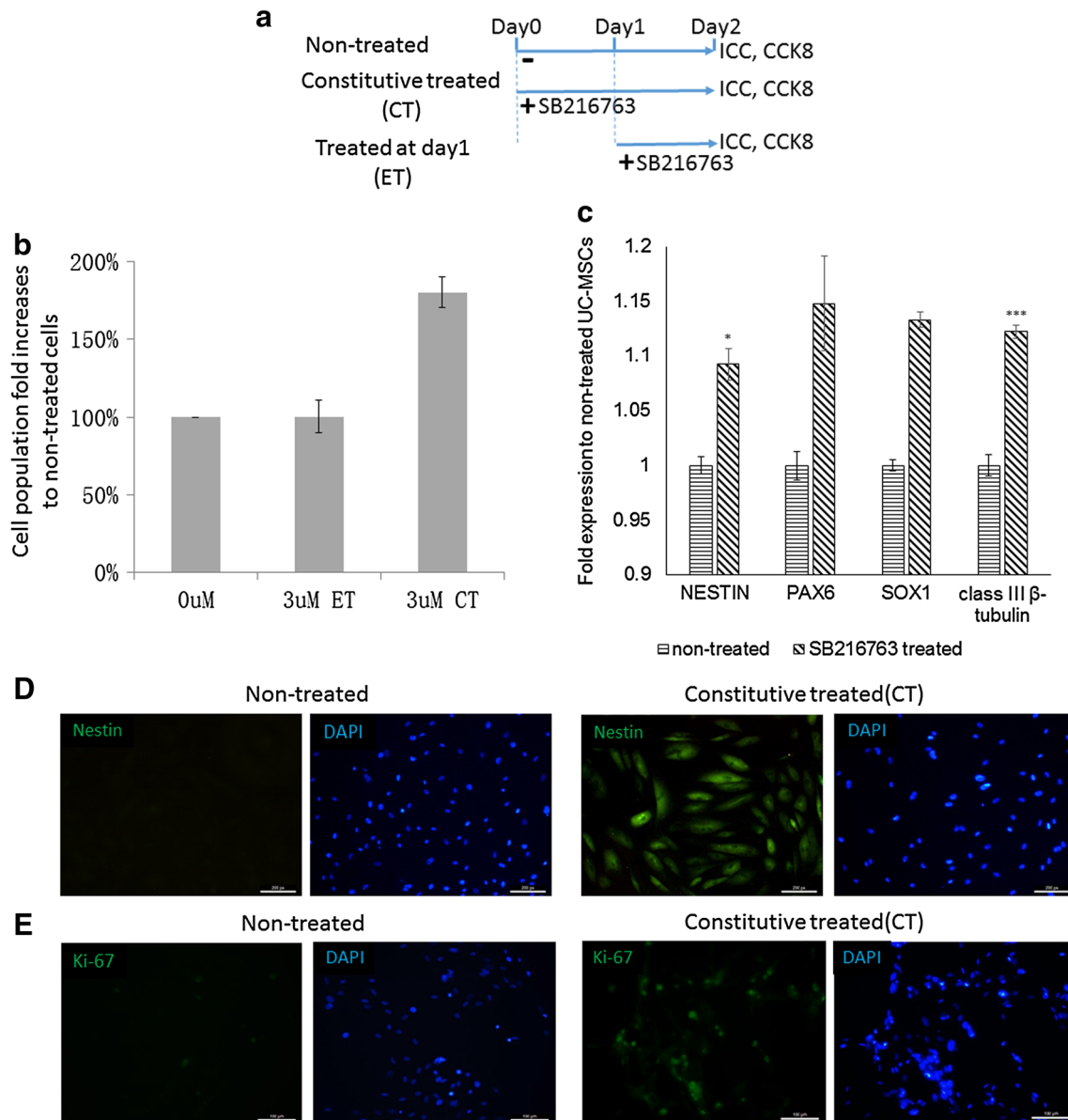
Fig. 2 Neural commitment of hUC-MSCs under "default condition". **a** Experimental design of time-course induction of UC-MSCs under default condition. **b** Percentage of living cells after treated under default condition. *ET* treated at day 1, *CT* constitutive treated. Data are the average of three assays, and *error bar* represents mean ± SEM. **c** Expression of the early neural specific markers, *NESTIN*, *PAX6*, *SOX1*, and *class III β-tubulin* in non-treated and constitutive-treated UC-MSCs showed that gene expressions have been stimulated by 3 µM SB216763. **d** Expression of NESTIN (anti-NESTIN, *green*) in cells which treated under default condition for 2 days. The nuclei were stained with DAPI. *Bar* 200 px (100 µm). **e** Expression of Ki-67 (anti-Ki-67, *green*) in non-treated and constitutive-treated UC-MSCs under "default condition". The nuclei were stained with DAPI. *Bar* 100 µm

replacement of serum with N2 supplement and the reduction of other signals by resuspending single hUC-MSCs in this defined medium, which consisted of DMEM/F12 and the N2 supplement. The N2 supplement has been widely used to culture primary hippocampal progenitors and neural stem cells [42, 44]. The adherent monoculture method was based on Ying's report that ESCs cultured in monolayer were able to convert ESCs into neuroectodermal precursors [45].

SB216763 enhanced the direct neural differentiation of hUC-MSCs and prolongs the expression of neural markers in differentiated cells

In this step, neural differentiation medium, which contain DMEM/F12/N2, was used to trigger the neural differentiation of hUC-MSCs. To detect β-catenin during neural differentiation of hUC-MSCs, the Western blot analysis was used; moreover, the intensity of each band was

analyzed by the ImageJ software. The results showed that the β-catenin protein expression was increased after treating cells with SB216763 at different time points during neural differentiation (Fig. 3). Indeed, we found that the expression of β-catenin was increased in a time-dependent manner (Fig. 3). Thus, the Western blot analysis results suggested that β-catenin was required during the process of neural differentiation triggered by the "neural differentiation medium".

To examine the neural differentiation of hUC-MSCs using this protocol, the profiles of NESTIN, class III β-tubulin, and GFAP at different time points were monitored by ICC (Fig. 4). A strong expression of cytoplasmic Nestin was detected in SB216763-treated cells after 6 h induction (Fig. 4a), but no such expression was seen in the untreated group. This finding suggested that SB216763 could efficiently promote the differentiation of hUC-MSCs into NPCs. The absence of obvious expression of NESTIN in the untreated groups after prolong culturing (Fig. 4a), suggested that DMEM/F12 plus N2 supplement might not be sufficient to induce the generation of NESTIN positive neural progenitors. The measurement of the expression of Class III β-tubulin in both untreated and SB216763-treated cells showed a strong expression of protein in both groups after 6 h of induction (Fig. 4b). However, the class III β-tubulin positive cells decreased dramatically in the untreated group after 1 day (Fig. 4b). This result suggested

that SB216763 could maintain the expression of class III β-tubulin in differentiated hUC-MSCs longer than in the untreated group. The expression of GFAP was clearly detected in the SB216763-treated groups, but not in non-treated group (Fig. 4c). Furthermore, similar to the NESTIN expression, the expression of GFAP only occurred in the SB216763-treated group and its expression was sustained more than 3 days.

Discussion

We have previously found that the Wnt signaling can promote the generation of neural precursor cells (NPCs) from mESCs, and in addition, it could stimulate the differentiation of NPCs into post-mitotic neurons. There are some chemicals to inhibit the activity of GSK, such as Lithium, SB216763, CHIR99021, and 6-BIO [41]. In this study, the small molecule SB216763 was used to investigate the effect of Wnt signaling on hUC-MSCs. SB216763 is widely used as an efficient GSK3β inhibitor, which can activate the Wnt-signaling pathway by increasing cytoplasmic β-catenin [5, 17]. It has been used in animal models in vivo and cell culture in vitro [12, 15], as well as in stem-cell research [2, 17]. SB216763 at a concentration of 10 μM maintained the proliferation of mESCs in vitro [5, 17]. In neural development, SB216763 at a concentration of 100–500 nM has been found to inhibit the development of oligodendrocyte through the Wnt/β-catenin-signaling pathway [34]. In addition, in vivo studies showed that SB216763 combined with ECM and chitosan scaffold could repair spinal cord injury in rat [16]. Moreover, SB216763 could cross the blood–brain barrier and inhibit GSK3 in some part of brain [19]. All these studies suggested that SB216763 might be used as a drug, in combination with stem cells, to treat neurodegenerative diseases. For this reason, we chose SB216763 as an activator of Wnt signaling in this research.

To characterize the gene expression in SB216763-treated hUC-MSCs, stem-cell-related markers and neural specific markers were examined. OCT4 is one of the key transcription factor for the development of inner cell mass (ICM) during the blastocyst stage of the embryo, and it has been found to regulate the self-renewal and pluripotency of embryonic stem cells and induced pluripotent stem cells (iPSCs) [33, 37, 40, 46]. The expression level of OCT4 also indicates the differentiation status of pluripotent stem cells, as the previous research found that half-decreased expression of OCT4 led to the spontaneous differentiation of embryonic stem cells [33]. Apart from pluripotent stem cells, OCT4 is also expressed in adult stem cells and certain cancer cells [9, 11, 38], which suggests that OCT4 may play an important role in cell differentiation and division.
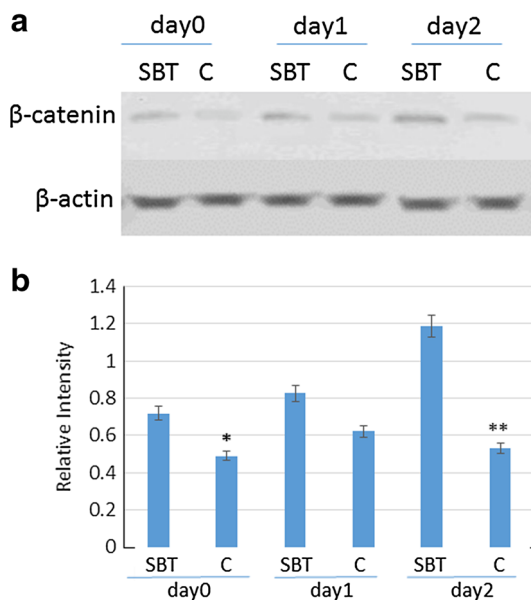

Fig. 3 Expression of β-catenin at different time points during the neural differentiation of hUC-MSCs under neural differentiation medium. **a** Western blot result showed that total β-catenin increased obviously in hUC-MSCs after treating with SB216763. **b** Histogram indicated the relative expression of β-catenin. The relative intensity was examined by the ImageJ software. *SBT* SB216763-treated group; **c** control, non-treated group

The expression of the primordial germ cell markers *NANOG* and *SSEA1*, which are highly expressed in pluripotent stem cells [4, 23, 36], was also evaluated in this research to assess the "stemness" status of hUC-MSCs. We found that SB216763 did not change the "stemness" status of hUC-MSCs, but enhanced the neural fate by increasing the expression of neural development-related genes.

For the neural differentiation of hUC-MSCs, two protocols were used in this study. The serum-free neural differentiation protocol based on the "default mechanism" was previously used to trigger neural specification of embryonic stem cells (ESCs) into neural progenitor cells [27, 39]. It has been reported that the neural commitment of ectoderm starts after withdrawal of all inhibitory signals, such as BMP4 and TGFβ [28]. In addition, the neural fate of ESCs emerged after dissociating cells in serum-free medium [43]. The ESCs cultured in a serum-free, feeder layer-free condition, referred to as the default condition,
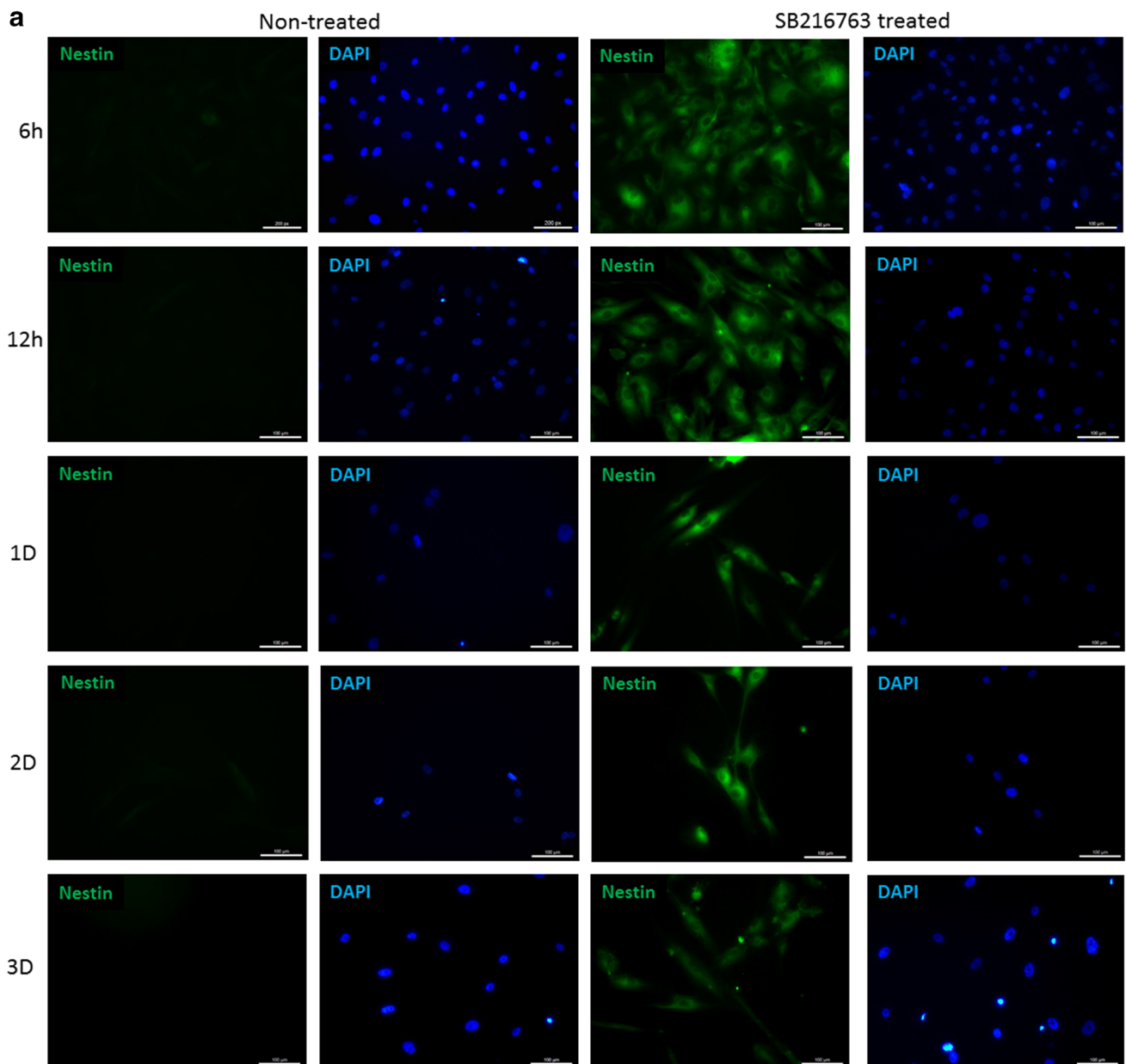

Fig. 4 Expression neuronal markers NESTIN, class III β-tubulin (TuJ1) and GFAP during the neural determination of UC-MSCs by immunocytochemistry staining. **a** Expression of NESTIN (anti-NESTIN, *green*) in non-treated and SB216763-treated groups at different time points during neural differentiation. **b** Expression of class III β-tubulin (anti-TuJ1, *green*) in non-treated and SB216763-treated groups at different time points during neural differentiation. **c** Expression of GFAP (anti-GFAP, *green*) in non-treated and SB216763-treated groups at different time points during neural differentiation. Data represent three independent experiments. *Bar* 100 μm (200 px)
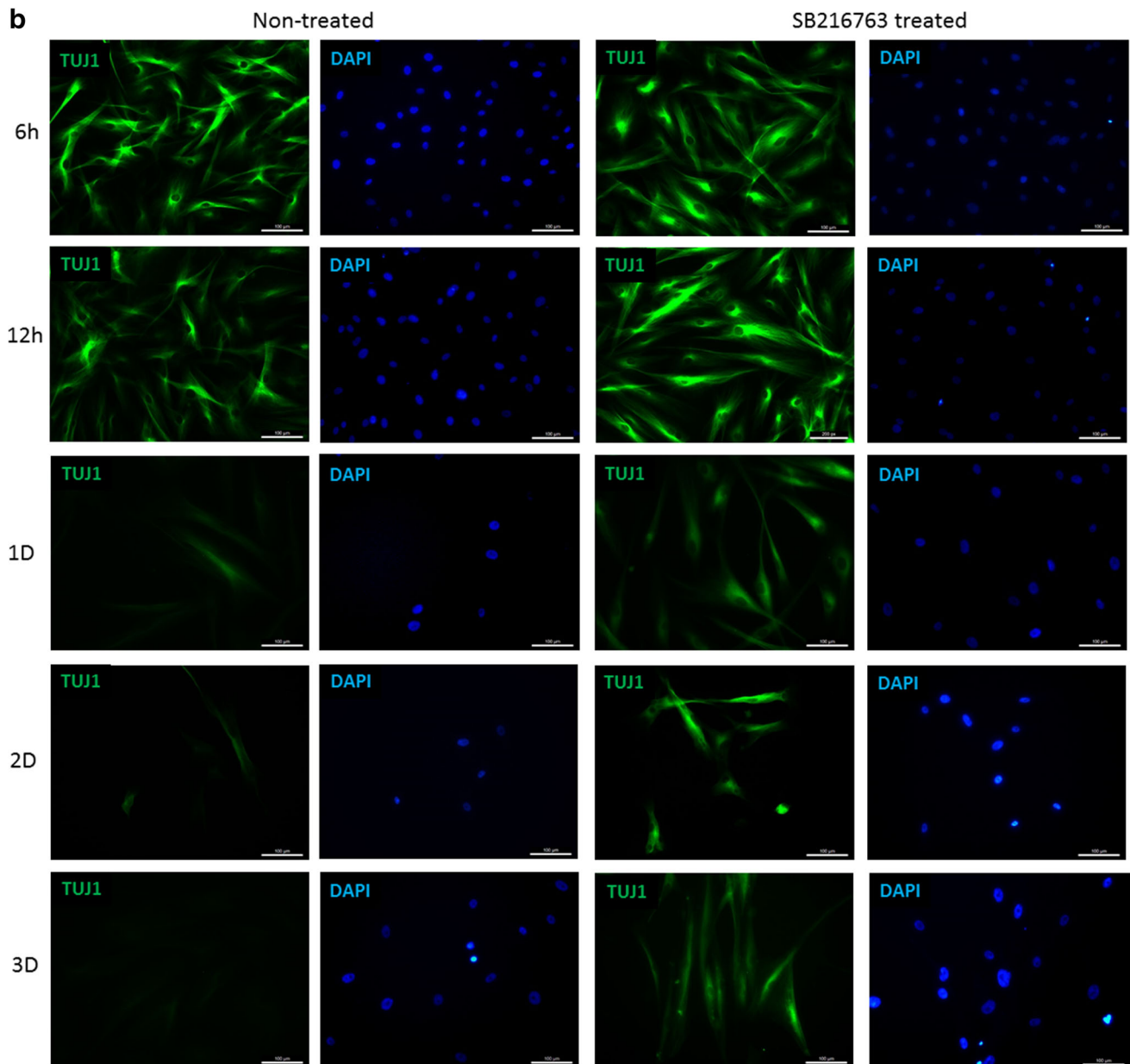
Fig. 4 continued

were able to generate sphere colonies, and further differentiated into neurons and glia [39]. We found that the cells which were cultured under the default condition could change their morphology, but not develop into neural progenitor cells. This suggested that suppressing inhibition signals were not sufficient to induce the neural differentiation of hUC-MSCs, and other factors were required to trigger the neural fate of hUC-MSCs. The second protocol we adopted in this research was based on the neuroectodermal-inducing conditions, which include the use of N2 and DMEM/F12 [10, 44, 45]. It is a commonly used protocol to differentiate ESCs into neural progenitor cells. We found that this direct neural differentiation protocol could

induce non-treated hUC-MSCs into class III-β-tubulin positive cells, but not NESTIN and GFAP positive cells. To explain this phenomenon, we found evidence from the previous reports that fibroblast-like cells, which have similar morphology to neural progenitor cells, could also express some early neural markers, such as class III β-tubulin, but fibroblast-like cells could not differentiate into functional neurons and glia cells [26]. This evidence suggested that the use of only DMEM/F12 plus N2 supplement medium was not sufficient to induce the neural differentiation of hUC-MSCs in a short time. However, different from the untreated group, SB216763-treated cells expressed class III β-tubulin, NESTIN, and GFAP after
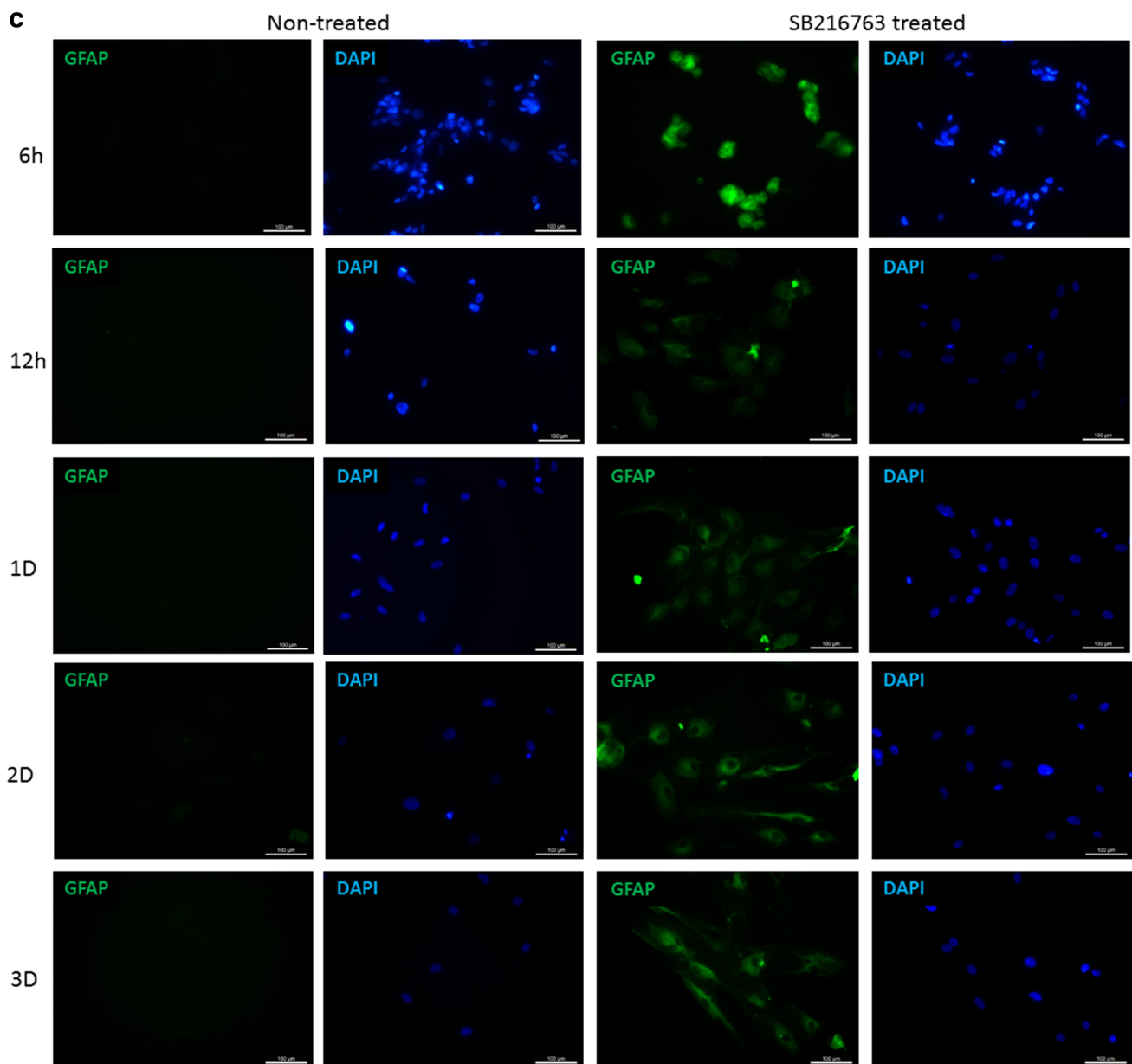
Fig. 4 continued

differentiation. It suggested that the Wnt/β-catenin-signaling pathway, which is able to promote the neural differentiation of ESCs (Kirby et al., [17], iPSCs, and adult neurogenesis [7, 32], might also be crucial for the neural differentiation of hUC-MSCs.

Conclusion

In conclusion, we developed a protocol to induce an efficient direct neural differentiation of hUC-MSCs using a defined medium in combination with the small molecule SB216763. The expression profiles of NESTIN,

class III β-tubulin, and GFAP showed that our protocol could efficiently induce the neural commitment of hUC-MSCs, and moreover, sustain the in vitro survival of hUC-MSCs-derived neural progenitor cells for a short period of time.

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