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RESEARCH ARTICLE

A Potential Differentiation of Adipose and Hair Follicle-derived Mesenchymal Stem Cells to Generate Neurons Induced with EGF, FGF, PDGF and Forskolin

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ABSTRACT:
Human Adipose Derived Mesenchymal Stem Cells (HADMSCs) and Human Hair Follicle Derived Mesenchymal Stem Cells (HHFDMSCs) have attracted great interest because of their multilineage differentiation potential, self-renewal properties, and their possible use of cell and gene therapies. This present study to investigate the neurogenic differentiation ability of hADMSCs and hHFDMSCs induced by Epidermal Growth Factors (EGF), Fibroblast Growth Factor (FGF), Platelet Derived Growth Factor (PDGF) and Forskolin. This study was true experimental with longitudinal study design. The sample size determined with minimal sample size formula and it was randomly chosen. These studies employed an *in vitro* design for the expansion and proliferation of Mesenchymal Stem Cells (MSCs) and examined the heterogeneity of these cells using the markers CD105, CD90, OCT4, and SOX2. MSCs from adipose tissue and hair follicles were induced with EGF, FGF, PDGF and Forskolin to differentiate and generate neurons. The capacity of MSCs to generate neurons were verified using glial fibrillary acidic protein, nestin, and β-tubulin III . The expression of neural markers and morphological changes in Mesenchymal stem cells from hADMSCs and hHFDMSCs were confirmed. hADMSCs and hHFDMSCs share a similar capacity to differentiate and generate neurons, which is beneficial for the development of neuronal restoration for future therapies for patients suffering from neurological diseases.

KEYWORDS: Human Adipose Mesenchymal Stem Cells, Human Hair Follicle Mesenchymal Stem Cells, Proliferation and differentiation, Growth Factor, Neurons.

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INTRODUCTION:

Mesenchymal Stem Cells (MSCs) are multipotent stem cells that can be obtained from a variety of tissues such as adipose tissue, amniotic fluid, bone marrow[1]. Adipose

Tissue (AT) has been an abundant source of MSCs and easily accessible with minimal patient discomfort. It lacks donor limitation, and obtaining these stem cells has a low risk of side effects. These cells are fibroblast-like cells capable of multipotent differentiation, which have been found in different species and have been termed Human Adipose Derived Mesenchymal Stem Cells (hADMSCs)[2]. Likewise, AT, Human Hair Follicle-Derived Mesenchymal Stem Cells (hHFDMSCs) also are easily accessible as a potential source of MSCs. HADMSCs and hHFDMSCs provide an opportunity to use autologous MSCs transplantation in the field of regenerative medicine[3].

The neurosurgical field focuses on physical restoration, repairing and replacing damaged cells and tissues[3]. The MSCs possess the ability to self-renew, secrete trophic and multipotent factors which have potential to facilitate the development of autologous tissue transplantation. Although both hADMSCs and hHFDMSCs have been used in plastic surgery as MSCs continuing to develop into and generate various cell types, manipulation of the MSCs microenvironment or niche is still required[4]. Understanding other sites of the MSCs micro environment surrounding HFDMSCs is crucial. Hair Follicles (HFs) cells are mammalian skin cells. HFs have important role to in maintaining the body's mechanical protective barrier, physiological tissue renewal and regeneration after injury[5,6].

HADMSCs and hHFDMSCs have attracted great interest because of their multilineage differentiation potential, self-renewal properties, and their possible use of cell and gene therapies. ADMSCs and HFDMSCs exhibit differentiation potentials that are similar to those of another MSCs such as multi-lineage differentiation into adipocyte, osteocyte, and chondrocyte[2]. Differentiation ability of ADMSCs into various cell lineages including cartilage, fat, bone etc., are so much affected by aging. As a result, the use of MSCs from older donors is lower than younger donors. Furthermore, proliferation and population doubling time in old MSCs slowed compared with the young MSCs ones. It is crucial to maintain the proliferation and differentiation capacity of MSCs[1]. Additionally, hADMSCs have higher and greater multipotent rates in culture compared with Bone Marrow Mesenchymal Stem Cells (BMSCs)[7]. Moreover, hADMSCs have been found not only to differentiate into adipocytes but also to into various other cell type such as myocytes, osteoblasts, chondrocytes, and neuronal cells[8,9]. HFDMSCs also offer the potential for being used for a variety of tissue defect treatment[10,11].

MSCs are non-embryonic cells with multipotent properties which have the ability to differentiate into various cell types but are more limited in that potential in comparison to pluripotent stem cells[12]. Significant strides have been made regarding the use of multipotent MSCs as a therapeutic modality and therapies can be safely and efficaciously used as a source population of multipotent cells[13,14]. HADMSCs and hHFDMSCs require the optimization of their isolation in compliance with regulatory standards and an improved understanding of their behaviors in niches is required.

The aims of these studies are to investigate the potential differentiation abilities of hADMSCs and hHFDMSCs into neurons that can subsequently be used in neurosurgery, Parkinson's disease, dermatology venerology treatment and understanding the properties of both cells using different resources to achieve the desired outcomes and minimize the side effects of these therapies[15]. This present study was performed to investigate the ability of isolated Mesenchymal stem cells from AT and HF to differentiate and generate neurons.

MATERIAL AND METHODS:

Ethical considerations:

This project complied with the national guidelines and was approved by the institutional ethics committee and regarding the research purpose using human tissues from the Ethics Research Committee, Faculty of Medicine, Airlangga University and Doctor Soetomo General Hospital Surabaya, East Java, Indonesia (Number: 067/Panke.KKE/VI/2014). The research was conducted at an experimental laboratory within the Stem Cell and Tissue Engineering Development Centre, Airlangga University, Surabaya, East Java, Indonesia. This study was true experimental with descriptive observational and longitudinal study design. The sample size determined with minimal sample size formula and it was randomly chosen.

Culturing Adipose Derived Mesenchymal Stem Cells:

HADMSCs isolate were obtained from Stem Cell Research and Development Center, Universitas Airlangga, Surabaya, Indonesia. HADMSCs isolated was subsequently re-suspended in α-MEM supplemented with 20% fetal bovine serum (FBS), 500 IU penicillin and 500µg streptomycin (Mediatech, Manassas, VA, USA) as control media. Cells were counted and plated in T75 uncoated flask or 10-cm petri dish (Sigma Aldrich, USA) with 1×10^6 cells concentration counted using cell counter (Becton Dickson, USA) were expanded until the 3rd passage and were passaged after they reached 90% confluence in normoxia preconditioning (O_2 21%) inside the Incubator (Thermofisher, USA).[9,12]

Culturing Hair Follicle Derived Mesenchymal Stem Cells:

HFDMSCs isolate were obtained from Stem Cell

Research and Development Center, Universitas Airlangga, Surabaya, Indonesia. HFDMSCs were then plated in a 5-cm petri dish with 3 ml of α-MEM supplemented with 20% foetal bovine serum (FBS), 500 IU penicillin and 500 µg streptomycin (Mediatech, Manassas, VA, USA) as complete medium. Cells were characterized in the 3rd passage.[9,12]

Phenotyping of the stem cells:

The standard procedure for MSC phenotyping is FACS analysis for several positive and negative markers according to the International Society for Cellular Therapy position statement.[16] The cells were characterized according to the human mesenchymal stem cell CD105, CD90, and CD45 markers and labelled with immunofluorescence green label fluorescent isothiocyanate (FITC). The characterizations were performed to ensure that the growing cell population was Mesenchymal stem cell. Monolayer cells were detached using trypsin (0.025%); next, after washing and counting counted using cell counter (Becton Dickson, USA) approximately 5000 cells were dropped in a volume of 10 µl PBS into a COOKE® well and incubated for 1 hour at 37ºC. After fixing with acetone, washing in -20ºC PBS with 1% serum, and reacting with monoclonal antibody anti human, anti-CD105 (Becton Dickson, US, Cat no 563264), anti-CD90 (Becton Dickson, US, Cat no 5662385) and anti–CD45 antibodies anti human with concentration 1:200 (Becton Dickson, US, Cat no 340910), the cells were analyzed under a fluorescence microscope (BX63, Olympus, US).[9,12,17] Additionally, FACS Calibur flowcytometry (Becton Dickson, USA) were used to identify the CD90 and CD45 phenotypic marker proteins on the superficial membranes.[18]

Chemical Induction of Adipose Derived Mesenchymal Stem Cells and Hair Follicle Derived Mesenchymal Stem Cells:

ADMSCs and HFDMSCs were also chemically induced to become neuron-like cells with serum-free DMEM/F12 (with high glucose; Gibco/BRL) containing one of the following:

Anti-fungal: Amphotericin B 1%; Penicillin Strep 1%; FBS 10%; EGF 20ng/ml; FGF 40ng/ml for 1-7 days medium only. In Addition, for days 8 to days 21 we made the DMEM/F12 containing one of the following: Anti-fungal: Amphotericin B 1%; Penicillin Strep 1%; FBS 10%; EGF 20ng/ml; FGF 40ng/ml; Forskolin: 4µg/ml. The cells were washed with DMEM, and the cell culture medium containing one of the above listed compounds was added for 21 days. Differentiation culture medium was changed every 2 days.

Proliferation and differentiation assay:

4',6-diamidino-2-phenylindole (DAPI) staining (D1306, Thermofisher, USA) was used to analyze the differentiation and proliferation abilities of hADMSCs and hHFDMSCs. The neurogenic ability ADMSCs and HFDMSCs were analysed using GFAP and nestin then visualised with fluorescence microscope (BX63, Olympus, US**)** MSCs were cultured in a 24-well microplate, washed once in PBS, and fixed using -20°C acetone. Finally, the MSCs were examined for the neuron-like cell markers with monoclonal antibody anti-human GFAP (cat no. G3893), nestin (cat no. N5413), bisBenzimide H 33342 trihydrochloride / Hoechst stain (cat no. 23491-52-3), and β-tubulin III (cat no. T8660) (Sigma Aldrich, USA) then visualised using fluorescence microscope with 200x magnification (Olympus, USA).

Trophic activities of Mesenchymal stem cells:

In these studies, the activity of MSCs culture was examined to know various GF and cytokines secretion as depicted in Figure 6. In human Mesenchymal stem cells, growth and stromagenesis factors such as interleukin-3, interleukin-6, interleukin-11, co-stimulating factor, transforming growth factor-β, and leukemia inhibitory factor were analyzed after 48h in culture medium then changed with fresh medium change. The culture medium contained with high-glucose DMEM containing 10% fetal bovine serum from a selected batch (Sigma Aldrich, US). As clearly indicated in Table 2, each column exhibited cytokines and GFs secretion analyzed with quantitative Enzyme-Linked Immunosorbent Assay (ELISA) kits; LIF Cat no. E1111Hu; IL6 Cat no E0090Hu; IL11 Cat no E0101Hu; CSF E0166Hu; TGF-β Cat no E0067; IL-3 E0093Hu, Bioassay Technology Laboratory)**.** Substrate, HT-PRD, was diluted in dilution buffer (25 mM Tris-HCl, pH 7.4 and 100 mM NaCl) to a concentration of 2 ng/µl and used to coat a 96-well plate (BD Falcon #353072) with 100µl per well (200 ng/well unless otherwise indicated) at 4°C overnight. Unbound materials were washed away with dilution buffer and wells were blocked with 150µl blocking buffer (2% BSA, 1X PBS, and 0.25% Tween 20) at room temperature for 60 min. After blocking, wells were washed extensively with dilution buffer before subjecting to phosphorylation. We routinely use 1:3000 dilutions for ABx purified (~1.5 mg/ml) and 1:2000 dilutions of commercial secondary antibody for the assay. ELISA result analyzed and quantified by spectrophotometer (SPECTRONIC 200, Thermofisher, US).

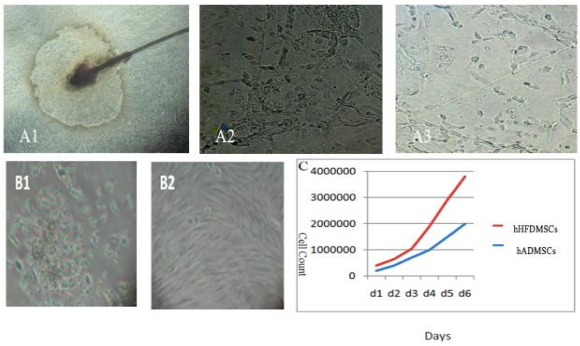

Figure 1. Isolate of MSCs from hair follicles as shown in A1, which depicts stem cell growth around a hair bulk shown in A2 and A3, which depicts colonies of stem cells. B1 depicts a colony of stem cell isolated from adipose tissue, and B2 depicts stem cell growth in the 1st passage. The expansion of the hADMSCs and hHFMSCs was done. Stem cells in the 3rd passage was cultured in petri dishes, and the proliferation of the stem cells was then calculated using a hemocytometer. The red line indicates the total cell count from the hair follicles, and the blue line indicates the total count of stem cells that were isolated from the adipose tissue shown in C.

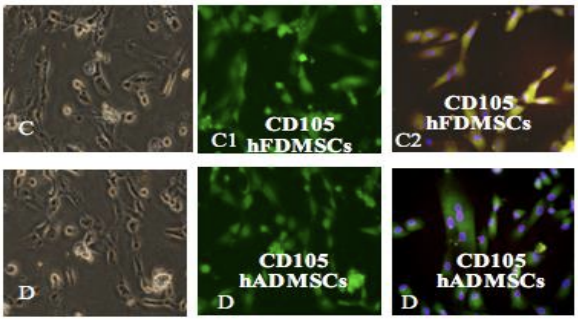

Figure 2. Characterizations of the hADMSCs and hHFDMSCs based on analyses of CD90 and CD105 labelled FITC and DAPI. A. hHFDMSCs was stained with CD90-labelled FITC and A1 was stained with CD90-labelled DAPI; B. hADMSCs were stained with CD90-labelled FITC and B1 were stained with CD90-labelled DAPI; C. hHFDMSCs was stained with CD105-labelled FITC shown in C1 and C2 was stained with CD105-labelled DAPI; D. hADMSCs were stained with CD105-labelled FITC shown in D1 and D2 were stained with CD105-labelled DAPI. hADMSCs and hHFDMSCs were examined under a fluorescence microscope without a filter and with a filter.

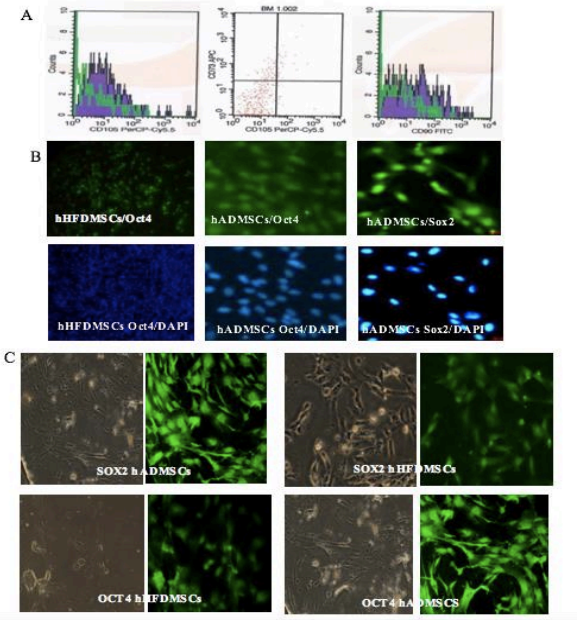

Figure 3. The heterogeneity of the stem cells isolated from the 3rd passage hADMSCs were analyzed based on the markers OCT4 and SOX2-labelled FITC and DAPI. (A). Flowcytometry with the markers CD105 and CD90 were applied to the hHFDMSCs and hADMSCs Green indicates CD105 and CD90; (B) Immunofluorescence confirm pluripotency and differentiation marker of SOX2, and OCT in both hADMSCs and hHFMSCs; C. hADMSCs and hHFDMSCs were examined under a fluorescence microscope without a filter and with a filter.

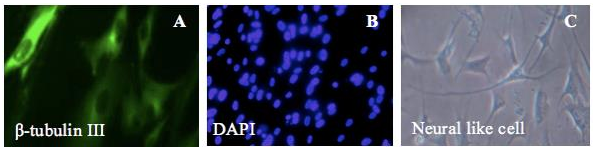

Figure 4. Analyses of the differentiation of HFSC-derived MSCs into neuron-likes using complete medium with growth factors (i.e., FGF, EGF, PDGF, and forskolin). A. The cells were analyzed using the marker β-tubulin III. B. The cells were stained using DAPI. C. Neuron-like cells were analyzed under an inverted microscope.

Table 1. Inducing hADPMSCs and hHFMSCs using growth factors.

Cell type	Passage	Induced protein	Pathways	Incubation time	Neural phenotype
ADP-MSCs	P3	Forskolin, FGF, EGF, PDGF	cAMP, NT, RA, SHH.	21 days	GFAP, Hoechst, Nestin
HF-MSCs	P3	Forskolin, FGF, EGF, PDGF	cAMP-PKA CREB	21 days	GFAP, Hoechst, Nestin, β-Tubulin III

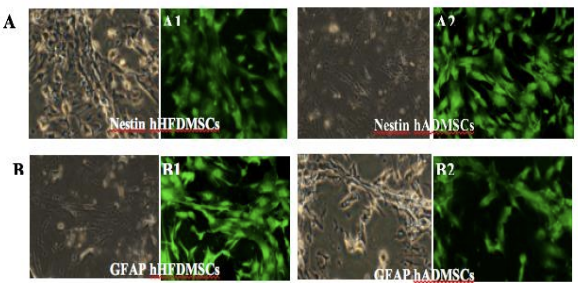

Figure 5. The differentiation of hADMSCs and hHFDSMSCs were identified using GFAP and Nestin labelled FITC.

Table 2. Simplified summary of the data regarding the potential differentiation of adipose tissue- and hair follicle-derived MSCs to neurogenesis over 48 h on conditioned media based on the secretion of bioactive factors.

Bioactive	Adipose derived MSCs		Hair follicle derive MSCs	
	Growth	Neurogenesis	Growth	Neurogenesis
LIF	+	+++	+	++
IL-6	+	+++	+	+++
IL-11	+	+++	+	+
CSF	+	++	+	++
TGF-β	+	+	+	+
IL-3	0	0	0	0

*Information: + positively expressed, ++ more positively expressed, +++ greater positively expressed.

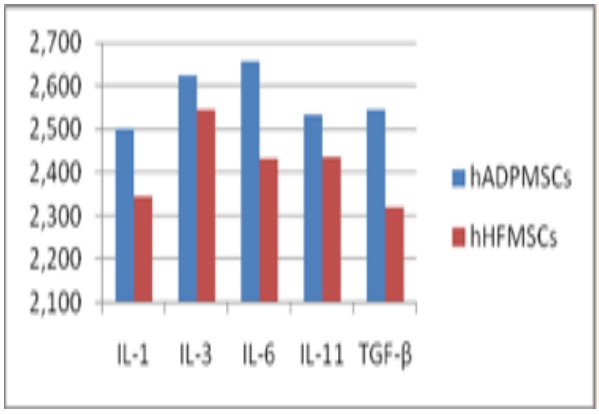

Figure 6. Secretion of bioactive factors from hADPMSCs and hHFMSCs in the supernatant media after 48 h of culture in the 3rd passage.

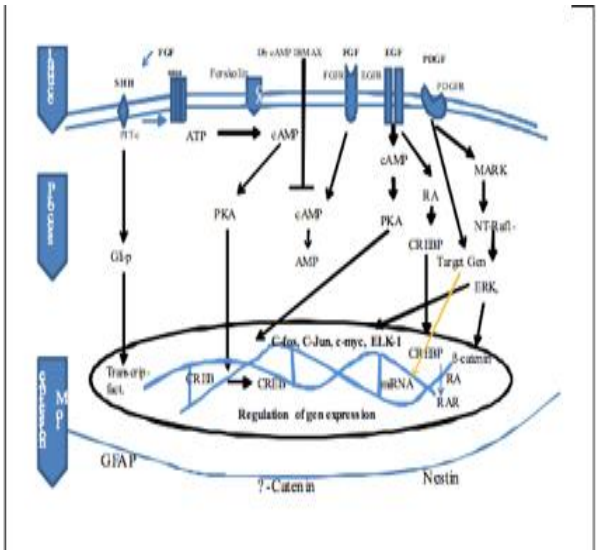

Figure 7. Proposed Mechanism of the microenvironment effects of MSC-derived factors in MSCs differentiation ability induced by EGF, FGF, PDGF, and Forskolin.

Here, we describe the results of our efforts to thaw hADMSCs and hHFDMSCs (see Figure 1). The characterizations of hADMSCs and hHFDMSCs based on immunotyping on the proliferation and differentiation abilities of both of these MSC resources indicated in the legends, the figure panels correspond to the phenotypic characterizations of the multipotent cells based on immunocytochemistry with emphases on the human stem cell markers CD105, CD90, CD45 and OCT4, SOX2 as proliferation and pluripotent marker. The values for these markers were interpreted based on the fluorescence from the superficial membranes (see Figure 2).

To analyse the heterogeneity of the MSCs, the cells were also characterized with the pluripotent stem cell markers Oct4- and Sox2-labelled FITC and DAPI with subsequent multipotent characterization by flow cytometry using the markers CD105 and CD90. The flowcytometry result only for descriptive data CD105 positive CD45 negative CD90 positive, so we can conclude that MSCs non HSCs (see Figure 3).

hADMSCs and hHFDMSCs have the properties of a high capacity for differentiation, including differentiation into multipotent stem cells. hADMSCs and hHFDMSCs can differentiate into various cells type thus into specific cell types. Foregoing studies have indicated that MSCs can differentiate become cardiomyocytes, osteocytes as well as chondroblasts. In the present studies, MSCs developed from adipose tissue and hair follicles were differentiated into neurons (see Figure 4).

Both the derived hADPMSCs and hHFDMSCs were induced with growth factors, and they were then incubated for same time and identified using GFAP, Hoechst staining and nestin. Growth factors utilize different pathways to induce cells differentiation, nevertheless both types of cells expressed GFAP, Hoechst, and nestin (see Table 1 and Figure 5).

It is clear that the MSCs isolated from both of the different resources exhibited similar phenotype-specific markers neuron-like cells. However, they showed different pathways. The analyses of the bioactive factors used some types of cytokine group markers. Different concentrations of bioactive factors were released, for example, the secretions of bioactive below quantified using ELISA (Optical Density) (see Tabel 2 and Figure 6). Proposed mechanism the microenvironmental effects of Neurogenic MSC differentiation induced by EGF, FGF, PDGF, and forskolin (Figure 7).

In these studies, the development of human hADMSCs and hHFMSCs into neurons induced by GFs, such as FGF and EGF, and Forskolin which can enhance differentiation into specific neuron cells, was examined.[15] Moreover, we used EGF, FGF, PDGF and Forskolin in these studies. hADMSCs regenerative potential has been demonstrated over many years through several techniques, like adipose tissue grafting from fat to replace soft tissue which contains various cells including MSCs that support tissue neo-vascularization and healing through the GF secretion mechanisms.[16,17] Therefore, hADMSCs can generate neurons that exhibit neural differentiation.[18,19]

The majority of research into neuronal injury has focused on regenerating host nerve cells such as Schwann cells (SCs). SCs are vital and play an important role in providing trophic factors and axon healing

support.[16,20] The present studies focused on analyzing the potential of hADMSCs to generate neurons, as hADMSCs can be obtained without invasive surgery, and are very simple to collect from adipose tissue. The present study focused on analysing the potential of hADMSCs generate neurons because these can be exploited without invasive care and are easily collected from adipose tissue. MSCs isolated from human adipose and hair follicles have beneficial proliferation and heterogeneity properties over those of multipotent and pluripotent stem cells. These findings were demonstrated by MSCs colonies and CD105, CD90, Oct4 and Sox2 expression.[21]

Analysis of the hHFMSCs that differentiate into neuron-like cells revealed β-tubulin III expression indicating that hHFMSCs offer substantial benefits in terms of neurongenic differentiation, although this ability does not match that of Embryonic Stem Cells (ESCs) or induced Pluripotent Stem Cells (iPSCs).[22] hHFDMSCs have certain advantages in regenerating cell and tissue damage due to their multipotent differentiation ability. Burn victim treatment featuring new human hHFDMSCs and gene therapy can gradually replace damaged cells and tissues.[2,10] Moreover, hHFDMSCs can induce the generating of new follicles in androgen alopecia patients.[22] The results of these studies indicated that hHFDMSCs and hADMSCs generate neurons through their neurogenic differentiation ability and precursor molecular mechanisms. hADMSCs and hHFMSCs released bioactive molecules, which include CSF, LIF, IL-6, and IL-11 but exclude TGF-β. These findings indicate that these cells promoted neurogenesis, especially the hADPMSCs. The bioactive molecules released by the hHFMSCs were similar to those released by the hADPMSCs, with the exception of IL-6, which did not strongly influence neurogenesis. These growth factors can influence molecular signalling and pathways, such as SHH, PIT-c, cAMP, RA, PKA, MARK, and CREB, and can subsequently activate transcription factors and gene expression regulation and then finally be expressed in addition to GFAP, β-Catenin, Nestin, SHH, hedgehog, cAMP and CREB; PKA, protein kinase A; MARK, matrix extracellular-regulated kinases; ERK, extracellular-regulated kinases; Growth Factor Associate Protein (GFAP) was suggested.[23]

Based on gene microarray studies that analysed the transcriptomes of undifferentiated human ADPMSCs and Bone Marrow Mesenchymal Stem Cells (BMSCs) through 28 genes analysis and two cells types were not significantly different.[24] Affymetrix gene chips was performed to compare hADMSCs and BMSCs. Affymetrix gene chips has determined that hADMSCs and BMSCs share a common transcriptome. Based on several previous studies and comparisons with the present study, we suggest that hADMSCs and hHFDMSCs are derived from Mesenchymal stem cell which possess identical potentials to generate neurons.[25] However, compared to pluripotent stem cells expressed OCT4 and SOX2 associated gene markers, hADMSCs and hHFMSCs shown less similar to BMSCs mRNA based its potential to differentiate.[12] These data are similar to those previous study, mentioned that all of the MSCs expressed embryonic stem cell markers, Oct-4, Rex-1, and Sox-2 for at least 10 passages. MSCs types showed similar multipotent differentiation ability. However, only the hBMSCs or hADSCs retain greater differentiation efficiency at higher passage.[26]

The evidence from previous studies would suggest the activation of cyclic-Adenosine-Monophosphate (cAMP) has been promoted using FGF, EGF, PDGF, and Forskolin. cAMP induced MSCs to differentiate into neural lineages. Forskolin (which activates adenylyl-cyclase), dibutyryl-cAMP (db-cAMP) and 3-isobutyl-1-methylxanthine (IBMX) increase cAMP levels. Increased level of cAMP inhibits phosphodiesterases. 8-bromo-cAMP activates PKA by phosphodiesterases. cAMP induced Mesenchymal Stem Cells in neural lineages Forskolin (which activates adenylyl-cyclase), dibutyryl-cAMP (db-cAMP) and 3-isobutyl-1-methylxanthine (IBMX) increase cAMP levels that inhibit phosphodiesterases. 8-bromo-cAMP activates PKA and is long-acting due to its resistance to degradation by phosphodiesterases. The cAMP cytoplasmic target is PKA which mediates neurogenic differentiation. Lepski stated that BMSCs demonstrate neurogenic differentiation ability which depends on the PKA pathway.[27] IL-1, IL-3, IL-6 and IL-11, which play an important role in the differentiation of cells, were released into the CSF by MSCs. Previous studies have argued that PKA inhibits the differentiation process which is induced by Brain-Derived Neurotrophic Factor (BDNF).[28,29]

HADMSCs and hHFDMSCs with FGF are attached with SHH for 12 days after incubation, that differentiates dopaminergic neurons and increases Tyrosine Hydroxylase (TH) expression and electrophysiological features.[30] HADMSCs and hHFDMSCs possessed neuronal markers and gene downregulation which are involved in cell cycle like Cyclin-Dependent Kinase 2 (CDK2) and Proliferating Cell Nuclear Antigen (PCNA) that are indicators of post-mitotic fate.[31,32,33] HADMSCs and hHFDMSCs share a similar capacity to differentiate and generate neurons, which is beneficial for the development of neuronal restoration for future therapies. HADMSCs and hHFMSCs are suggested for neuronal restoration in the future therapies for patients suffering from neurological diseases.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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