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Small molecules enhance neurogenic differentiation of dental-derived adult stem cells

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ABSTRACT

Objective: Dental-derived stem cells originate from the embryonic neural crest, and exhibit high neurogenic potential. This study aimed to investigate whether a cocktail of eight small molecules (Valproic acid, CHIR99021, Repsox, Forskolin, SP600125, GO6983, Y-27632 and Dorsomorphin) can enhance the in vitro neurogenic differentiation of dental pulp stem cells (DPSCs), stem cells from apical papilla (SCAPs) and gingival mesenchymal stem cells (GMSCs), as a preliminary step towards clinical applications.

Materials and Methods: Neural induction was carried out with a small molecule cocktail based two-step culture protocol, over a total duration of 14 days. At the 8 and 14 day timepoints, the cells were analyzed for expression of neural markers with immunocytochemistry, qRT-PCR and Western Blot. The Fluo 4-AM calcium flux assay was also performed after a further 14 days of neural maturation.

Results: More pronounced morphological changes characteristic of the neural lineage (i.e. neuritogenesis) were observed in all three cell types treated with small molecules, as compared to the untreated controls. This was corroborated by the immunocytochemistry, qRT-PCR and western blot data, which showed upregulated expression of several early and mature neural markers in all three cell types treated with small molecules, versus the corresponding untreated controls. Finally, the Fluo-4 AM calcium flux assay showed consistently higher calcium transient (F/F_o) peaks for the small molecule-treated versus untreated control groups.

Conclusions: Small molecules can enhance the neurogenic differentiation of DPSCs, SCAPs and GMSCs, which offer much potential for therapeutic applications.

1. Introduction

Neural stem/progenitor cells (NSCs) are considered an ideal cell source for treatment of neurodegenerative diseases and other neural lesions. However, to obtain sufficient numbers of transplantable NSCs for translational applications is extremely challenging. Human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) both demonstrate high propensities for neural differentiation (McComish & Caldwell, 2018). Nevertheless, these cell types are greatly disadvantaged by their tumorigenic potential, ethical controversy surrounding the isolation of hESCs, and safety concerns on the use of viral vectors and genetic modification associated with reprogramming of somatic cells into iPSCs ((McComish & Caldwell, 2018). Mesenchymal stem cells (MSCs) extracted from bone marrow and adipose tissues also demonstrate some capacity for neural differentiation, albeit at much lower efficiencies than hESCs or iPSCs (Volkman & Offen, 2017).

Dental-derived adult stem cells from the oral cavity possess high neurogenic potential because of their embryonic neural crest origin (Parisi & Manfredi, 2016). Autologous dental stem cells can be easily isolated from the clinical waste (i.e. extracted deciduous and wisdom tooth, gingival tissues) of routine dental treatment without imposing additional discomfort to the patient, and then cryopreserved and stored. Subsequently these cells can be re-cultivated and induced into the neural lineage prior to transplantation into the same patient when needed. These cells would not only be immune-compatible, but would also be free of viral vectors, transgenic manipulation and genomic

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Table 1
Composition of culture milieu for neural induction in the small molecule and untreated control groups.

Duration	Basal Medium (Both Groups)	Other Supplements (Both Groups)	Growth Factors (Both Groups)	Small Molecule Group	Untreated Control Group
Initial 8 days	DMEM/ F12: Neurobasal (1:1)	0.5% (v/v) N2 + 1% (v/v) B27	20 ng/mL bFGF	0.5 mM VPA, + 3 μM CHIR99021 + 1 μM Repsox, + 10 μM Forskolin, + 10 μM SP600125, + 5 μM GO6983, + 5 μM Y-27632 + 100 μM cAMP	100 μM cAMP
Next 6 days	DMEM/ F12: Neurobasal (1:1)	0.5% (v/v) N2 + 1% (v/v) B27	20 ng/mL bFGF + 20 ng/mL BDNF + 20 ng/mL GDNF + 20 ng/mL NT3	3 μM CHIR99021, + 10 μM Forskolin, + 1 μM Dorsomorphin + 100 μM cAMP	100 μM cAMP

modification, and are thus more readily translatable to clinical therapy.

To date, diverse dental stem cells, such as dental pulp stem cells (DPSCs) (Gronthos, Mankani, Brahim, Robey, & Shi, 2000), stem cells from apical papilla (SCAPs) (Huang et al., 2008), and gingival mesenchymal stem cells (GMSCs) (Mitrano et al., 2010), have been isolated. Some of these dental stem cells have even been reported to express pluripotency markers including SOX2, NANOG, and OCT4, which are not usually expressed by other adult stem cells (Athanassiou-Papaefthymiou, Papagerakis, & Papagerakis, 2015; Lima et al., 2017). Animal transplantation studies have already demonstrated the positive neuroregenerative potential of DPSCs (Wang et al., 2017; Zhang et al., 2018) and SCAPs (De Berdt et al., 2015; Yang, Li, Sun, Guo, & Tian, 2017). Although the neurogenic potential of GMSCs have been much less studied compared to DPSCs and SCAPs, these cells are of great interest in the field of regenerative medicine because of their unique capacity for fetal-like scar-free healing with minimal inflammation (Venkatesh, Kumar, & Alur, 2017; Zhang, Nguyen, Yu, & Le, 2012), which may be attributed to their unique immunomodulatory properties (Zhang et al., 2009). Hence, this study will focus on the neurogenic differentiation of DPSCs, SCAPs and GMSCs.

The seminal study of Hu et al. (2015) demonstrated that human adult dermal fibroblasts can be directly reprogrammed into the neural lineage by a chemical cocktail of eight small molecules (Valproic acid, CHIR99021, Repsox, Forskolin, SP600125, GO6983, Y-27632, Dorsomorphin), without the need to pass through the neural progenitor stage. These chemically induced neuronal cells resembled neurons derived from human iPSCs and human induced neurons (hiNs), with similar cell morphology, gene expression, and electrophysiological properties (Hu et al., 2015). The major advantage of this approach is that there is no need to use viral vectors, transgenic manipulation, or genomic modification.

It was previously reported that the originating cell type could influence efficiency of reprogramming into iPSCs and subsequent differentiation into the desired cell lineage (Phetfong et al., 2016). Because DPSCs, SCAPs and GMSCs, all originate from the embryonic neural crest, and display high baseline expression of both canonical neural and 'stemness' markers (OCT-4, SOX2, cMYC, KLF4) (Athanassiou-Papaefthymiou et al., 2015; Kang et al., 2016; Lima et al., 2017), these may be a better cell source for the generation of neural lineage cells, as compared to the dermal fibroblasts utilized by Hu et al. (2015). Hence, this study will investigate whether the aforementioned cocktail of small molecules could enhance the in vitro differentiation of DPSCs, SCAPs and GMSCs into the neural lineage, which in turn can potentially be utilized for therapeutic applications.

2. Materials and methods

2.1. Chemical reagents, culture media and supplements

Unless otherwise stated, all chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA), all culture media and supplements were obtained from Life Technologies (Carlsbad, CA, USA), and all plastic labware consumables were obtained from Becton-Dickinson (Franklin Lakes, NJ, USA).

2.2. Cell cultures

The DPSCs, SCAPs and GMSCs utilized in this study were monocultures that were each derived from single donors. The DPSCs utilized in this study were isolated from the extracted third molar of a young adult patient below 25 years of age, and were of the same batch as that utilized in our previous study (Heng et al., 2018). The DPSCs thus derived were characterized by flow cytometric analysis of the expressions of CD45, CD73, CD90, and CD105 as well as multi-lineage differentiation assays (i.e., osteogenic, adipogenic, and neurogenic induction), as previously described (Heng et al., 2018). Human SCAPs were obtained as a gift from Dr. Anibal Diogenes, Department of Endodontics, University of Texas Health Science Center at San Antonio, USA (Yuan et al., 2016). Human GMSCs were kindly gifted by Prof. Shao Hua Ge, Department of Periodontology, School of Stomatology of Shandong University, China (Du, Yang, & Ge, 2015). All cells were cultured in T75 culture flasks with alpha minimum essential medium (α-MEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin antibiotic solution at 37 °C within a humidified 5% CO_2 incubator. The culture media were refreshed every 3–4 days, and confluent monolayers were dissociated with 0.5% (w/v) trypsin-EDTA for serial passage.

2.3. Neural induction of DPSCs, SCAPs and GMSCs with cocktail of small molecules

DPSCs, SCAPs and GMSCs were seeded onto either 6-well or 12-well

culture plates (10,000 cells/cm^2) and cultured in normal growth medium. Neural induction of these dental-derived adult stem cells will be carried out with a small-molecule based two-step culture protocol devised by Hu et al. (2015), over a duration of 14 days (Table 1). Altogether, each cell type was subjected to four differentiation experiments to collect samples for the four different analyses of this study: (i) qRT-PCR, (ii) immunocytochemistry, (iii) Western blot, and (iv) the Fluo-4 AM calcium flux assay. Briefly, when the cells have reached 40%–60% confluence, the culture milieu will be changed to neuronal induction medium (DMEM/F12: Neurobasal [1:1] with 0.5% [v/v] N2, 1% [v/v] B27, 100 μM cAMP, 20 ng/mL bFGF) supplemented with the chemical cocktail VCRFSGY (0.5 mM VPA, 3 μM CHIR99021, 1 μM Repsox, 10 μM Forskolin, 10 μM SP600125, 5 μM GO6983, 5 μM Y-27632). Subsequently, after 8 days of culture, the milieu will be switched to neuronal maturation medium (DMEM/F12: Neurobasal [1:1] with 0.5% [v/v] N2, 1% [v/v] B27, 100 μM cAMP, 20 ng/mL bFGF, 20 ng/mL BDNF, 20 ng/mL GDNF, 20 ng/mL NT3) supplemented with the chemical cocktail CFD (3 μM CHIR99021, 10 μM Forskolin, 1 μM Dorsomorphin), and subjected to a further 6 days of culture, with a single change of culture medium after 3 days. The untreated control will be cultured in the same neural induction medium without small molecules (DMEM/F12: Neurobasal [1:1] with 0.5% [v/v] N2, 1% [v/v] B27, 100 μM cAMP), but with the same dosages of growth factors (20 ng/mL bFGF for the first 8 days, followed subsequently with 20 ng/mL bFGF + 20 ng/mL BDNF + 20 ng/mL GDNF + 20 ng/mL NT3 for the next 6 days). Cell samples were harvested after 8 and 14 days of neural induction culture for qRT-PCR, immunocytochemistry and Western blot analyses, or subjected to a further 14 days of neural maturation for the Fluo-4 AM calcium flux assay.

2.4. qRT-PCR analysis of neural marker expression

Total RNA of the cultured DPSCs was extracted with the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) followed by reverse-transcription with SuperScript VILO Master Mix (Life Technologies, Grand Island, NY, USA). Quantitative real-time PCR (qRT-PCR) analysis was performed with the StepOne Real-Time PCR System (Applied Biosystems, Grand Island, NY, USA) utilizing SYBR Premix Ex Taq II (Cat No. RR820A, Takara Bio, Shiga, Japan). The primer sequences of the neural markers that were utilized for the qRT-PCR analysis were identical to that utilized in our previous study, as shown in Table 2, with Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) being utilized as the endogenous reference control gene. The following amplification parameters were utilized for the qRT-PCR analyses: two minutes at 50 °C, 20 s at 95 °C, and 40 cycles of 3 s at 95 °C followed by 30 s at 60 °C. The $2^{-\Delta\Delta Ct}$ method was used to compute the relative cycle threshold (Ct) values for each gene, which were then normalized against the endogenous GAPDH gene expression. The fold-differences in gene expression by the different cell type that were subjected to neural induction in the various experimental groups were normalized with respect to the same cell type in the corresponding untreated control groups at the same time point. Altogether, there were 3 experimental replicates for each gene analysed by qRT-PCR.

Table 2
Primer sequences utilized in qRT-PCR.

Gene		Primer sequences	
Immature neural markers	Nestin	F	5'-AGCCCTGACCACTCCAGTTTAG-3'
		R	5'-CCCTCTATGGCTGTTTCTTTCTCT-3'
	Musashi1	F	5'-GAGCTTACAGCCATTCCTCTCAC-3'
		R	5'-GCGCTGATGTAACTGCTGACC-3'
	βIII-Tubulin	F	5'-AGACCTACTGCATCGACAACGAGG-3'
		R	5'-GCTCATGGTGGCCGATACCAGG-3'
	MASH1	F	5'-AAGAGCAACTGGGACCTGAGTCAA-3'
		R	5'-AGCAAGAACTTTCAGCTGTGCGTG-3'
	NGN2	F	5'-CGCATCAAGAAGACCCGTAG-3'
		R	5'-GTGAGTGCCCAGATGTAGTTGTG-3'
Mature neural markers	NSE	F	5'-GTCCCACGTGTCTTCCACTT-3'
		R	5'-TGGGATCTACAGCCACATGA-3'
	NeuN	F	5′-GCGGCTACACGTCTCCAACATC-3′
		R	5′-ATCGTCCCATTCAGCTTCTCCC-3′
	NFM	F	5'-GTCAAGATGGCTCTGGATATAGAAATC-3'
		R	5'-TACAGTGGCCCAGTGATGCTT-3'
	MAP2	F	5'-TTGGTGCCGAGTGAGAAGAA-3'
		R	5'-GGTCTGGCAGTGGTTGGTTAA-3'

2.5. Immunocytochemistry for the detection of MAP2, NeuN and NSE

After 14 days of neural induction in the presence or absence of the small molecules, the cells were fixed with 4% (v/v) paraformaldehyde for 20 min, followed by permeabilization with 0.1% (w/v) Triton X-100 for 10 min, and blocking in PBS with 1% (w/v) BSA for 2 h. The fixed cells were then incubated with primary antibodies (1:200 dilutions): mouse anti-MAP2 (Cat. No. 13-1500, Invitrogen, Carlsbad, CA, USA) or rabbit anti-NeuN (Cat. No. 702022, Invitrogen, Carlsbad, CA, USA) or mouse anti-NSE (Cat. No. MA5-17072, Invitrogen, Carlsbad, CA, USA) for 2 h at room temperature. Excess primary antibodies were removed by washing in 1% (w/v) BSA/PBS, and the samples were then incubated with the corresponding secondary antibodies (1:200 dilutions), i.e., goat anti-mouse secondary antibody conjugated to Alexa fluor 488(Cat No. ab150117, Abcam, Cambridge, UK) or goat anti-rabbit secondary antibody conjugated to tetramethylrhodamine (TRITC; Cat No. ab6718, Abcam, Cambridge, UK), for 2 h in the dark at room temperature. After the removal of the excess secondary antibodies by washing in 1% (w/v) BSA/PBS, DAPI was used to stain the cell nuclei, and the samples were imaged under fluorescence microscopy (Olympus, Tokyo, Japan) under the specific excitation/emission wavelengths for TRITC (540/570 nm) and Alexa Fluor 488 (490/520 nm).

2.6. Western blot

After 14 days of neural induction culture, the cells were lysed in M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, Rockford, IL, USA) supplemented with a protease inhibitor cocktail (Thermo-Fisher Scientific, Carlsbad, CA, USA) and incubated on ice for 30 min. Homogenized samples were centrifuged to remove insoluble material (14,000 *g* for 15 min at 4 °C). The extracted cellular proteins were quantified with a bicinchoninic acid assay kit (Pierce, Rockford, IL, USA) and 30 μg of protein were electrophoresed in 10% SDS-PAGE gels (Bio-Rad, Hercules, CA, USA), followed by transfer onto a 0.45-μm Immun-Blot polyvinylidene fluoride membrane (GE Healthcare Life Sciences, Little Chalfont, UK). The blotted membranes were then blocked with 5% (w/v) skimmed milk at room temperature for 1 h before incubation with appropriate primary and secondary antibodies. Mouse anti-NSE primary antibody was obtained from Invitrogen (Cat. No. MA5-17072, Life Technologies, Carlsbad, CA, USA). The secondary antibody was horseradish peroxidase–conjugated goat anti-mouse immunoglobulin G (Cell Signaling Technology, Danvers, MA), and the Pierce ECL Western Blotting Substrate (Thermo-Fisher Scientific, Carlsbad, CA, USA) was used to visualize blots. Depending on the specific manufacturer's instructions, primary and secondary antibodies utilized for Western blots were diluted within a range of 1:500 to 1:1000. For each particular experiment, Western blots were performed in triplicates, with one representative gel image for each experiment being presented in the results.

2.7. Fluo-4 AM calcium flux assay

After 14 days of neural induction culture, the cells in both the small molecule and untreated control groups were further cultured for 14 days in neural maturation medium comprised of DMEM/F12: Neurobasal (1:1) supplemented with 0.5% (v/v) N2, 1% (v/v) B27, 100 μM cAMP, 20 ng/ml bFGF, 20 ng/ml BDNF, 20 ng/ml GDNF, 20 ng/

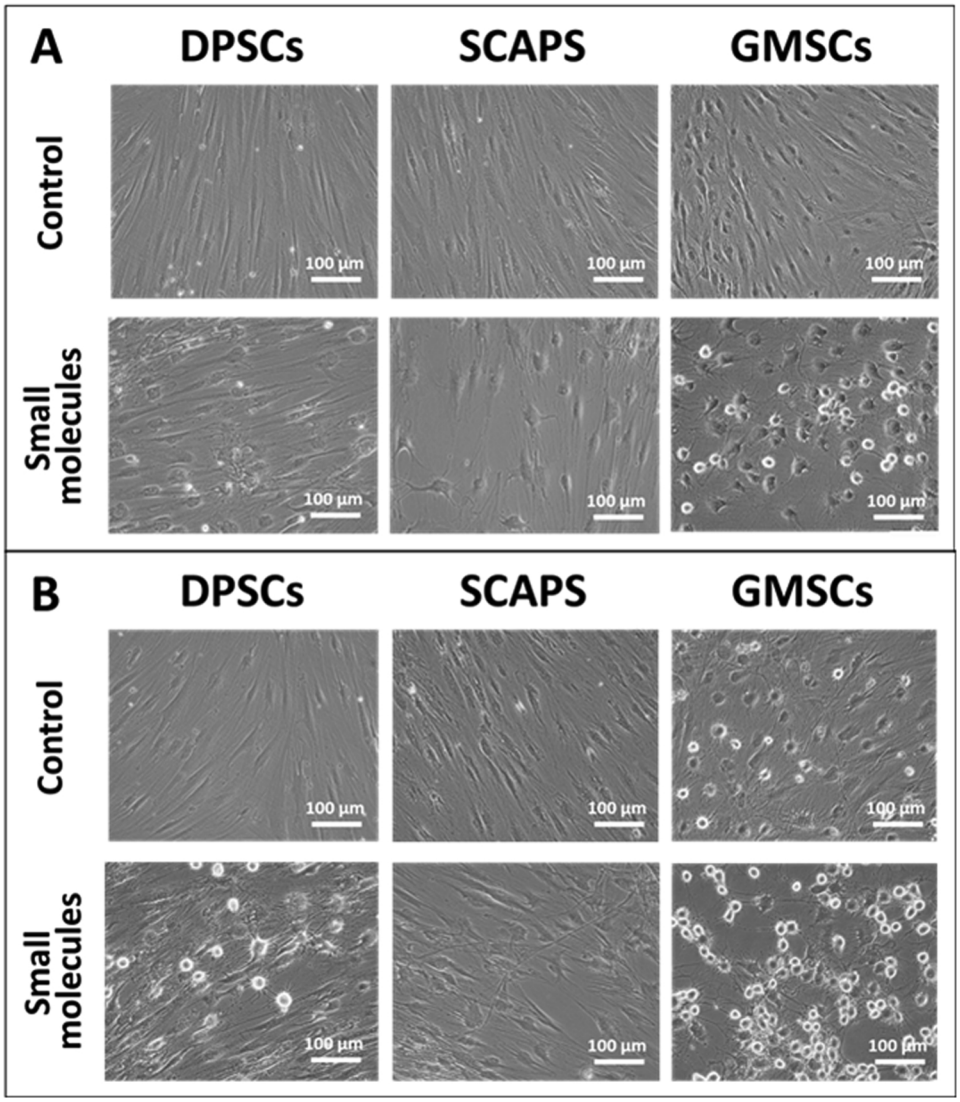

Fig. 1. Morphological changes in DPSCs, SCAPs and GMSCs treated with small molecules versus the corresponding untreated control at the (A) Day 8, and (B) Day 14 timepoints.

ml NT3 and 1% (v/v) penicillin/streptomycin, prior to physiological evaluation with the Fluo-4 AM Calcium flux assay, in accordance with the study of Hu et al. (2015). Subsequently, the cells were then washed three times with Hanks' Balanced Salt Solution (HBSS) and then stained with 3 µM Fluo-4 AM loading solution (Cat. no. F14201, Sigma-Aldrich, St. Louis, MO, USA) for between 30–60 min at 37 °C. Excess dye solution was then removed and the cells were finally washed three times in HBSS, prior to exposure to a 50 mM KCl solution. Time-lapse fluorescence images (excitation/emission wavelength of 490 nm/520 nm respectively) were captured before and after exposure to the 50 mM KCl solution at 10 s intervals, for a total duration of 11 min. The data (total pixel intensity within a defined area for each image) was analyzed using the ImageJ software (National Institues of Health, Bethesda, MD, USA). The Ca^{2+} response was expressed as relative change in the fluorescence emission intensity above the normalized baseline, prior to exposure to the 50 mM KCl solution (F/F_0).

2.8. Statistical analysis

For each differentiation experiment, all assays were carried out on triplicate samples collected from three separate culture wells of multi-well culture plates. The data are expressed as the means ± the standard deviations, and statistically significant differences between the datasets were evaluated with Student's *t*-test using the SPSS 19.0 Statistics Software (SPSS, Chicago, IL, USA). The threshold for statistical significance was set at $P < 0.05$.

3. Results

3.1. Morphological changes upon neural induction with small molecules

The DPSCs, SCAPs and GMSCs were subjected to a two-step neural induction culture protocol (Hu et al., 2015) over a duration of 14 days (first stage lasting 8 days, followed by a second stage lasting a further 6 days). Light microscopy images of the three different adult stem cell types with and without small molecule treatment, were captured at the end of the first and second stages of the neural induction protocol (Fig. 1A on Day 8 and Fig. 1B on Day 14, respectively). It was observed that at both time-points, all three different adult stem cells treated with small molecules exhibited marked differences in cellular morphology with respect to the corresponding untreated controls. In particular, the cells adopted a more rounded morphology that displayed more neurite outgrowths upon being treated with the cocktail of small molecules. By contrast, the untreated controls retained a more fibroblastic-like spindle-shaped morphology typical of adult mesenchymal stem cells.

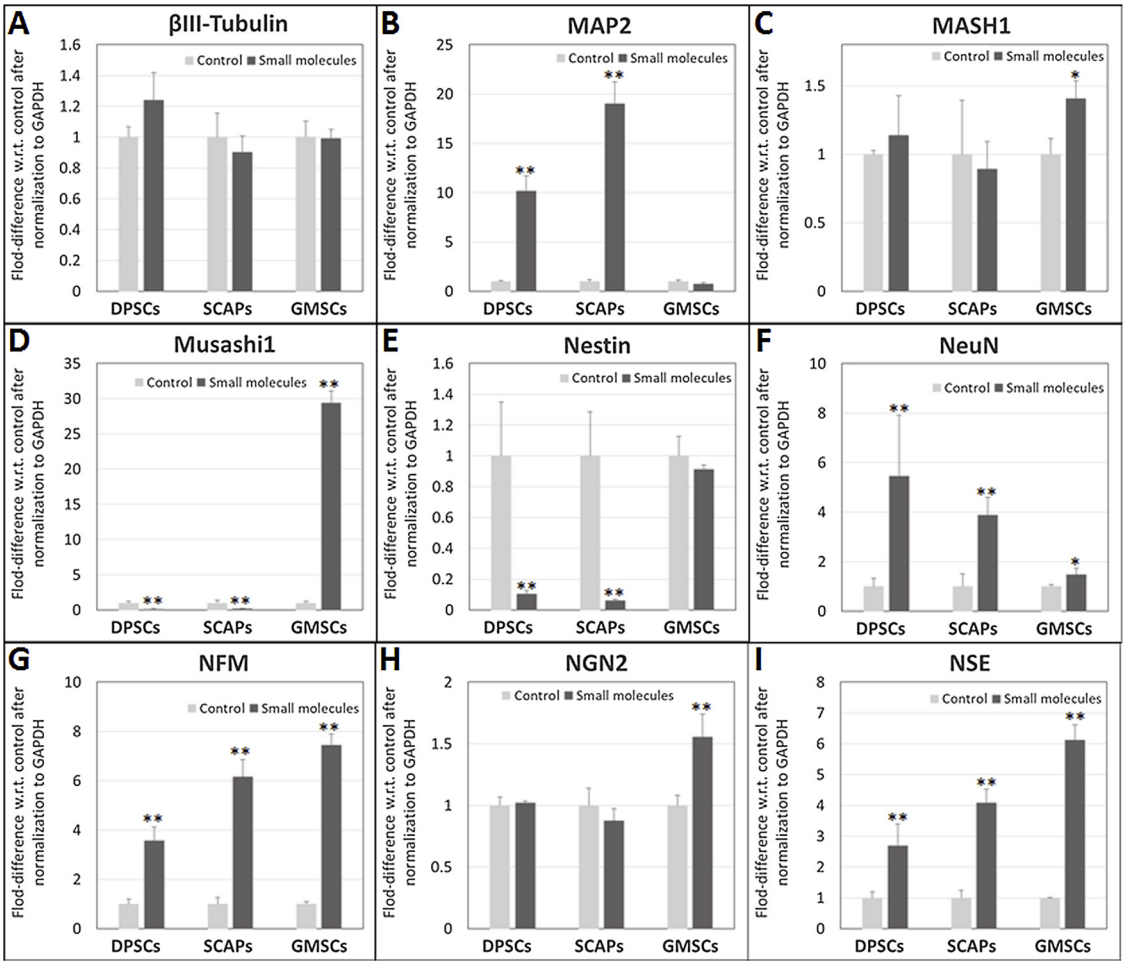

Fig. 2. Quantitative reverse transcription - polymerase chain reaction analyses of neural marker expression by DPSCs, SCAPs, and GMSCs after treatment with small molecules for 8 days, in comparison to the untreated control. Fold-differences in expression with respect to the untreated controls is shown for (A) βIII-tubulin, (B) MAP2, (C) MASH1, (D) Musashi1, (E) Nestin, (F) NeuN, (G) NFM, (H) NGN2 and (I) NSE. Student's *t*-test, *: $P < 0.05$, **: $P < 0.01$.

3.2. qRT-PCR analyses of neural marker expression

qRT-PCR analyses of neural marker expression by DPSCs, SCAPs and GMSCs were carried out at the end of the first (Day 8) and second stages (Day 14) of the neural induction protocol. At the Day 8 timepoint (Fig. 2), DPSCs, SCAPs and GMSCs treated with small molecules displayed significantly upregulated expression of NFM, NeuN and NSE with respect to the untreated control. MAP2 expression was observed to be significantly upregulated in both DPSCs and SCAPs treated with small molecules, but not GMSCs on Day 8. By contrast, NGN2 and MASH1 were observed to be significantly upregulated only in GMSCs treated with small molecules, but not in DPSCs and SCAPs. Both DPSCs and SCAPs treated with small molecules exhibited significantly reduced expression of Musashi1 and Nestin, as compared to the untreated control, whereas GMSCs treated with small molecules displayed significantly increased expression of Musashi1 but no significant changes in the expression of Nestin. There were however no significant changes in the expression of βIII-tubulin in all three different adult stem cell types after 8 days of neural induction with small molecules.

At the Day 14 timepoint (Fig. 3), DPSCs, SCAPs and GMSCs treated with small molecules displayed significantly increased expression of NFM and NSE with respect to the untreated control. MAP2 and NeuN expression were observed to be significantly upregulated in both DPSCs and SCAPs treated with small molecules, but not in GMSCs on Day 14. By contrast, NGN2 and βIII-Tubulin were observed to be significantly upregulated only in GMSCs treated with small molecules, but not in DPSCs and SCAPs. Similar to the results on Day 8, both DPSCs and SCAPs treated with small molecules exhibited significantly reduced expression of Musashi1 and Nestin, as compared to the untreated control on Day 14; whereas there were no significant changes in the expression of these two genes by GMSCs. Both DPSCs and GMSCs treated with small molecules exhibited significant upregulation in the expression of MASH1, whereas no significant changes were observed in SCAPs.

3.3. Immunocytochemistry

The qualitative immunocytochemistry results (Figs. 4–6) corroborated the qRT-PCR data (Figs. 3 & 4). Both DPSCs and SCAPS treated with small molecules exhibited increased immunofluorescent staining for MAP2 (Figs. 4A & 5A respectively) and NeuN (Figs. 4B & 5B respectively), as compared to the untreated control. By contrast, there appeared to be hardly any discernible difference in the staining intensity of MAP2 and NeuN, in the case of GMSCs treated with small molecules versus the untreated control (Figs. 4C & 5C respectively). However, all three different adult stem cell types that were treated with small molecules exhibited increased immunofluorescent staining for NSE, as compared to the untreated control, as seen in Fig. 6. All these results were thus consistent with the qRT-PCR data.

3.4. Western blot

The Western blot results corroborated the qRT-PCR data. As seen in Fig. 7, all three different adult stem cell types that were treated with

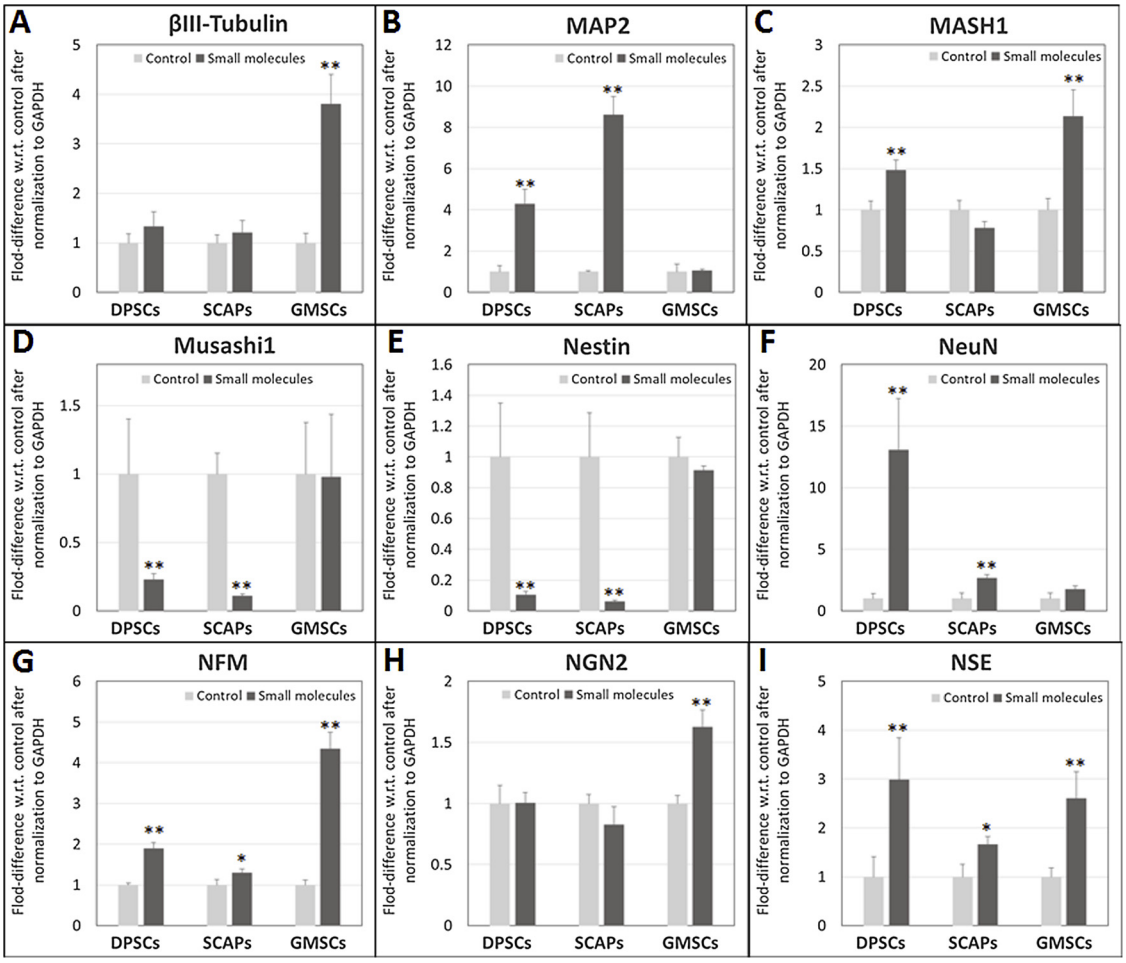

Fig. 3. Quantitative reverse transcription - polymerase chain reaction analyses of neural marker expression by DPSCs, SCAPs, and GMSCs after treatment with small molecules for 14 days, in comparison to the untreated control. Fold-differences in expression with respect to untreated controls is shown for (A) βIII-tubulin, (B) MAP2, (C) MASH1, (D) Musashi1, (E) Nestin, (F) NeuN, (G) NFM, (H) NGN2 and (I) NSE. Student's *t*-test, *: $P < 0.05$, **: $P < 0.01$.

small molecules exhibited increased expression of NSE at the protein level, as compared to the untreated control, at both the Day 8 (Fig. 7A) and Day 14 (Fig. 7B) timepoints.

3.5. Fluo-4 AM calcium flux assay

The Fluo-4 AM calcium flux assay was performed to compare the physiological functionality of the putative neuronal lineages derived from DPSCs, GMSCs and SCAPs upon induction with small molecules, versus the corresponding untreated controls. The results (Fig. 8) showed that the neuronal lineages derived from induction with small molecules for all three different cell type, exhibited consistently higher calcium transient peaks (F/F_o) than the corresponding untreated controls, upon exposure to 50 mM KCl. This thus indicates that induction with the cocktail small molecules enhanced the physiological functionality of the neuronal lineages derived from dental stem cells.

4. Discussion

To date, the majority of animal transplantation studies on the neuroregenerative potential of dental stem cells was based on the grafting of undifferentiated stem cells at the neural lesion site. Whilst many of these studies have reported positive efficacy of undifferentiated dental stem cells in facilitating healing and regeneration of neural deficits, there is still much room for improvement (Heng, Lim, Wu, & Zhang, 2016). In particular, undifferentiated stem cells might undergo spontaneous differentiation into undesired lineages that could hinder neuronal regeneration, such as the formation of fibrotic scar tissue (Heng et al., 2016). Pre-differentiation or pre-commitment of dental stem cells into neural lineages in vitro prior to transplantation may also enhance expression of neuronal adhesion molecules and surface receptors, which could facilitate integration with the host nervous system, thereby improving clinical efficacy of the transplantation (Heng et al., 2016). Moreover, development of efficient in vitro protocols for neurogenic differentiation of stem cells can have diverse non-therapeutic applications. It can be utilized for in vitro toxicological and pharmacological screening of neuroactive drugs and industrial chemicals, and for the in vitro modeling of neurodevelopmental and neurodegenerative disorders (Heng et al., 2016; Yap et al., 2015). Hence, this study investigated the use of a two-step small molecule-based protocol developed by Hu et al. (2015), to enhance the commitment and differentiation of DPSCs, SCAPs and GMSCs into the neural lineage in vitro.

The study of Hu et al. (2015) reported that their formulated cocktail of small molecules reprogrammed dermal fibroblasts into the neural lineage via simultaneous downregulation of fibroblast-specific genes accompanied with concomitant upregulation of endogenous neuronal transcriptional factors within these cells, as shown by their microarray data. Previous studies have reported that the recombinant overexpression of certain neural-specific transcription factors can reprogram mouse or human somatic cells into neuronal cells (Caiazzo et al., 2011; Pang et al., 2011). Indeed, the microarray data of Hu et al. (2015) demonstrated conclusively that the gene expression profiles of their chemically-reprogrammed cells more closely matched that of control

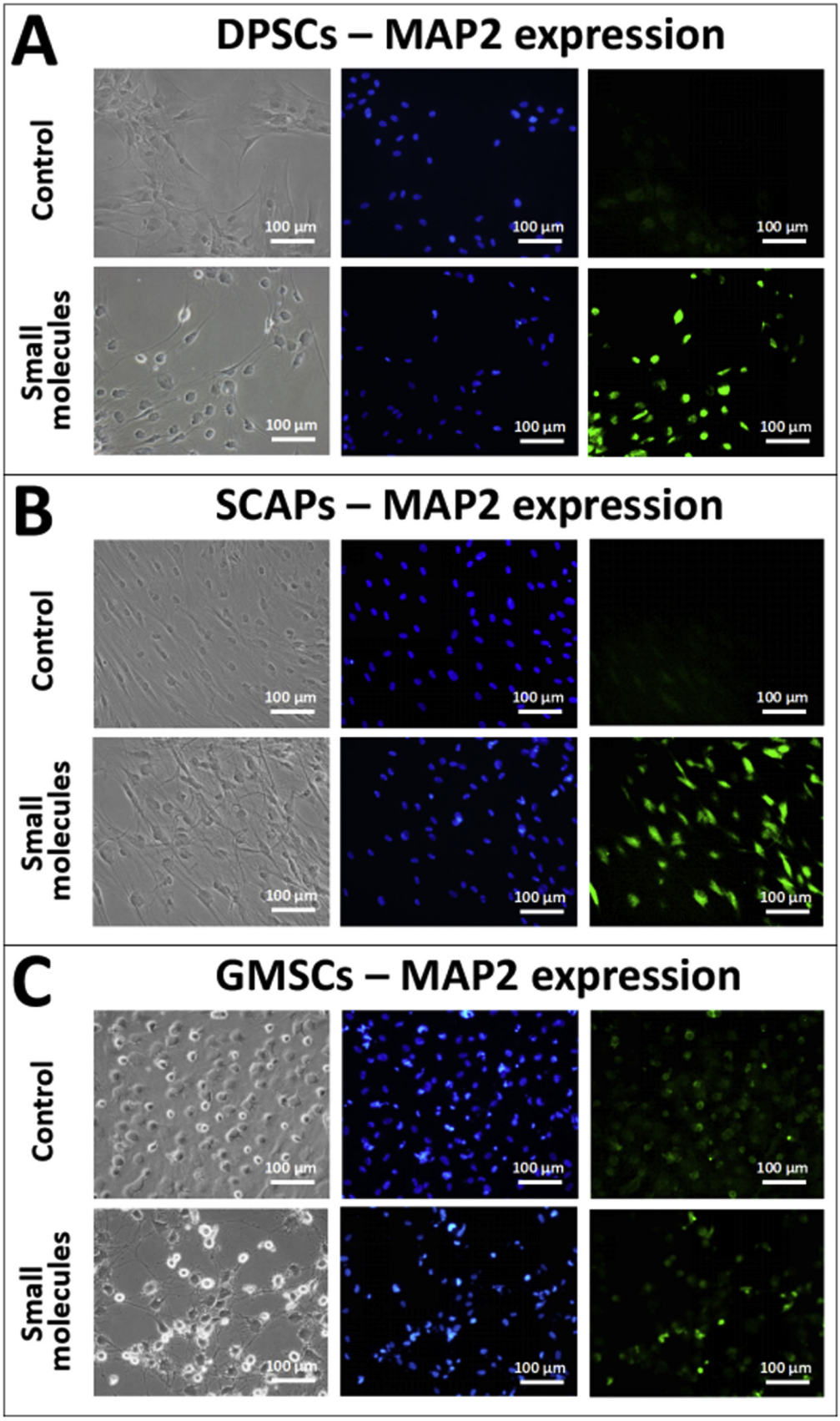

Fig. 4. Immunocytochemistry for detection of MAP2 expression by (A) DPSCs, (B) SCAPs, and (C) GMSCs, after 14 days of neural induction.

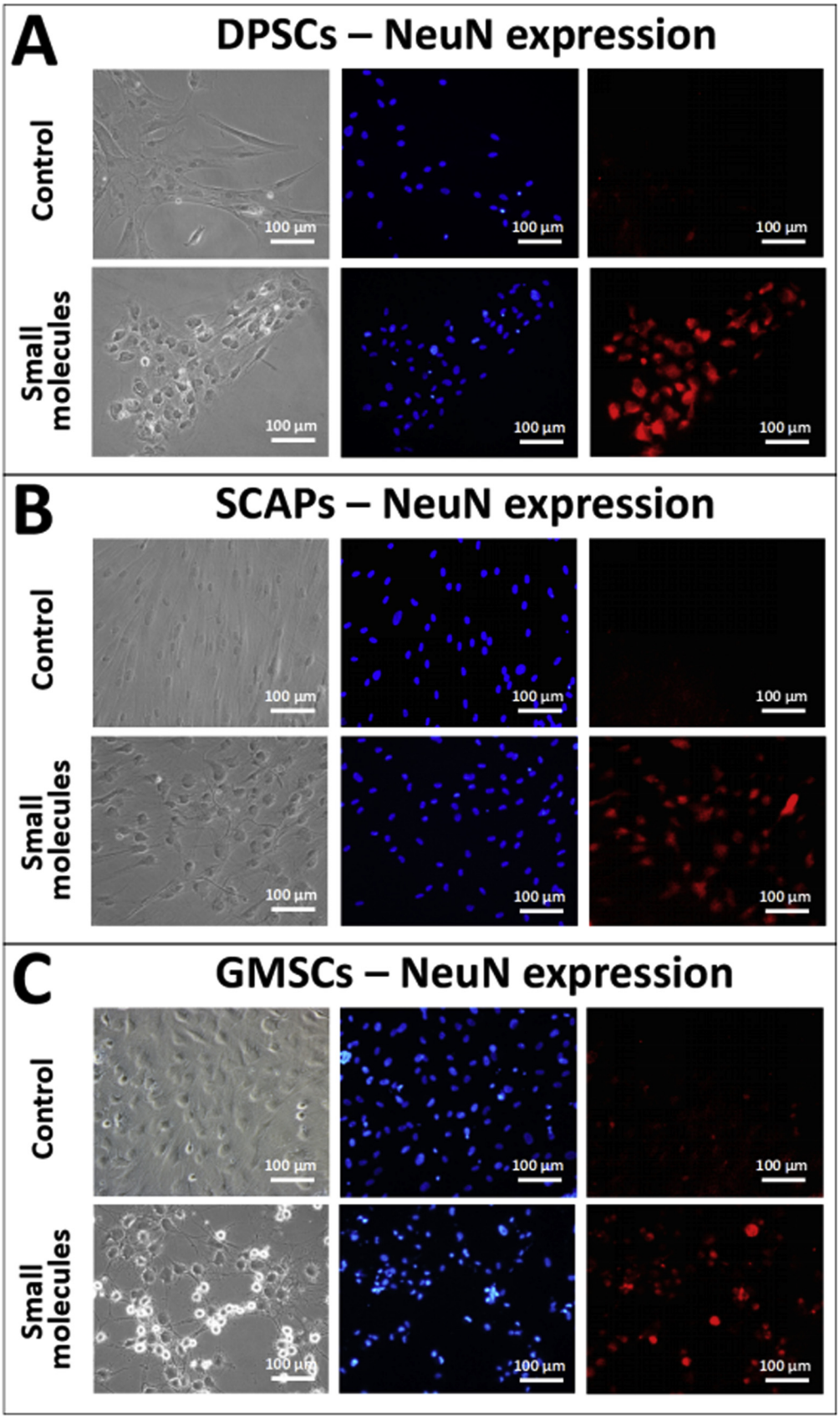

Fig. 5. Immunocytochemistry for detection of NeuN expression by (A) DPSCs, (B) SCAPs, and (C) GMSCs, after 14 days of neural induction.

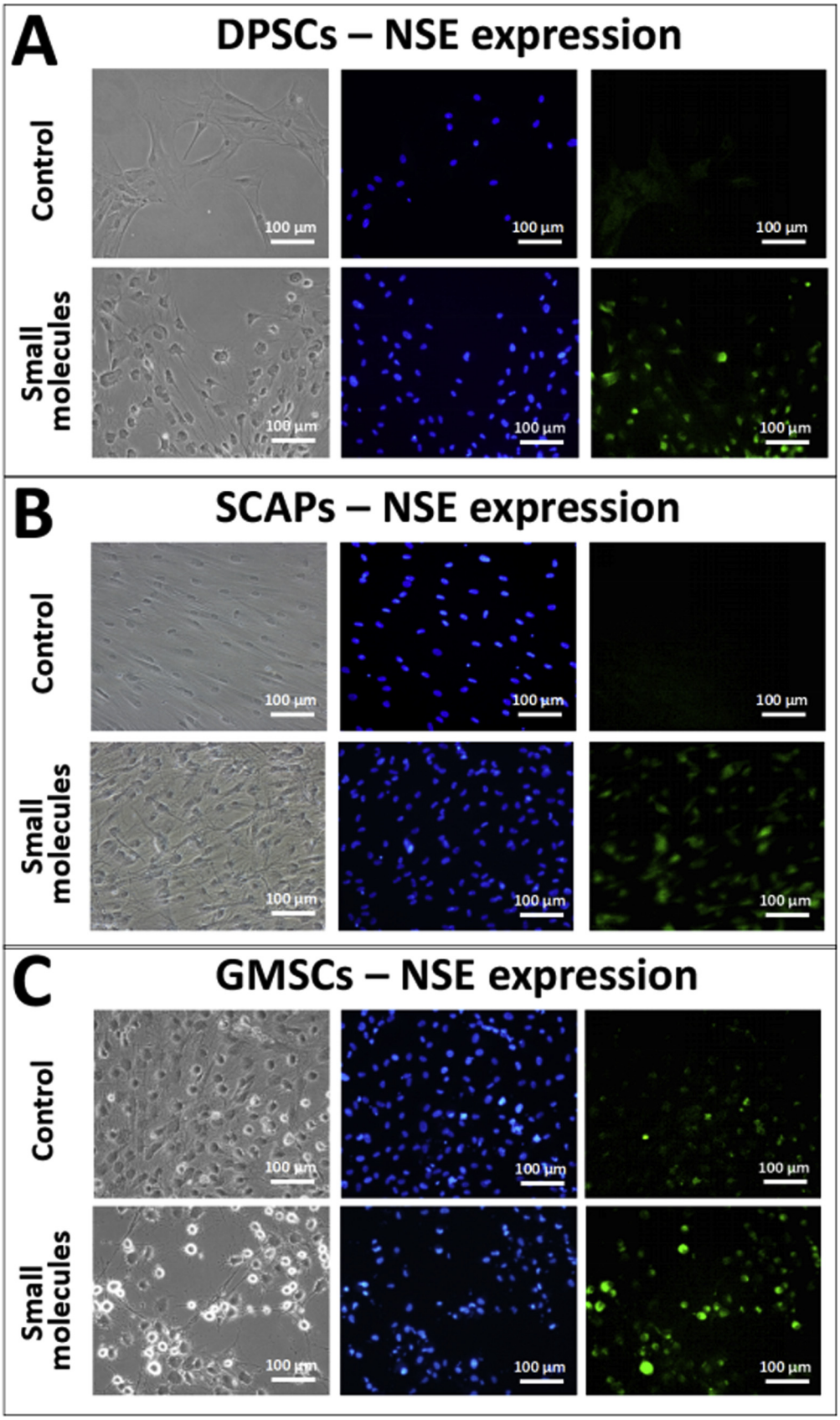

Fig. 6. Immunocytochemistry for detection of NSE expression by (A) DPSCs, (B) SCAPs, and (C) GMSCs, after 14 days of neural induction.

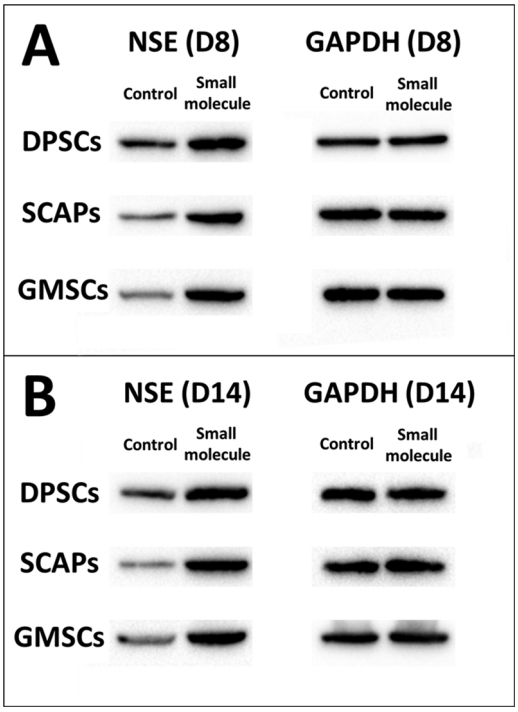

Fig. 7. Western blot analyses for detection of NSE expression at the protein level by DPSCs, SCAPs, and GMSCs at the (A) Day 8, and (B) Day 14 timepoints.

primary neurons than that of the untreated adult dermal fibroblasts. It is thought that the cocktail of small molecules formulated by Hu et al. (2015) successfully reprogrammed adult dermal fibroblasts into the neural lineage by achieving a fine-tuned modulation of multiple signaling pathways. The physiological effects and signaling pathways modulated by each small molecule of the chemical cocktail utilized in this study and that of Hu et al. (2015), are briefly summarized in Table 3.

The results of this study showed conclusively that the small molecule cocktail significantly enhanced the differentiation of DPSCs, SCAPs and GMSCs into the neural lineage at both the Day 8 and Day 14 timepoints, as seen by the morphological changes, qRT-PCR, immunocytochemistry and Western blot data. The physiological functions of the various mature and immature neural markers analyzed in this study are summarized in Table 4. It was observed that the changes in gene expression profile induced in DPSCs by the small molecule cocktail was more similar to SCAPs, as compared to GMSCs. As seen in the qRT-PCR data, there was upregulation of the mature neural markers NeuN, NFM, NSE and MAP2 in both DPSCs and SCAPs at both the Day 8 and Day 14 timepoints, together with concomitant downregulation of the early upstream neural markers Musashi1 and Nestin at both timepoints. Indeed, maturation of the neural lineage is well-known to be associated with the downregulation of early upstream neural markers such as Musashi1 and Nestin (Gioia et al., 2014; Park et al., 2010). By contrast, GMSCs demonstrated upregulation of a mixture of both mature neural markers – NeuN (D8 only), NFM (D8 & D14), NSE (D8 & D14), as well as immature neural markers – Musashi1 (D8 only), βIII-Tubulin (D14 only), NGN2 (both D8 and D14), and MASH1 (D8 and D14). This in turn could imply that treatment with the small molecule cocktail may result in a lesser degree of neural maturation in GMSCs, as compared to DPSCs

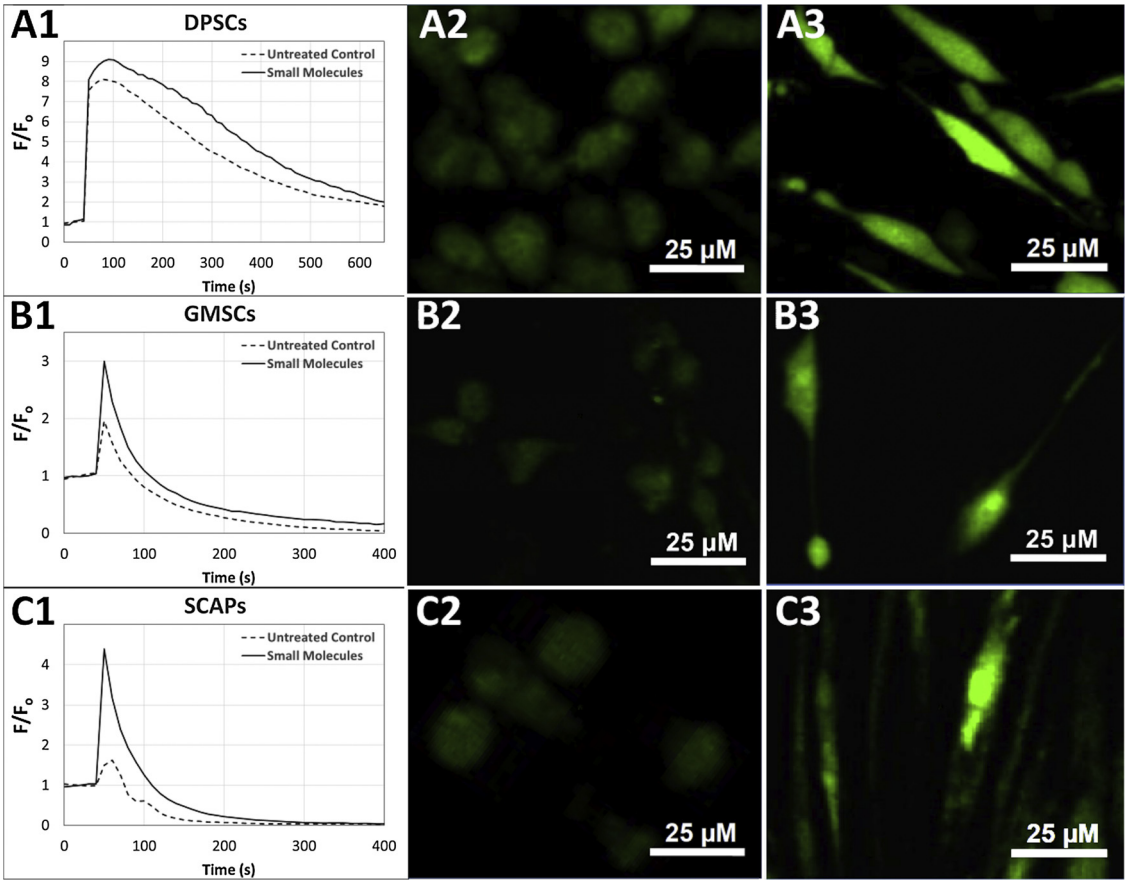

Fig. 8. Fluo-4 AM calcium flux assay to assess physiological functionality of (A1) DPSCs, (B1) GMSCs, and (C1) SCAPs, after a further 14 days of neural maturation. (A2 & A3) Respective fluorescent images of control and small molecule treated DPSCs at peak f/fo. (B2 & B3) Respective fluorescent images of control and small molecule treated GMSCs at peak f/fo. (C2 & C3) Respective fluorescent images of control and small molecule treated SCAPs at peak f/fo.

Table 3
Physiological effects of small molecules.

Small Molecule	Physiological effects / Signalling pathways modulated	Key References
Valproic acid (VPA)	Induction of cells into a more plastic state for cell fate transition through epigenetic modification via inhibition of histone deacetylases	Huangfu et al. (2008)
Forskolin	Enhance the effects of recombinant NGN2 overexpression in reprogramming human fibroblasts into cholinergic neurons.	Liu et al. (2013)
SP600125	Facilitate neural reprogramming of human fibroblasts that were transfected with OCT4 alone	Zhu et al. (2014)
GO6983	Inhibition of Protein Kinase C, which improved plasticity of naive human pluripotent stem cells	Gafni et al. (2013)
Y-27632	Facilitate survival of neural lineage cells	Lamas et al. (2014) Li, Meng, Krawetz, and Liu, (2008)
Repsox	Inhibition of TGF-β pathways, which have been reported to enhance direct reprogramming of human fibroblasts into the neural lineage through recombinant overexpression of ASCL1 and NGN2	Ladewig et al. (2012)
CHIR99021	Inhibition of GSK-3 kinases, which have been reported to enhance direct reprogramming of human fibroblasts into the neural lineage through recombinant overexpression of ASCL1 and NGN2	Ladewig et al. (2012)
Dorsomorphin	Inhibition of the BMP signalling pathway by targeting the type I receptors ALK2, ALK3, and ALK6.	Morizane, Doi, Kikuchi, Nishimura, and Takahashi, (2011) Zhou et al. (2010)

Table 4
Physiological functions of mature and immature neural markers.

Gene		Physiological function	Key references
Immature neural markers	Nestin	Neural stem cell marker	Bernal and Arranz (2018)
	Musashi1	Upstream transcription factor that initiates differentiation and transition of neural stem cells to neural progenitors	MacNicol, Hardy, Spencer, and MacNicol, (2015)
	βIII-Tubulin	Early marker of the differentiation of neural precursors	Memberg and Hall (1995)
	MASH1	Transiently expressed by early neuroepithelial and neural crest cells	Lo, Johnson, Wuenschell, Saito, and Anderson, (1991)
	NGN2	Marker of proliferating neural progenitors	Ozen et al. (2007)
Mature neural markers	NSE	Brain-specific glycolytic enzyme expressed in mature neurons	Isgrò, Bottoni, and Scatena, (2015)
	NeuN	Nuclear antigen that is strongly expressed in post-mitotic mature neurons	Gusel'nikova and Korzhevskiy (2015)
	NFM	Cytoskeletal protein expressed by mature neurons, which is associated with electrophysiological maturation	Steinschneider et al. (1996)
	MAP2	Stabilizes microtubules in the dendrites of post-mitotic mature neurons	Soltani et al. (2005)

and SCAPs.

We hypothesize that the small molecule cocktail induced less mature neural differentiation in GMSCs due to their higher proliferative capacity compared to DPSCs and SCAPs. In a recent study by Angelopoulos, Brizuela, and Khoury, (2018), GMSCs exhibited significantly higher proliferation rates than haploidentical DPSCs derived from the same donor. A number of previous studies had conclusively demonstrated an inverse relationship between proliferation and neural maturation, such that cells with a higher proliferative capacity had lesser propensity for neural maturation (Gao et al., 2016; Horie et al., 2004; Kong et al., 2015). Nevertheless in this study, we did not attempt to directly compare the proliferation of GMSCs, DPSCs and SCAPs, because these were all derived from different donors with variable ages, gender and medical conditions. It must also be noted that DPSCs and SCAPs are phenotypically more similar to each other, as compared to GMSCs, as both these cell types originate from the dental papilla of the developing tooth germ (Zhu, Zhang, Gu, Liu, & Zhou, 2018), unlike GMSCs. Hence, it would thus be expected that DPSCs and SCAPs respond more similarly to the small molecule cocktail under neural inducing conditions, as compared to GMSCs.

Immunocytochemistry data for the detection of MAP2, NeuN and NSE at the Day 14 timepoint further corroborated the qRT-PCR data. Because NSE is the only neural marker that is consistently being upregulated in all three different cell types at both timepoints after treatment with the small molecule cocktail; it was thus selected for Western blot analysis, which in turn corroborated both the qRT-PCR and immunocytochemistry data. Finally, the Fluo-4 AM calcium flux assay demonstrated that the neuronal lineages derived from induction with small molecules for all three different cell type, exhibited consistently higher calcium transient peaks (F/F_o) than the corresponding untreated controls, upon exposure to 50 mM KCl. This would thus indicate that induction with the cocktail small molecules enhanced the physiological functionality of the dental stem cell-derived neuronal lineages. Nevertheless, it must be noted that the calcium flux data is insufficient to confirm maturity of neuronal differentiation under small molecule induction. Only the generation of action potential and evidence of sodium currents with the patch clamp technique can provide such proof of mature neuronal differentiation. This will be carried out in our future study together with transplantation in live animal models; because the main focus of this study is to demonstrate that small molecules can enhance the neuronal differentiation of dental stem cells.

Over the course of neural induction with the small molecule cocktail, we observed a more significant level of cell death in all three cell types, as compared to the untreated control. This is expected since it is well-established that apoptosis of immature neural cells widely occurs during neural differentiation (Eizenberg et al., 1996; Esdar, Milasta, Maelicke, & Herget, 2001; Raff et al., 1993), which is required to avoid hyperproliferation of brain tissue during early embryonic development (Blaschke, Staley, & Chun, 1996; Sommer & Rao, 2002). Hence, enhanced neural differentiation/maturation induced by the small molecule cocktail would thus translate to higher levels of cell death. There is also the possibility that some of the small molecules may exert cytotoxic effects on the cells. Nevertheless, under the neural inducing conditions of this study, it would be difficult to discern whether apoptosis is being triggered by cytotoxic stress, or by the process of neural maturation itself. In this study, we did not attempt to directly compare the apoptosis of GMSCs, DPSCs and SCAPs during neural differentiation, because all three cell types were derived from different donors with variable ages, gender and medical conditions. This will be the subject of our future investigations if we are able to get hold of haploidentical DPSCs, SCAPs and GMSCs from the same donor, which would make the three different cell types more directly comparable

In conclusion, this study demonstrated that the small molecule cocktail formulated by Hu et al. (2015) for neurogenic reprogramming of adult dermal fibroblasts can also enhance the commitment and differentiation of DPSCs, SCAPs and GMSCs into the neural lineage. Changes in the gene expression profile induced by the small molecule cocktail were more similar between DPSCs and SCAPs, which both exhibited upregulation of mature neural markers together with concomitant downregulation of immature neural markers. By contrast, GMSCs treated with the small molecule cocktail exhibited upregulation of a mixture of mature and immature neural markers, which could possibly indicate that it is being induced to a less mature state compared to DPSCs and SCAPs. Neuroregenerative capacity of the neural lineages derived from DPSCs, SCAPs and GMSCs needs to be further investigated by transplantation studies in live animal models.

Conflict of interest disclosures

Authors declare no conflict of interest.

Ethical approval

Ethical approval for this study was obtained from the Institutional Review Board of The University of Hong Kong.

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