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A Combination of 8 Bromo CyclicGMP with Neural Inducing Agents Could Efficiently Convert Human Adipose Stem Cells to Neural Progenitors

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ABSTRACT

Human adipose stem cells (hASCs) among other stem cells offer a glimpse of possibilities in neurodegenerative disease treatment. By that fact, researchers have been developing neural transdifferentiation methods of hASCs over the last decades. Various types of inducing agents were applied to improve the protocol. NO-cGMP signaling pathway is known to play a vital role in regulation of cell growth, survival, differentiation, proliferation, migration, axon guidance and many other processes. cGMP, as the mediator, helps support NO regulation of cell survival, differentiation and neuroprotective regulation. Previous research demonstrated that applying NO donor agents in neural differentiation medium could convert hESCs into neural- like cells. However, neural differentiation through this specific signaling pathway on hASCs has not yet been investigated. In this study, 8 Bromo- cyclic GMP (8Br- cGMP) was added to neural induction cocktails to improve the neural transdifferentiation efficiency of hASCs. Results demonstrated that hASCs under this condition exhibited higher expression of neural genes and could further differentiate into mature neuronal and glial cells. Our findings highlighted that differentiated hASCs displayed neuronal progenitor profile, yet remained their plasticity as they can differentiate toward glial cells. Future studies employing in vivo transplantation models and their underlying mechanisms are warranted.

Keywords: Adipose stem cells, Mesenchymal, Neural differentiation, NO-cGMP, Stem cells, 8 Bromo cyclicGMP

INTRODUCTION

Stem cells have been considered as a promising tool in cell-based therapy. Stem cell therapy aims to induce the neuro-restorative process that is essential to support recovery after a loss of function. Human embryonic stem cells (hESCs) have seemed to be the most powerful tool for cell therapy in animal models, but several challenges need to be resolved before translating ESCs application in humans. ESCs are genetically and epigenetically unstable which could cause teratoma formation upon transplantation. Another source is neural stem cells (NSCs) which have unlimited differentiation potential in vitro and potential of producing all kinds of brain-specific cell types that make them a feasible candidate in the therapy (Bain et al., 1995; Lee et al., 2000). However, due to their accessible difficulty, limited source of cells, and weak immune rejection potential, utilizing these cells in cell-based therapy would be challenging.

Due to less ethical controversy, mesenchymal stem cells (MSCs) have been recently brought up against those cells. These cells hold many features which are easily collected, abundant availability, extensively expansion in vitro, and the ability to repair the tissue by differentiating itself to replace injured cells and/or by creating an environment favorable for the tissue repair by endogenous cells (Izadpanah et al., 2005). Moreover, increasing evidence on the neuroectodermal lineage commitment of mesenchymal stem cells (MSCs) has raised a good appeal of MSCs in cell-based therapies (Deng et al., 2001; Jiang et al., 2002; Sanchez-Ramos et al., 2000, Woodbury et al., 2002). Among many sources of MSCs, adipose tissue seems to be a promising source of cells since they are available in large quantities (over hundreds of million cells per individual), easily isolated and can be cultured for several months in vitro with low levels of senescence (Zuk et al., 2001; Zuk et al., 2002). Investigating the utilization and application of these cells would be beneficial in cell-based therapy.

Nitric oxide (NO) is a short-lived diatomic free radical species that is synthesized by nitric oxide synthases (NOS). At low nanomolar concentrations of NO, activation of soluble guanylyl cyclase (sGC: the major NO receptor) triggers intracellular cyclic GMP level elevation (Arnold et al., 1977; Katsuki et al., 1977). This signaling pathway, also known as NO-cGMP

signaling pathway, is involved in diverse physiological processes, such as smooth muscle relaxation, neurotransmission, blood pressure regulation, inhibition of platelet aggregation and immunomodulation. Its roles at cellular level are to regulate cell growth, survival, differentiation, proliferation, migration, axon guidance and many other processes through a variety of downstream signaling cascades depending on cell type specific regulation. cGMP, as the mediator, help support NO regulate cell survival, differentiation and neuroprotective regulation (Thippeswamy and Morris, 1997, 2001; Ciani et al., 2002). 8 bromo cyclic GMP (8Br-cGMP), NO donor, has been extensively investigated in neural differentiation of many types of cell, but the evidence of applying this agent in human MSCs is rarely seen. The effect of this NO donor on neural differentiation of hASCs remained questionable. In this study, the effect of 8 bromo cyclic GMP was investigated whether they could induce the differentiation of hASCs into neural-like cells that hold both neural phenotypic and genotypic profiles.

MATERIAL AND METHODS

Isolation and expansion of hASCs

Isolation of hASCs was carried out using previously procedures (Gimble and Guilak, 2003; Zuk et al., 2001). This work was approved by the Ethics Committee for Researches Involving Haman Subjects, Suranaree University of Technology and Maharat Nakorn Ratchasima Hospital Institutional Review Board (MNRH IRB). Briefly, visceral adipose tissue was collected with written and informed consent from three donors who underwent surgery. The tissue then was stored in 4°C sterile phosphate buffered saline without calcium and magnesium (PBS) and transferred to the laboratory. Then, adipose tissue was immersed in 75% ethanol for 30 sec under sterile manner. The tissue was manually minced into small fragments in PBS at 37˚C and digested by collagenase type I with stirring magnetic bar in a water bath at 37˚C for 60 min. The cell suspension was centrifuged at 400g for 5 min and the supernatant was discarded. The cells were seeded and expanded in media containing alpha modification of Eagle's medium (α-MEM; GIBCO-BRL) supplemented with 10% fetal bovine serum (FBS; Hyclone) and 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen), and then incubated at 37°C under humidified atmosphere of 5% CO_2 in air. The medium was changed three times a week. After characterization, the most promising hASCs cell line was used for the experiment in the present study.

Immunocytochemistry analysis

Passage 4 of hASCs at 70% confluence were fixed by 4% PFA (Sigma) at room temperature for 15 min. They were washed with PBS and incubated with blocking buffer consisting of 10% normal goat serum at room temperature for 2h. After that, they were incubated at 4°C overnight with primary rabbit antibodies raised against CD45, CD73, CD90 and CD105; with primary mouse antibodies raised again CD34 and Vimentin (all antibodies' dilution factor was 1:200 and purchased from Santa Cruz Biotechnology, California, USA). Alexa fluor® 488 goat anti rabbit IgG (1:1000; Invitrogen) were used for 2h at room temperature. Cells were then counterstained with 1 mg/ml 40, 6-diamino-2-phenylindole (DAPI; Sigma) for 10 minutes. They were observed and photographed under a fluorescent microscope (Olympus, model BH2-RFL-T3).

Multipotency analysis

Isolated cells were induced to differentiate into mesodermal lineages which are osteoblasts, adipocytes and chondrocytes. hASCs were induced to osteoblasts under the hMSCs culture medium without any fetal bovine serum and supplemented with 100 nM dexamethasone, 0.2 mM L-ascorbate-2-phosphate, 2 mM L-glutamine, 10 mM β-glycerophosphate, 100 units/ml penicillin and 100 µg/ml streptomycin (all from Sigma –Aldrich). hASCs differentiated to adipocytes were under the medium without fetal bovine serum and supplemented with 10 µg/ml insulin, 100 µM indomethacin, 1 µM dexamethasone, 0.5 mM isobutyl methylxanthine (IBMX), 100 units/ml penicillin and 100 µg/ml streptomycin (all from Sigma –Aldrich). For chondrocytes differentiation, hASCs were under the medium with 2% fetal bovine serum and supplemented with 0.05 mM ascorbate 2-phosphate, 40 µg/ml L-proline, 1 mM sodium pyruvate, 100 nM dexamethasone, 100 units/ml penicillin and 100 µg/ml streptomycin (all from Sigma –Aldrich). Additionally, 10 ng/ml TGF-β3 (R&D Systems, Minnesota, USA) and 1% ITS-X (Gibco®, Invitrogen) were also supplemented. The medium was subsequently changed every other day for three weeks. The culture was then fixed in 4% PFA followed by Alizarin Red S, Oil Red O and Alcian blue 8GX (all from Sigma –Aldrich) staining for 30 minutes, respectively. Morphology and stained cells were observed under inverted microscope (Olympus, model BH2-RFL-T3).

Neural transdifferentiation of hASCs

hASCs were converted to neural cells by a modified protocol of Johe et al. (Johe et al, 1996). The culture dish was coated with 10µg/ml Poly-L-

Lysine (Cultrex) and 5µg/ml mouse laminin (Cultrex). hASCs at P5 were under N2B27 basal medium which is a mixture of DMEM/F12 (Gibco) and Neurobasal (Gibco) medium (1:1) supplemented with 1%N2 (v/v) and 2%B27 (v/v) supplementation (Gibco). To find the optimal concentration of 8Br-cGMP (Sigma), various concentrations (0µM, 10µM and 100µM) of 8Br-cGMP were added to induction medium for 14 days. Additional supplementations which are 20ng/ml epidermal growth factor (EGF, Sigma), 20ng/ml basic fibroblast growth factor (bFGF, Sigma), 100ng/ml human noggin (Prospec), 10µM SB431542 (Calbiochem), 10µM retinoic acid (Sigma), 5U/ml Heparin (Sigma), 100U/ml penicillin and 100µg/ml streptomycin (Invitrogen) were added in every conditions. Induction medium was changed every two days for 14 days. hASCs under N2B27 basal medium supplemented with 0.5% DMSO (v/v, Sigma) and DMEM/F12 medium conditions, serving as controls in this study, were cultured along with 8Br-cGMP treated groups. hASCs at D7 and D14 were characterized and examined. Optimal condition was selected for terminally differentiate to mature neurons and glia using NeuroCult™ NS-A differentiation kit for human cells (Stem Cell, USA) for 10 days. Neural like cells were analyzed by immunocytochemistry and RT-qPCR analyses. Every experiment was done in triplicate independently.

Characterization of neural induced hASCs (NI-hASCs)
Immunocytochemistry cell analysis

NI-hASCs at d7, d14 and mature neural cells cultured on 8-well cell culture chamber slide (SPL Life Sciences) were fixed in 4% PFA, permeabilized with 0.2% Triton X-100 (Sigma, USA) in PBS at room temperature for 10 min, and blocked with 10% normal goat serum (Zymed, USA) at room temperature for 60 min. Cells were incubated at 4˚C overnight with the following primary antibodies: mouse a-Nestin (Abcam, 1:200), mouse a-Sox2 (R&D systems, 1:50), rabbit a-Glial-Fibrillary-Acidic-Protein (GFAP, Abcam, 1:200), mouse a-Beta III tubulin (TUJ1, Abcam, 1:200), mouse a-Neurofilament-Light (NF-L, Abcam, 1:200), mouse a-Olig2 (Abcam, 1:100) and goat a-Choline acetyltransferase (ChAT, R&D systems, 1:100). Alexa fluor® 488 goat anti rabbit IgG (1:1000; Invitrogen), Alexa fluor® 488 goat anti mouse IgG (1:1000; Invitrogen) and Alexa fluor® 488 donkey anti goat IgG (1:1000; Invitrogen) were used based on primary antibody reactive species for 2 hours at room temperature. NI-hASCs were then counterstained with 1 mg/ml 40, 6-diamino-2-phenylindole (DAPI;

Sigma) for 10 minutes. Finally, the cells were observed and photographed under a fluorescent microscope (Nikon, model Eclipse-Ti-S1 DRi-UJ-D).

RT-qPCR Analysis (Biorad CFB3240G Chromo4 Thermocycle)

Total RNA extraction was performed using Total RNA extraction mini kit (RBC Bioscience). DNaseI set (amplification grade, life technologies) was used for the removal of genomic DNA in the RNA sample. Then, RNA was reverse-transcribed in the presence of oligo-dT primer using iScriptTM Reverse Transcription Supermix for RT-qPCR (Bio-Rad). Primers that are specific to different types of neural cells are in Table S1 (supplement data). The optimum annealing temperature for each primer pair was determined experimentally. The KAPA SYBR® Fast qPCR Kit (KAPA Biosystems) was used for all RT-PCRs as per the manufacturer's instructions with the addition of cDNA or RNase-free water (negative control). mRNA values for each gene were normalized to *β-actin*. The Ct value was calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schimittgen, 2001).

Neurite analysis of NI-hASCs

The morphology of NI-hASCs treated with various 8Br-cGMP concentrations for 7 days was analyzed using In Cell Analyzer 1000 Cellular Imaging and Analysis System (GE Healthcare Life Science). Neurite outgrowth automated scoring was performed on the samples immunostainned with TUJ1 together with DAPI as previously described by Ramm et al., 2003 with some modification. Differentiated cells that were positive for TUJ1/DAPI were selected based on specific criteria. Well that contains more than 2,000 cells was selected and cells were analyzed in 12 different fields (N=12).

Neuronal and glial differentiation of 10µM 8Br-cGMP induced NI-hASCs

NI-hASCs with optimal 8Br-cGMP were cultured under NeuroCult™ NS-A Differentiation medium (STEMCELL™ Technologies) for another 10 days as recommended by the instruction manual. To differentiate NI-hASCs into neurons, 10ng/ml Nerve Growth Factor (NGF) was added into the differentiation medium as well as 10ng/ml Platelet Derived Growth Factor-AA (PDGF-AA) for glial differentiation. NI-hASCs under NGF, PDGF, and normal NS-A differentiation medium were used to examine their gene expression pattern and phenotype by RT-qPCR and immunocytochemistry analyses.

Statistical analysis

All experiment was performed in triplicate. Neurite outgrowth analysis was measured in 6 replications. For RT-qPCR analysis, three independent samples were replicated in the analysis. Statistical analysis was performed using SPSS 17.0 (SPSS, Inc., USA). RT-qPCR was analyzed by two-way ANOVA. Immunopositive cell and neurite outgrowth analyses were determined using one-way ANOVA. The level of significance was set at $p < 0.05$. Post hoc comparisons between all groups were performed using the Turkey multiple comparison test. Data are represented as mean ± SD. In every analysis, Difference was considered significant at $*p < 0.05$, statistically significant at $**p < 0.01$ and statistically significant at $***p<0.001$.

RESULTS

Isolated hASCs display mesenchymal stem cells phenotypes

Isolated hASCs display a fibroblast like morphology (Fig. 1a). They were positive for adhesion molecules CD73, CD105 and extracellular matrix protein CD90 (Fig. 1d-f), but negative for CD34 and CD45 (Fig. 1b-c). They were able to differentiate toward osteoblasts, adipocytes, and chondrocytes (Fig. 1h-j) under specific *in vitro* differentiating conditions for 21 days. These results could identify that hASCs isolated in this study were stromal multipotent cells that could differentiate toward cells under mesodermal lineage.

10µM treatment for 7d displayed neural morphology and are highly positive for SOX2, TUJ1 and NF-L

hASCs isolated from adipose tissue in this study were neurally induced by 8Br-cGMP supplementation at 0µM, 10µM, 100µM. After exposure to neural induction medium for a few days, NI-hASCs exhibited rapid morphological changes and looked different when compared to the controls. Most differentiated cells retract their cytoplasm and emit cellular protrusions (Fig. 2a); some cells form spherical cell body (Fig. 2b); some cells remain large and flat just like normal hASCs. The majority of NI-hASCs population in 10µM (Fig. 2c) appears as sharp, elongated bi- or multipolar cells with primary and secondary processes after induction for a week. Nestin, Sox2, TUJ1 and NF-L staining were used to determine the stage of neural cells induced from hASCs. NI-hASCs in all groups were positive for Nestin, Sox2, TUJ1 and NF-L after one week of 8Br-cGMP exposure. $Sox2^+$ and $TUJ1^+$ percentage of 10µM 8Br-cGMP condition were distinctively higher than other

conditions (79.08%±11.31% and 51.98%±18.16%, respectively). NI-hASCs under 10μM 8Br-cGMP condition were also highly positive for Nestin (83.42%±3.42%) and NF-L (98.34%±0.76%), but not significantly different than other treatments. The morphology of the controls (Fig. 2h and 2i) was slightly different when compared to hASCs under normal culture condition (Fig. 2j). hASCs in normal culture condition were weakly positive for Nestin, Sox2 and TUJ1 (10.41%±1.67%, 34.52%±7.66% and 18.71%±9.99%, respectively), but the only control that was positive for NF-L (18.62%±14.06%) was DMSO. After 14 days of induction, NI-hASCs population number was roughly decreasing but their morphology remains similarly to those at d7. Large number of cell detachment could be observed in all treatments at this point.

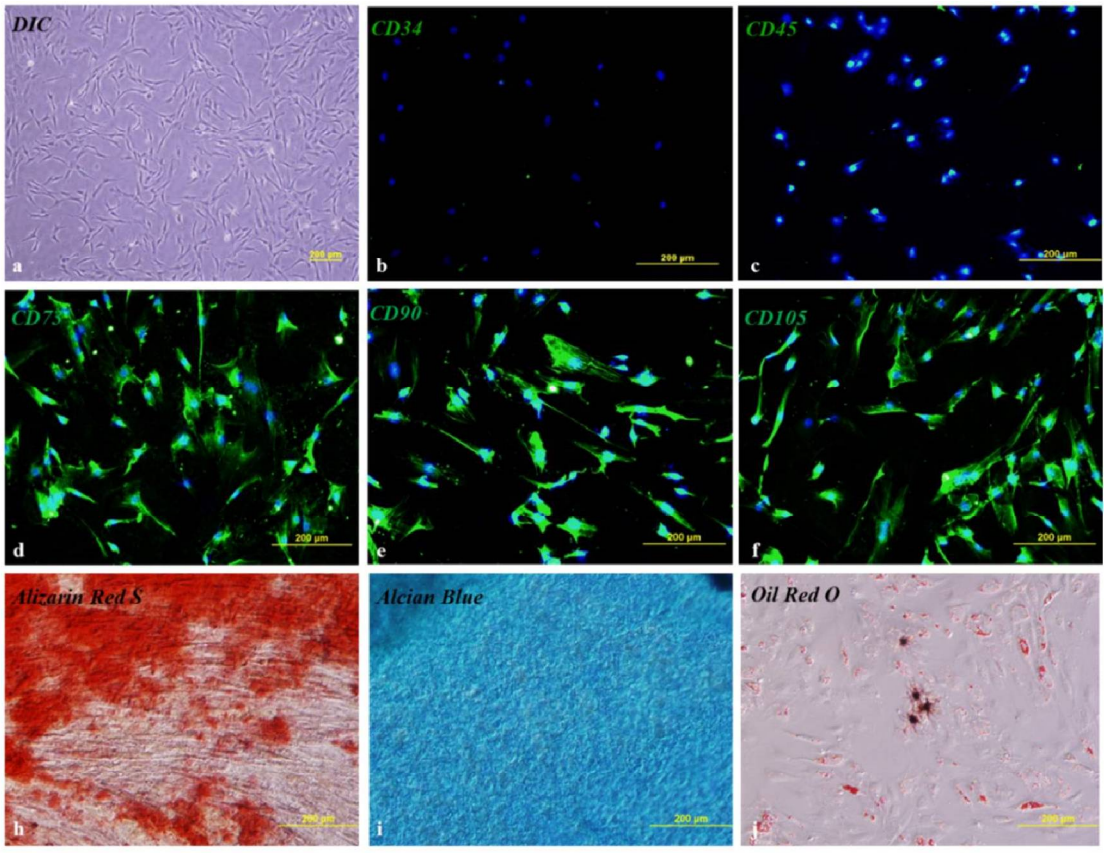

Figure 1. Morphological and immunocytochemical characterization of hASCs (a-f). Multipotent potential of hASCs was investigated under standard osteogenic (h), chondrogenic (i) and adipogenic (j) differentiation medium for three weeks. Calcium minerization,

extracellular matrix proteoglycan accumulation and lipid droplets formation were detected under the microscope (h-j). Image a was taken under 40x magnification. Image b-j were taken under 200x magnification.

10µM treatment could produce significantly higher number of TUJ1+ NI-hASCs with neurites

Neurite outgrowth analysis of NI-hASCs was performed under In Cell Analyzer 1000 Cellular Imaging and Analysis System (GE Healthcare Life Science). $TUJ1^+$ NI-hASCs under specific criteria were selected for the analysis for more accurate interpretation. 10µM 8Br-cGMP showed the highest number of $TUJ1^+$ NI-hASCs with neurites and was significantly greater ($p<0.001$) than other conditions (Fig. 3b). Their cell body area and number of neurite/cell were significantly higher ($p<0.05$) than the control (Fig. 3c and 3e). Total neurite length of untreated and 10µM 8Br-cGMP was about at 50µm with 10µM 8Br-cGMP slightly greater than the untreated (Fig. 3d). Their lengths were higher than the 100µM 8Br-cGMP treated group and the control but not statistically significant. Total neurite length per cell and mean neurite length were found to be not statistically different across all conditions (Fig. 3f and 3g). There weren't any $TUJ1^+$ populations with neurite under defined criteria presented in DMEM condition.

Neuroectodermal promoting activity was significantly higher in 10µM treatment at d7 of induction

To find the most optimal condition of neural cells differentiation, mRNA expression level of neuroectodermal development genes in NI-hASCs of each condition was measured at d7 and d14. The expression level was reported relatively to DMEM/F12 condition (the control). Most of neural gene expression in 10µM 8Br-cGMP condition were upregulated at one week of induction (Fig. 4a) and started to downregulate overtime. Surprisingly, the only gene that was upregulated at d14 was *TUJ1* (Fig. 4b) but other genes were decreasing. *SOX2*, the neural progenitor marker, mRNA level of each condition was not significantly different; still the highest expression level was of 10µM 8Br-cGMP treated group.

qPCR analysis at d7 was examined closely by genes due to much neuroectodermal promoting activity. Each condition was analyzed comparatively with neural cell derived from human embryonic carcinoma cells (NPC-EC) as the positive control previously reported by Peter W. Andrews. The morphology of NPC-EC was distinctively different from the

experimental group (Fig. 4d and 4e) but displayed similar neural profile to previous study (Andrews, 1984). Genes examined in this study were referred to early progenitor (*Nestin* and *SOX2*) and neuronal developing (*MASH1*, *GAP43, MAP2*, *TUJ1* and *NF-L*) stages of neuroectodermal development. *Nestin* expression level in both of 0µM and 10µM treatment was significantly higher than other conditions (Fig. 4c). *GAP43, MASH1, MAP2* and *NF-L* mRNA levels of 10µM 8Br-cGMP condition were significantly higher than other conditions, but *MAP2* and *TUJ1* expression levels were not different from NPC-EC. These markers are associated with neuronal maturation and neurite outgrowth. This incidence was confirmed by the highest total length of neurite (Fig. 3d) and the highest number of neurite (Fig. 3e) in 10µM 8Br-cGMP. Even though most of neural markers were suppressed and significantly lower in 100µM 8Br-cGMP, about 20% of $TUJ1^{+}$ NI-hASCs with slightly lower total neurite length existed (Fig. 3b and 3d).

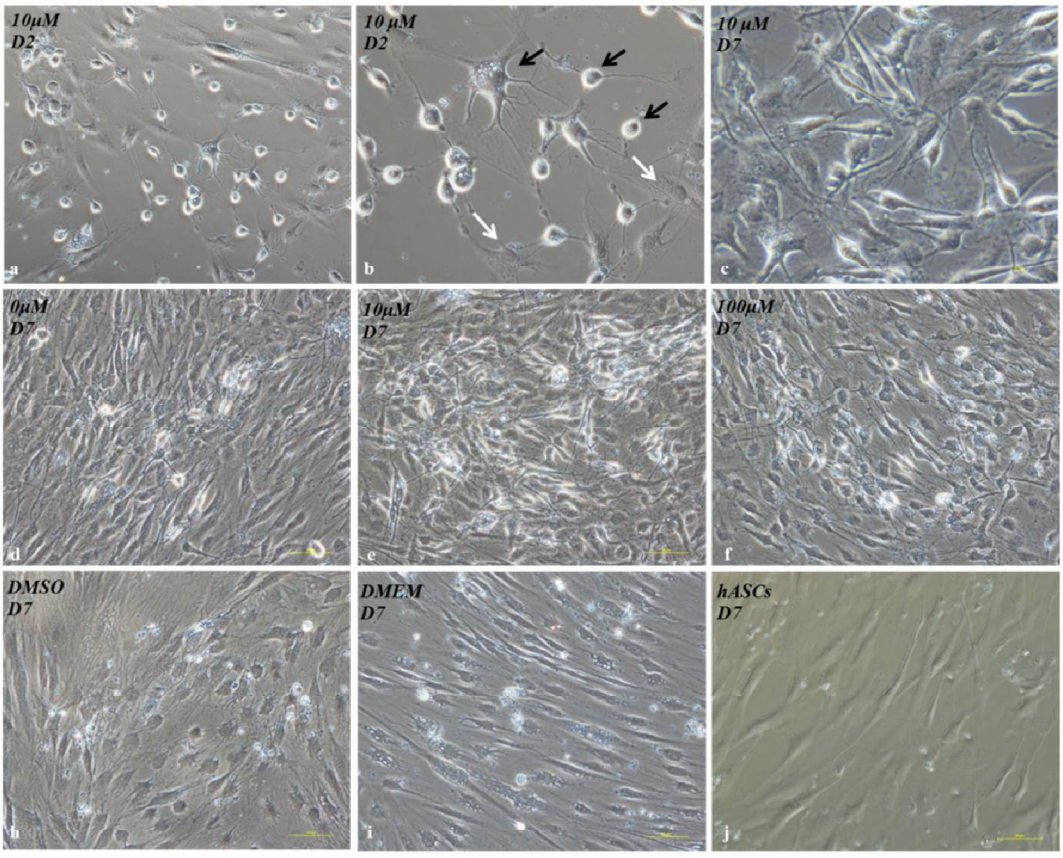

Figure 2. Morphological changes exhibited by hASCs exposed to 8 Bromo cyclicGMP and other neural inducing agents. As early as 2 days

postinduction (a-b), cell with neural-like structure were observed (black arrow). Those cells developed rounded cell bodies with several branching extensions. Some cells remained spindle –shape structure (white arrow) like hASCs under the controls (h-i) and non inducing hASCs (j). Image a and d-j were taken under 200x magnification. Image b-c were taken under 400x magnification.

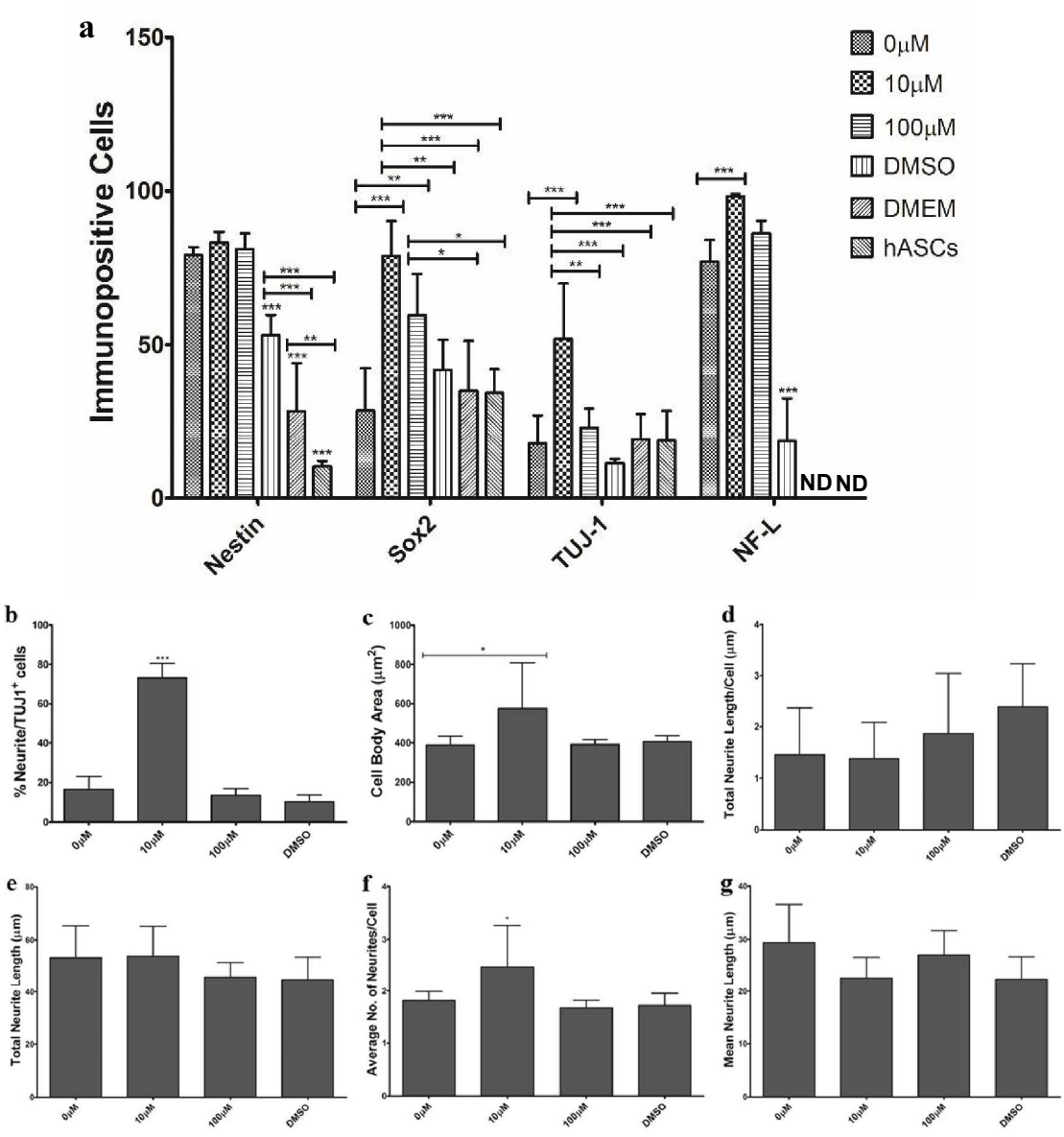

Figure 3. Immunopositive (a) and neurite outgrowth (b-g) analyses of NI-hASCs after 7 days of induction. NF-L expression in DMEM and

hASCs could not be detected. All treatments were counted in twelve different fields (N=12). Statistical significance is indicated (one-way ANOVA, *post hoc* comparisons between all groups were performed using the Turkey test, *p<0.05, **p<0.01, ***p<0.001).

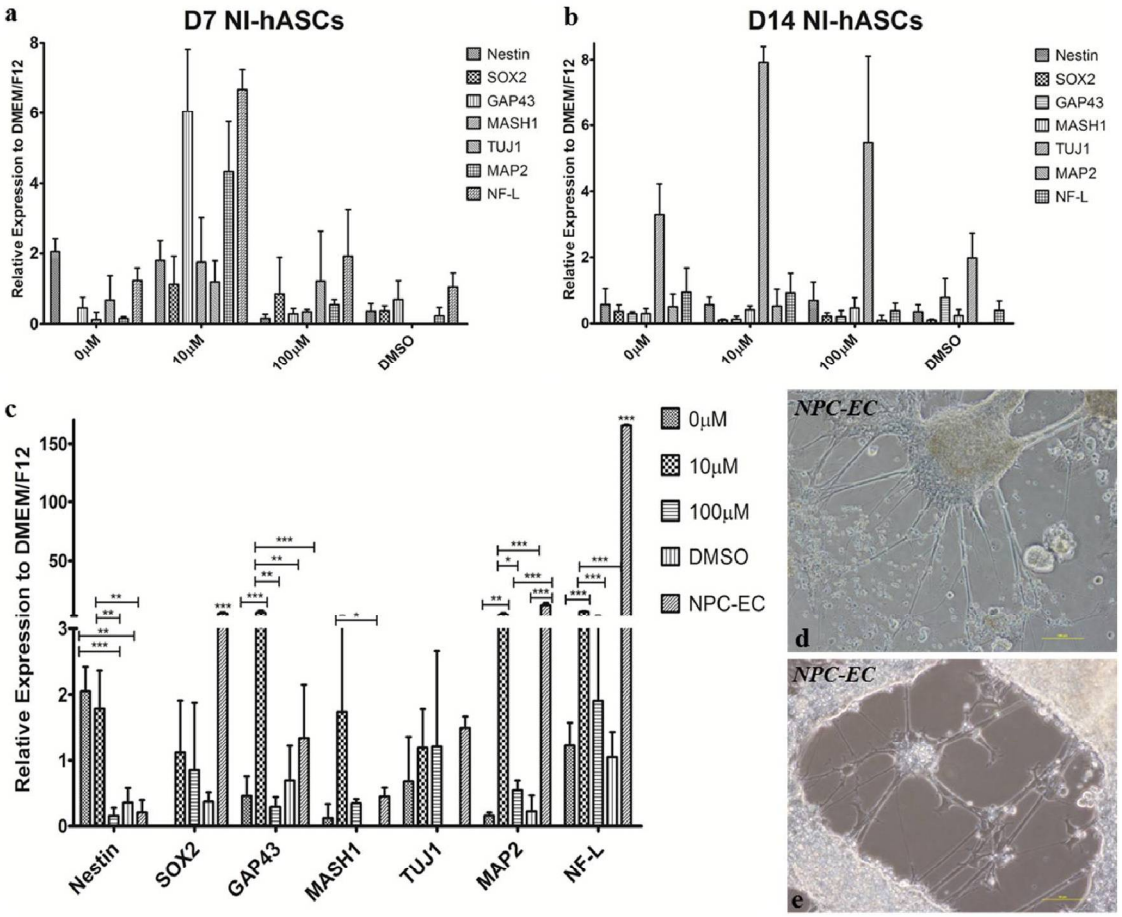

Figure 4. Relative gene expression of NI-hASCs to DMEM condition at d7 (a and c) and d14 (b). Each of 3 independent samples in all treatments was performed in replicate. Statistical significance is indicated (two-way ANOVA, *post hoc* comparisons between all groups were performed using the Turkey test, *p<0.05, **p<0.01, ***p<0.001). Neural cell derived from human EC cells were used as a positive control (d-e). Image d was taken under 200x magnification and e was taken under 40x magnification.

10µM 8Br-cGMP NI-hASCs could differentiate toward neuronal and glial lineage under maturation inducing condition

NI-hASCs under 10µM 8Br-cGMP condition were differentiated toward neuronal and glial cells to test their progenitor plasticity. NeuroCult™ NS-A Differentiation Kit was used to differentiate NI-hASCs

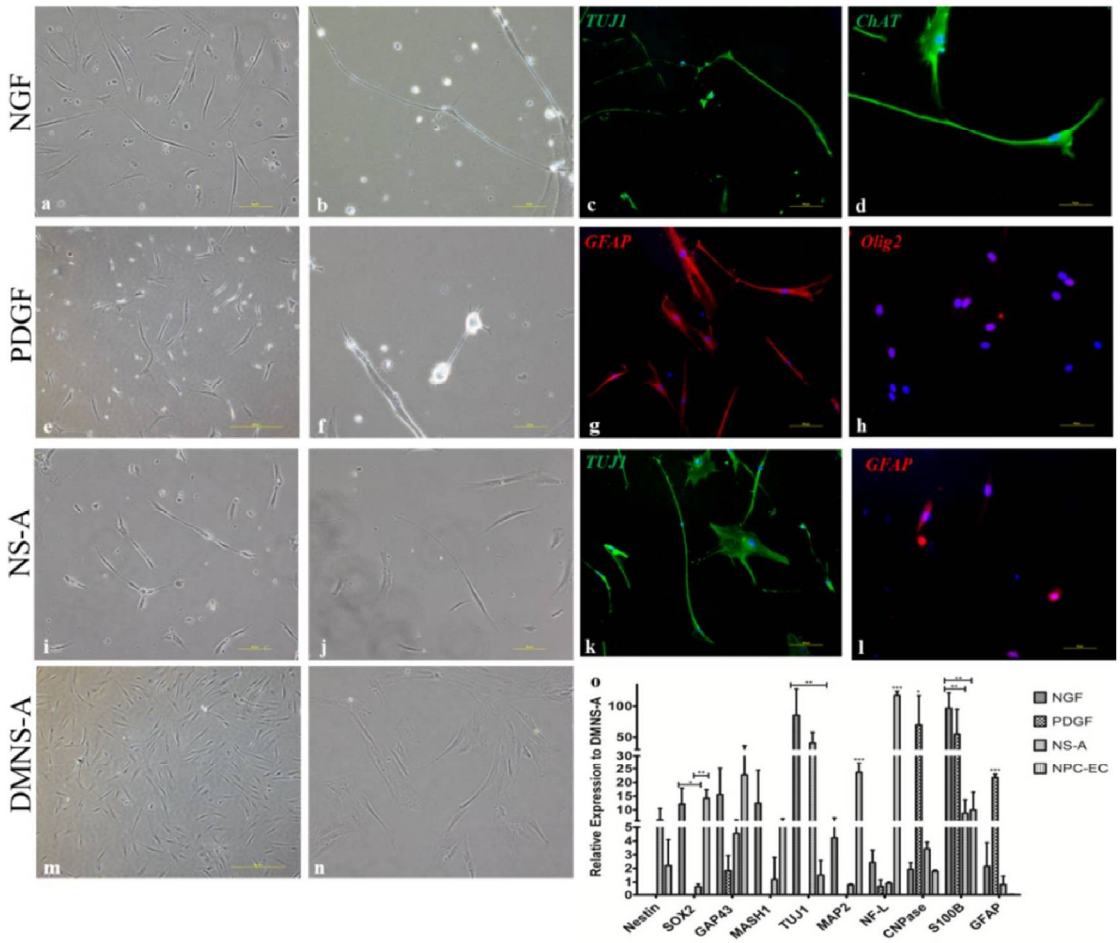

Figure 5. Morphological, Immunochemical staining and qPCR analyses of differentiated NI-hASCs toward neuronal (a-d) and glial (e-h) cells under specific induction medium. Each of 3 independent samples in all treatments was performed in replicate. Statistical significance is indicated (two-way ANOVA, *post hoc* comparisons between all groups were performed using the Turkey test, *p<0.05, **p<0.01, ***p<0.001). Images in the first and the third column were taken under 200x magnification and images in the second and the fourth column were taken under 400x magnification.

as a standard differentiation protocol. Differentiated NI-hASCs display distinctive morphology when compared to hASCs cultured under NS-A medium (Fig. 5m and 5n). The morphology of NI-hASCs under NGF condition consisted of larger soma and longer axon with neural ends on each neuronal-like cell (Fig. 5b). Overall, they were positive for mature neuronal markers, TUJ1 (Fig. 5c) and ChAT (Fig 5d). *TUJ1* mRNA level was significantly higher than other conditions (Fig. 5o), roughly 50 folds greater than NI-hASCs without NGF. Apart from that, *S100B* mRNA level was almost a hundred folds higher ($p<0.01$) than the controls and about 50 folds higher than PDGF condition. Other mature neuron related genes (*GAP43*, *MASH1*, *MAP2* and *NF-L*) were expressed in terminally differentiated cells as well. However, *NF-L* mRNA level of NGF condition was slightly downregulating and significantly lower than that of NPC-EC. NI-hASCs morphology under PDGF and normal NS-A condition were smaller with a shorter axonal protrusion. A large number of glial-like cells could be observed under PDGF condition (Fig. 5e-f). Those populations were positive for glial cells markers, GFAP (Fig. 5g) and Olig2 (Fig. 5h), and demonstrated significant upregulating level of *GFAP* and *CNPase* (Fig. 5o), suggesting that those populations could be glial and oligo progenitor cells. The lack of *MAP2* and *TUJ1* expression in PDGF condition also confirmed that these cells are committed to glial lineage. Neuronal-like cells with a shorter neurite extension in the normal NS-A condition were immunopositive for TUJ1 (Fig. 5k) but not positive for GFAP (Fig. 5l).

DISCUSSION

hASCs are considered as a promising tool in stem cell therapy due to many features that triumph hMSCs from other sources (Safford and Rice 2005). hASCs from adipose tissue can be obtained easily with minimal invasive procedure and high hASCs yield content (about 1×10^9 stem cells from 10 ml of adipose tissue) (Mizuno 2010; Zuk 2010; Ra et al. 2011). Furthermore, they also hold mesodermal and ectodermal differentiation features. (Gronthos et al. 2001; Halvorsen et al. 2001; Sen et al. 2001; Erickson et al. 2002; Safford et al. 2002; Zuk et al. 2002; Ashjian et al. 2003). In the present study, isolated hASCs displayed distinctive mesenchymal stem cell phenotypes and expressed stem cell markers reported in other human MSC sources previously (Pittenger et al., 1999; Zuk et al., 2002; Gimble and Guilak, 2003; Katz et al., 2005; Strem et al., 2005; Kocaoemer et al., 2007;

Bieback et al., 2009). They are usually positive for surface markers CD9, CD10, CD13, CD29, CD44, CD49d, CD49e, CD54, CD55, CD59, CD73, CD90, CD105, CD146, CD166, and STRO-1, and are negative for hematopoietic lineage markers CD11b, CD14, CD19, CD34, and CD45 (Zuk et al., 2002; Strem et al., 2005; Wagner et al., 2005; Krampera et al., 2007; Schaffler and Buchler, 2007; de Villiers et al., 2009) The hASCs in this study expressed MSC-specific cell type markers including CD73, CD90, and CD105, but did not express CD34 and CD45. They could also differentiate into adipocytes, osteoblasts, and chondrocytes, indicating that hASCs were of mesenchymal origin (Zuk et al., 2002; Casteilla and Dani, 2006).

Many studies demonstrated that hASCs could be successfully induced to differentiate into neural cells (Zuk et al., 2002; Ashjian et al., 2003; Jang et al., 2010; Kompisch et al., 2010; Zavan et al., 2013). Comparative studies of ASCs and MSCs from other sources recommended that ASCs held a better potential for neural differentiation (Kwon et al., 2011; Mostafavi et al., 2014). Various protocols used to convert hASCs into neural cells include exposure of butylated hydroxyanisole and dimethyl sulfoxide (Zuk et al. 2002; Safford et al. 2002), a cocktail of butylated hydroxyanisole, insulin, hydrocortisone and valproic acid (Saffford et al., 2002; Guilak et al. 2006) or a cocktail of indomethacin, insulin and isobutylmethylxanthine (Ashijan et al., 2003; Fujimura et al., 2005; Ning et al., 2006). Agents that increase intracellular cAMP levels (Ashjian et al. 2003; De Ugarte et al. 2003), 5-azacytidine (Kang et al., 2003; Lee et al., 2008), mitogen bFGF and EGF together with forskolin (Nagase et al., 2007; Jang et al., 2010, Zavan et al., 2010; Razavi et al., 2012), various glial growth factors (Kingham et al., 2007), and brain-derived neurotrophic factor (BDNF) with retinoic acid (Anghileri et al., 2008) could also induce neural differentiation of hASCs *in vitro*. NO signaling pathway is one of the most studied and significant signaling pathways in many cells and tissues, most particularly in nervous system. Many reports have found that NO donor could convert many types of cell to neural-like cells (Feng et al., 2002; Estephane and Anctil, 2009; Sulz et al., 2009; Li et al., 2010; Muller et al., 2010). Those donors include sodium nitroprusside (SNP), S-nitroso-*N*-acetylpenicillamine (SNAP), 8 bromo cyclic GMP (8Br-cGMP) and amino-3-morpholinyl-1,2,3-oxadiazolium chloride (SIN-1). NO/cGMP signaling pathway plays a vital role in neuronal cell survival and differentiation (Contestabile and Ciani, 2004). Evidence demonstrated that combining RA with NO donor could enhance cell proliferation and neural precursors differentiation towards functional neurons (Estephane and Anctil, 2009; Li et al., 2010). However, the only study using 8Br-cGMP to induce neural

differentiation is found in H9 hESCs (Li et al., 2010). We demonstrate for the first time that 8Br-cGMP could efficiently induce neural differentiation of hASCs. Our results suggest that these neural progenitor cells obtained by our protocol have been committed to neural cell lineages and could differentiate toward neuronal and glial cells.

Undifferentiated hASCs showed positive expression of some neural markers. Nestin and Sox2 are typically highly expressed in neural stem cells and neural progenitors. hASCs in this study were weakly positive for Nestin, Sox2 and TUJ1. Previous reports on hASCs (Ashijan et al., 2003; Mareschi et al., 2006; Jang et al., 2010; Zavan et al., 2010) and hMSCs from other sources (Tondreau et al., 2004; Bertani et al., 2005; Croft and Przyborski, 2009; Wetzig et al., 2013) both of rat and human showed positive expression for these markers as well. Even though hASCs in this study also expressed TUJ1 which is the putative neuronal marker, Pontius et al. (2003) reported that TUJ1 expression could be broader distributed in non-neural tissue in human due to transcriptional activity of the gene *tubb3* (Pontius et al., 2003). Other evidence reported that hASCs also expressed low levels of Fibronectin, MAP2, GFAP and S100 (Bertani et al., 2005; Jang et al., 2010), indicating a native neural differentiation potential of these cells. Surprisingly, in our study Nestin and NF-L positive cells ratio in DMSO condition was significantly higher than other controls. Other studies also found that DMSO treated cells were weakly positive for neural markers (Lu et al., 2004; Neuhuber et al., 2004; Bertani et al., 2005; Tao et al., 2005), but does not significantly change the levels of neural-related markers already expressed by MSCs. The incidence might be due to fast-onset cytoskeletal changes caused by cellular toxicity, not from differentiation process.

After exposure to differentiation cocktail, NI-hASCs rapidly change their morphology within a few days. The morphology and expression profile are similar to those in previous reports (Zuk et al. 2002; AshiJan et al., 2003; Jang et al., 2010; Kompisch et al. 2010; Zavan et al., 2010). Then, differentiated cells population was increased overtime, but started to decrease at d14. Apoptosis of undifferentiated cells might be induced as reported in the previous study of neural differentiation in hMSCs (Sanchez-Ramos et al., 2000; Sanchez-Ramos, 2002; Jin et al., 2003; Padovan et al., 2003; Wislet-Gendebien et al., 2003; Zuk et al., 2003; Hermann et al., 2004; Kang et al., 2004; Wislet-Gendebien et al., 2005;). Our results suggest that induction within a week is the most suitable time. 10μM 8Br-cGMP NI-hASCs expressed increasing immunoreactivities for neuronal markers Sox2, Tuj1 and NF-L as well as the increased mRNA expression of *TUJ1*, *MAP2*, *NF-L*,

MASH1, *GAP43* compared to primary hASCs. *MAP2* and *TUJ1* mRNA expression is not significantly different from NPC-EC, the positive control in this study, suggesting that 10µM 8Br-cGMP NI-hASCs possess characteristics of neuronal-like cells. *SOX2* mRNA level of each condition was not significantly different; still the highest expression level was of 10µM 8Br-cGMP treated group. *SOX2* is neural stem cells and pluripotent markers. The upregulation of *SOX2* could affect the pluripotency of stem cells, stimulate differentiation to the ectoderm lineage, and improve induction efficiency (Thomson et al., 2011; Wang et al., 2012). Therefore, *SOX2* upregulation in each condition suggested that each condition was under the stage of differentiation. The significant upregulating of *TUJ1*, *MAP2*, *NF-L*, *MASH1*, *GAP43* mRNA levels are associated with neuronal maturation and neurite outgrowth. Among those genes, the relationship between cGMP pathway and *GAP43* expression has been demonstrated, as 8Br-cGMP fully prevented *GAP43* and *synapsin I* reduction in cells that were depleted neurite outgrown related gene. They suggested that their regulation levels are cGMP independent (Chen et al., 2004; López-Jiménez et al., 2009). *GAP43* is an integral membrane protein associated with the cytoplasmic surface of axonal growth cones in developing neurons, and it is generally considered as an intrinsic determinant of neurite outgrowth and plasticity (Skene, 1989; Lalli et al., 2005). When this gene is altered, neurite outgrowth and axonal pathfinding are affected both during development and in cultured cells. The significance uprising of this mRNA level in 10µM 8Br-cGMP is consistent with the highest total length of neurite (Fig 3.12C) and significant highest number of neurite (Fig 3.12D). Even though most of neural markers were suppressed and significantly lower in 100µM 8Br-cGMP, about 20% of $TUJ1^{+}$ NI-hASCs with slightly lower total neurite length existed. The underlying mechanism could be involved with the activity of RhoA and GTPase controlled by cGMP/cGK signaling in cell shape control and neurite outgrowth (Sauzeau et al., 2003).

Inducing environment might be favorable for neuronal differentiation as the role of bFGF in neural regulation is to generate neural precursor cells with a greater capacity for neuronal differentiation whereas EGF and specific neurotrophic factor are reported to restrict astrocyte lineages (Romero-Ramos et al., 2002; Schultz and Lucas, 2006; Xu et al., 2008, 2009). A combination of Noggin and SB431542, BMP and activin/nodal signaling inhibitor used to efficiently convert hESCs and iPS cells to neural cells, may force hASCs to commit under neuroectodermal lineage (Pera et al., 2004; Chiba et al., 2008; Chambers et al., 2009). Our results suggest that laminin combined with 8br-

cGMP at a certain amount could enhance neurite outgrowth as laminin has differential action in the proliferation, survival, and differentiation of cultured neuronal cells (Romero-Ramos et al., 2002; Schultz and Lucas, 2006). Mruthyunjaya et al (2010) reported that laminin also stimulates neurite outgrowth and upregulates *NF-L*, *MAP2* and *NeuroD1* mRNA level in hBMSCs (Mruthyunjaya et al., 2011). Our experiment demonstrated consistent results as *GAP43, NF-L*, *MAP2* and *TUJ1* mRNA level, immunoprofile and neurite assay results of 10µM 8Br-cGMP are significantly higher than any other groups. Retinoic acid (RA) supports cell proliferation, differentiation and maturation in brain development. RA may cause high NF-L positive expression of NI-hASCs as previous report showed that RA acts on the up-regulation of NF-L expression in MSCs derived from the umbilical cord blood and from bone marrow (Scintu et al., 2006). The presence of serum in the medium is reported to be essential for glial gene expression in MSCs (Chu et al.,2006). Our NI-hASCs were induced under serum-free medium which might explain the absence of the glial markers and suggest that the medium might be favorable for cells to differentiate towards neuronal lineage.

Even though qPCR profiles of NI-hASCs seem to commit to be neurons, they could also differentiate to neuronal and glial cells when cultured under standard NeuroCult™ NS-A Differentiation Kit. NI-hASCs under NGF differentiation medium could form synapse structure (Fig. 5a-b) and are positive for ChAT, a cholinergic neuronal marker. qPCR results suggested that differentiated NI-hASCs are in their maturation process as *S100B* plays a vital role in neurite extension and axon proliferation. *TUJ1* and *MAP2* are markers exclusively expressed in immature neuron and mature postmitotic neurons, respectively. However, no electrophysiological test was performed to validate their functional property. Other glial populations were found under PDGF condition as they were positive for olig2 and GFAP. The lack of *TUJ1* and *MAP2* and significant GFAP and CNPase mRNA levels confirm that they are committed to glial lineage. Thus, we successfully convert hASCs to neuronal and glial cells but further investigation is required on their functional properties.

CONCLUSION

NI- hASCs under 10µM 8Br- cGMP treatment display neuronal-committed progenitor profiles, yet remains their plasticity toward other neural fates. They could differentiate toward cholinergic neurons and oligoglial cell upon induction in defined medium. The findings of this study were to provide

an efficient alternative way of differentiating hASCs into neural progenitor cells which could be beneficial for basic and clinical research in cell-based therapy in the future.

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REFERENCES

Andrews PW. 1984. Retinoic acid induces neuronal differentiation of a cloned human embryonal carcinoma cell line in vitro. Dev Biol. 103(2):285-293.

Anghileri E, Marconi S, Pignatelli A, Cifelli P, Galie M, Sbarbati A, Krampera M, Belluzzi O, Bonetti B. 2008. Neuronal differentiation potential of human adipose-derived mesenchymal stem cells. Stem Cells Dev. 17(5):909-916.

Arnold WP, Mittal CK, Katsuki S, Murad F. 1977. Nitric oxide activates guanylate cyclase and increases guanosine 3'-5'-cyclic monophosphate levels in various tissue preparations. Proc Natl Acad Sci. 74:3203–3207.

Ashjian PH, Elbarbary AS, Edmonds B, DeUgarte D, Zhu M, Zuk PA, Lorenz HP, Benhaim P, Hedrick MH. 2003. In vitro differentiation of human processed lipoaspirate cells into early neural progenitors. Plast Reconstr Surg. 111:1922–1931.

Bain G, Kitchens D, Yao M, Huettner JE, Gottlieb DI. 1995. Embryonic Stem Cells Express Neuronal Properties in Vitro. Developmental Biology. 168:342-357.

Bertani N, Malatesta P, Volpi G, Sonego P, Perris R. 2005. Neurogenic potential of human mesenchymal stem cells revisited: analysis by immunostaining, time-lapse video and microarray. J Cell Sci. 118:3925–3936.

Bieback K, Hecker A, Kocaomer A, Lannert H, Schallmoser K, Strunk D, Kluter H. 2009. Human alternatives to fetal bovine serum for the

expansion of mesenchymal stromal cells from bone marrow. Stem Cells. 27:2331-2341.

Casteilla L, Dani C. 2006. Adipose tissue-derived cells: from physiology to regenerative medicine. Diabetes Metab. 32(5 Pt 1):393-401.

Chambers SM, Fasano CA, Papapetrou EP, Tomishima M, Sadelain M, Studer L. 2009. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nature Biotechnol. 27: 275–280.

Chen TJ, Huang CW, Wang DC, Chen SS. 2004. Co-induction of growth-associated protein GAP-43 and neuronal nitric oxide synthase in the cochlear nucleus following cochleotomy. Exp Brain Res. 158:151–162.

Chiba S, Lee YM, Zhou W, Freed CR. 2008. Noggin enhances dopamine neuron production from human embryonic stem cells and improves behavioral outcome after transplantation into Parkinsonian rats. Stem Cells. 26: 2810–2820

Chu MS, Chang CF, Yang CC, Bau YC, Ho LL, Hung SC. 2006. Signalling pathway in the induction of neurite outgrowth in human mesenchymal stem cells. Cell Signal. 18:519-530.

Ciani E, Guidi S, Della Valle G, Perini G, Bartesaghi R, Contestabile A. 2002. Nitric oxide protects neuroblastoma cells from apoptosis induced by serum deprivation through cAMP-response element-binding protein (CREB) activation. J. Biol. Chem. 277:49896–49902.

Contestabile A, Ciani E. 2004. Role of nitric oxide in the regulation of neuronal proliferation, survival and differentiation. Neurochemistry International. 45(6): 903-914.

Croft AP and Przyborski SA. 2006. Formation of neurons by non-neural adult stem cells: potential mechanism implicates an artifact of growth in culture. Stem Cells. 24(8): 1841–1851.

De Ugarte DA, Morizono K, Elbarbary A, Alfonso Z, Zuk PA, Zhu M, Dragoo JL, Ashjian P, Thomas B, Benhaim P, Chen I, Fraser J, Hedrick MH. 2003. Comparison of multi-lineage cells from human adipose tissue and bone marrow. Cells Tissues Organs. 174: 101–109.

De Villiers JA, Houreld N, Abrahamse H. 2009. Adipose derived stem cells and smooth muscle cells: implications for regenerative medicine. Stem Cell Rev. 5(3): 256-265.

Deng W, Obrocka M, Fischer I, Prockop DJ. 2001. In Vitro Differentiation of Human Marrow Stromal Cells into Early Progenitors of Neural Cells

by Conditions That Increase Intracellular Cyclic AMP. Biochemical and Biophysical Research Communications. 282: 148-152.

Erickson GR, Gimble JM, Franklin DM, Rice HE, Awad H, Guilak F. 2002. Chondrogenic Potential of Adipose Tissue-Derived Stromal Cells in Vitro and in Vivo. Biochemical and Biophysical Research Communications. 290(2): 763-769.

Estephane D. and Anctil M. 2010. Retinoic Acid and Nitric Oxide Promote Cell Proliferation and Differentially Induce Neuronal Differentiation In Vitro in the Cnidarian *Renilla koellikeri*. Develop Neurobiol. 70: 842–852.

Feng Z, Li L, Ng PY, Porter AG. 2002. Neuronal Differentiation and Protection from Nitric Oxide-Induced Apoptosis Require c-Jun-Dependent Expression of NCAM140. Molecular and Cellular Biology. 22(15): 5357–5366.

Fujimura J, Ogawa R, Mizuno H, Fukunaga Y, Suzuki H. 2005. Neural differentiation of adipose-derived stem cells isolated from GFP transgenic mice. Biochem Biophys Res Commun. 333(1):116-121.

Gimble JM and Guilak F. 2003. Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. Cytotherapy. 5:362-369.

Gronthos S, et al. 2001. Surface protein characterization of human adipose tissue-derived stromal cells. J Cell Physiol. 189:54.

Guilak F, Lott KE, Awad HA, Cao Q, Hicok KC, Fermor B, Gimble JM. 2006. Clonal analysis of the differentiation potential of human adipose-derived adult stem cells. J Cell Physiol. 206:229–237.

Halvorsen Y-DC, Franklin D, Bond AL, Hitt DC, Auchter C, Boskey AL, Paschalis EP, Wilkison WO, Gimble JM. 2004. Extracellular Matrix Mineralization and Osteoblast Gene Expression by Human Adipose Tissue–Derived Stromal Cells. Tissue Engineering. 7(6): 729-741.

Hermann A, Gastl R, Liebau S, Popa MO, Fiedler J, Boehm BO, Maisel M, Lerche H, Schwarz J, Brenner R, Storch A. 2004. Efficient generation of neural stem cell-like cells from adult human bone marrow stromal cells. J Cell Sci. 117:4411-4422.

Izadpanah R, Joswig T, Tsien F, et al. 2005. Characterization of multipotent mesenchymalstem cells from the bone marrow of rhesus macaques. Stem Cells Dev. 14(4):440-451.

Jang S, Cho HH, Cho YB, Park JS, Jeong HS. 2010. Functional neural differentiation of human adipose tissue-derived stem cells using bFGF and forskolin. BMC Cell Biology. 11:25-38.

Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM. 2002. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature. 418:41-49.

Jin K, Mao XO, Batteur S, Sun Y, Greenberg DA. 2003. Induction of neuronal markers in bone marrow cells: differential effects of growth factors and patterns of intracellular expression. Exp Neurol. 184:78-89.

Johe KK, Hazel TG, Muller T, Dugich-Djordjevic MM, McKay RD. 1996. Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. Genes Dev. 10:3129 –3140.

Krampera M, Marconi S, Pasini A, Galie M, Rigotti G, Mosna F, Tinelli M, Lovato L, Anghileri E, Andreini A, Pizzolo G, Sbarbati A, Bonetti B. 2007. Induction of neural-like differentiation in human mesenchymal stem cells derived from bone marrow, fat, spleen and thymus. Bone. 40(2):382-390.

Kang SK, Lee DH, Bae YC, Kim HK, Baik SY, Jung JS. 2003. Improvement of neurological deficits by intracerebral transplantation of human adipose tissue-derived stromal cells after cerebral ischemia in rats. Exp Neurol. 183(2):355-366.

Kang SK, Putnam LA, Ylostalo J, Popescu IR, Dufour J, Belousov A, Bunnell BA. 2004. Neurogenesis of Rhesus adipose stromal cells. J Cell Sci. 117:4289-4299.

Katsuki S, Arnold W, Mittal C, Murad F. 1977. Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. J Cyclic Nucleotide Res. 3:23–35.

Katz AJ, Tholpady A, Tholpady SS, Shang H, Ogle RC. 2005. Cell surface and transcriptional characterization of human adipose-derived adherent stromal (hADAS) cells. Stem Cells. 23:412-423.

Kingham PJ, Kalbermatten DF, Mahay D, Armstrong SJ, Wiberg M, Terenghi G. 2007. Adipose derived stem cells differentiate into a Schwanncell phenotype and promote neurite outgrowth in vitro. Exp Neurol. 207(2):267-274.

Kocaoemer A, Kern S, Kluter H, Bieback K. 2007. Human AB serum and thrombin-activated platelet-rich plasma are suitable alternatives to fetal calf serum for the expansion of mesenchymal stem cells from adipose tissue. Stem Cells. 25: 1270-1278.

Kompisch KM, Lange C, Steinemann D, Skawran B, Schlegelberger B, Müller R, Schumacher U. 2010. Neurogenic transdifferentiation of human adipose-derived stem cells? A critical protocol reevaluation with special emphasis on cell proliferation and cell cycle alterations. Histochem Cell Biol. 134:453–468.

Kwon EB, Lee JY, Piao S, Kim IG, Ra JC, Lee JY. 2011. Comparison of human muscle-derived stem cells and human adipose-derived stem cells in neurogenic trans-differentiation. Korean J Urol. 52(12):852-857.

Lalli G, Hall A. 2005. Ral GTPases regulate neurite branching through GAP-43 and the exocyst complex. J Cell Biol. 171: 857–869.

Lee SH, Lumelsky N, Studer L, Auerbach JM, McKay RD. 2000. Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. Nature Biotechnology. 18:675-679.

Lee TH and Yoon JG. 2008. Intracerebral transplantation of human adipose tissue stromal cells after middle cerebral artery occlusion in rats. J Clin Neurosci. 15(8):907-912.

Li TJ, Somasundaram C, Bian K, Xiong W, Mahmooduddin F, Nath RK, Murad F. 2010. Nitric Oxide Signaling and Neural Stem Cell Differentiation in Peripheral Nerve Regeneration. Journal of Plastic Surgery. 10: 343-353.

Livak, KJ and Schmittgen TD. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta Ct}$ Method. Methods. 25:402-408.

López-Jiménez ME, Bartolomé-Martín D, Sánchez-Prieto J, Torres M. 2009. Suppression of guanylyl cyclase (β1 subunit) expression impairs neurite outgrowth and synapse maturation in cultured cerebellar granule cells. Cell Death and Differentiation. 16:1266–1278.

Lu P, Blesch A, Tuszynski MH. 2004. Induction of bone marrow stromal cells to neurons: differentiation, transdifferentiation, or artifact? J Neurosci Res. 77:174–191.

Mareschi K, Novara M, Rustichelli D, Ferrero I, Guido D, Carbone E, et al. 2006. Neural differentiation of human mesenchymal stem cells: evidence for expression of neural markers and eag K+ channel types. Exp Hematol. 34:1563–1572.

Mizuno H. and Hyakusoku H. 2010. Fat Grafting to the Breast and Adipose-Derived Stem Cells: Recent Scientific Consensus and Controversy. Aesthetic Surgery Journal. 30(3) 381-387.

Mostafavi FS, Razavi S, Mardani M, Esfandiari E, Esfahani HZ, Kazemi M. 2014. Comparative Study of Microtubule-associated Protein-2 and Glial Fibrillary Acidic Proteins during Neural Induction of Human Bone Marrow Mesenchymal Stem Cells and Adipose-Derived Stem Cells. Int J Prev Med. 5(5):584-95.

Mruthyunjaya S, Rumma M, Ravibhushan G, Anjali S, Padma S. 2011. c-Jun/AP-1 transcription factor regulates laminin-1-induced neurite outgrowth in human bone marrow mesenchymal stem cells: Role of multiple signaling pathways. FEBS Lett. 585:1915-1922.

Muller D, Greenland KJ, Speth RC, Middendorff R. 2010. Neuronal differentiation of NG108-15 cells has impact on nitric oxide- and membrane (natriuretic peptide receptor-A) cyclic GMP-generating proteins. Molecular and Cellular Endocrinology. 320:118–127.

Nagase T, Matsumoto D, Nagase M, Yoshimura K, Shigeura T, Inoue M, Hasegawa M, Yamagishi M, Machida M. 2007. Neurospheres from human adipose tissue transplanted into cultured mouse embryos can contribute to craniofacial morphogenesis: a preliminary report. J Craniofac Surg. 18:49–53.

National Institute of Neurological Disorders and Stroke. 2004. Parkinson's disease: challenges, progresses and promises. National Institute of Health. 5(5585):1-41.

Neuhuber B, Gallo G, Howard L, Kostura L, Mackay A, Fischer I. 2004. Reevaluation of in vitro differentiation protocols for bone marrow stromal cells: disruption of actin cytoskeleton induces rapid morphological changes and mimics neuronal phenotype. J Neurosci Res. 77:192–204.

Ning H, Lin G, Lue TF, Lin CS. 2006. Neuron-like differentiation of adipose tissue-derived stromal cells and vascular smooth muscle cells. Differentiation. 74(9-10):510-518.

Padovan CS, Jahn K, Birnbaum T, Reich P, Sostak P, Strupp M, Straube A. 2003. Expression of neuronal markers in differentiated marrow stromal cells and CD133+ stem-like cells. Cell Transplant. 12:839-848.

Pera MF, Andrade J, Houssami S, Reubinoff B, Trounson A, Stanley EG, Ward-van Oostwaard D, Mummery C. 2004. Regulation of human embryonic stem cell differentiation by BMP-2 and its antagonist noggin. J Cell Sci. 117:1269–1280.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999.

Multilineage potential of adult human mesenchymal stem cells. Science. 284: 143-147.

Pontius JU, Wagner L, Schuler GD. 2003. UniGene: a unified view of the transcriptome. In *The NCBI Handbook*. Bethesda MD: National Center for Biotechnology Information.

Ra JC, Shin IS, Kim SH, Kang SK, Kang BC, Lee HY, Kim YJ, Jo JY, Yoon EJ, Choi HJ, Kwon E. 2011. Safety of intravenous infusion of human adipose tissue- derived mesenchymal stem cells in animals and humans. Stem Cells Dev. 20(8):1297-308.

Ramm P, Alexandrov Y, Cholewinski A, Cybuch Y, Nadon R, Soltys BJ. 2003. Automated screening of neurite outgrowth. J Biomol Screen. 1:7-18.

Razavi S, Ahmadi N, Kazemi M, Mardani M, Esfandiari E. 2012. Efficient transdifferentiation of human adipose- derived stem cells into Schwann-like cells: a promise for treatment of demyelinating diseases. Advanced biomedical research. 1(1):12.

Romero- Ramos M, Vourc'h P, Young HE, Lucas PA, Wu Y, Chivatakarn O, et al. 2002. Neuronal differentiation of stem cells isolated from adult muscle. J Neurosci Res. 69:894-907.

Safford KM, Hicok KC, Safford SD, Halvorsen YD, Wilkison WO, Gimble JM, Rice HE. 2002. Neurogenic differentiation of murine and human adiposederived stromal cells. Biochem Biophys Res Commun. 294(2):371-379.

Safford KM, Rice HE. 2005 Stem cell therapy for neurologic disorders: therapeutic potential of adipose-derived stem cells. Curr Drug Targets. 6:57–62.

Sanchez-Ramos J, Song S, Cardozo-Pelaez F, Hazzi C, Stedeford T, Willing A, Freeman TB, Saporta S, Janssen W, Patel N, Cooper DR, Sanberg PR. 2000. Adult Bone Marrow Stromal Cells Differentiate into Neural Cells in Vitro. Experimental Neurology. 164:247-256.

Sanchez-Ramos JR. 2002. Neural cells derived from adult bone marroe and umbilical cord blood. J Neurosci Res. 69:880-893.

Sauzeau V, Rolli-Derkinderen M, Marionneau C, Loirand G, Pacaud P. 2003. RhoA expression is controlled by nitric oxide through cGMP-dependent protein kinase activation. J Biol Chem. 278: 9472–9480.

Schaffler A, Buchler C. 2007. Concise review: adipose tissue-derived stromal cells--basic and clinical implications for novel cell-based therapies. Stem Cells. 25(4):818-827.

Schultz SS, Lucas PA. 2006. Human stem cells isolated from adult skeletal muscle differentiate into neural phenotypes. J Neurosci Methods. 152:144-55.

Scintu F, Reali C, Pillai R, Badiali M, Sanna MA, Argiolu F, Ristaldi MS, Sogos V. 2006. Differentiation of human bone marrow stem cells into cells with a neural phenotype: diverse effects of two specific treatments. BMC Neurosci. 7:14-26.

Sen A, Lea-Currie YR, Sujkowska D, Franklin DM, Wilkison WO, Halvorsen Y-DC, Gimble JM. 2001. Adipogenic potential of human adipose derived stromal cells from multiple donors is heterogeneous. J Cell Biochem. 81: 312–319.

Skene JH. 1989. Axonal growth-associated proteins. Annu Rev Neurosci. 12:127–156.

Strem BM, Hicok KC, Zhu M, Wulur I, Alfonso Z, Schreiber RE, Fraser JK, Hedrick MH. 2005. Multipotential differentiation of adipose tissuederived stem cells. Keio J Med. 54:132 – 41.

Sulz L, Astorga G, Bellette B, Iturriaga R, Mackay-Sim A, Bacigalupo J. 2009. Nitric oxide regulates neurogenesis in adult olfactory epithelium in vitro. Nitric Oxide. 20:238–252.

Tao H, Rao R, Ma DD. 2005. Cytokine-induced stable neuronal differentiation of human bone marrow mesenchymal stem cells in a serum/feeder cell-free condition. Dev Growth Differ. 47:423–433.

Thippeswamy T, Morris R. 1997. Cyclic guanosine 3',5'-monophosphate-mediated neuroprotection by nitric oxide in dissociated cultures of rat dorsal root ganglion neurones. Brain Res. 774:116–122.

Thippeswamy T, Morris R. 2001. Evidence that nitric oxide-induced synthesis of cGMP occurs in a paracrine but not an autocrine fashion and that the site of its release can be regulated: studies in dorsal root ganglia in vivo and in vitro. Nitric Oxide. 5:105–115.

Thomson M, Liu SJ, Zou LN, Smith Z, Meissner A, Ramanathan S. 2011. Pluripotency factors in embryonic stem cells regulate differentiation into germ layers. Cell. 145, 875–889.

Tondreau T, Lagneaux L, Dejeneffe M, Massy M, Mortier C, Delforge A, Bron D. 2004. Bone marrow-derived mesenchymal stem cells already express specific neural proteins before any differentiation. Differentiation. 72:319-326.

Wagner W, Wein F, Seckinger A, Frankhauser M, Wirkner U, Krause U, Blake J, Schwager C, Eckstein V, Ansorge W, Ho AD. 2005. Comparative characteristics of mesenchymal stem cells from human

bone marrow, adipose tissue, and umbilical cord blood. Exp Hematol. 33(11):1402-1416.

Wang Z, Oron E, Nelson B, et al. 2012. Distinct lineage specification roles for NANOG, OCT4, and SOX2 in human embryonic stem cells. Cell Stem Cell. 10, 440–454.

Wislet-Gendebien S, Leprince P, Moonen G and Rogister B. 2003. Regulation of neural markers nestin and GFAP expression by cultivated bone marrow stromal cells. J Cell Sci. 116: 3295-3302.

Wislet-Gendebien S, Hans G, Leprince P, Rigo JM, Moonen G, Rogister B. 2005. Plasticity of cultured mesenchymal stem cells: switch from nestin-positive to excitable neuron-like phenotype. Stem Cells. 23:392-402.

Wetzig A, Alaiya A, Al- Alwan M, Pradez CB, Pulicat MS, Al- Mazrou A, Shinwari Z, Sleiman GM, Ghebeh H, Al- Humaidan H, Gaafar A, Kanaan I, Adra C. 2013. Differential marker expression by cultures rich in mesenchymal stem cells. BMC Cell Biol. 5(14):54-66.

Woodbury D, Reynolds K, Black IB. 2002. Adult bone marrow stromal stem cells express germline, ectodermal, endodermal, and mesodermal genes prior to neurogenesis. Journal of Neuroscience Research. 69:908-917.

Xu Y, Liu L, Li Y, Zhou C, Xiong F, Liu Z, et al. 2008. Myelin- forming ability of schwann cell- like cells induced from rat adipose- derived stem cells in vitro. Brain Res. 1239:49-55.

Xu Y, Liu Z, Liu L, Zhao C, Xiong F, Zhou C, et al. 2009. Neurospheres from rat adipose- derived stem cells could be induced into functional Schwann cell-like cells in vitro. BMC Neurosci. 9:21-31.

Zavan B, Michelotto L, Lancerotto L, Della-Puppa A, D'Avella D, Abatangelo G, et al. 2013. Neural potential of a stem cell population in the adipose and cutaneous tissues. Neurol Res. 32:47-54.

Zuk PA, Zhu M, Mizuno H, et al. 2001. Multilineage cells from human adipose tissue: implication for cell-based therapies. Tissue Eng, 7:211-228.

Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. 2002. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell. 13:4279–4295.

Zuk PA. 2010. The Adipose-derived Stem Cell: Looking Back and Looking Ahead. Kellogg D, ed. Molecular Biology of the Cell. 21(11):1783-1787.