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FOREWORD

This proceeding is a collection of papers presented at the Scientific and Plenary Meeting ASEA-UNINET 2016 held in Bukit Jimbaran Campus, Udayana University, Bali, Indonesia from 15th to 18th of February 2016. The committee has accepted 67 papers from 7 countries (Austria 3 papers; England 1 paper; Indonesia 51 papers; the Philippines 2 papers; Poland 1 paper; Vietnam 7 papers; Thailand 1 paper; Laos 1 paper).

We would like to extend our big thanks to Rector of Udayana University, Prof. Dr. dr. Ketut Suastika, Sp.PD-KEMD, Vice Rector for Academic Affairs, Prof. Dr. Drh. I Made Damriyasa, MS and Vice Rector for Cooperation and Information Affairs, Prof. Drs. I Made Suastra, Ph.D for their great support on this event. My special thank goes to committees of ASEA-UNINET and Indonesian organizing committee for the excellent collaboration.

We would like to express our sincere thankfulness to those who presented their papers at the conference. We also wish to thank the students of Udayana University who have assisted us with the typesetting for the format of the proceedings. More importantly, we express our gratitude to the board of reviewers who have worked hard in reviewing the submitted papers selected for the proceedings.

Jimbaran, 15 Februari 2016

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SETTING AND TYPESET
I Made Sena Darmasetiyawan, S.S., M.Hum
I Made Yoga Dwi Angga

COVER
I Putu Oka Pradnyana, S.Kom

TABLE OF CONTENT

DIFFERENTIATION POTENTIAL OF AMNION MEMBRANE AND DENTAL PULP DERIVED MESENCHYMAL STEM CELL TO GENERATE NEURON INDUCED WITH EGF, FGF, PDGF AND FORSKOLIN

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Abstract

Human multipotent stem cells, including human pluripotent stem cells, hold promise as novel therapeutic tools for neuron treatment because of their self-renewal capacity and ability to differentiate into neuron cells. Small and large molecules play important roles in each stage of neuron differentiation from amnion membrane and dental pulp. In these studies have been explored of human amnion membrane (hAMSCs) - and dental pulp derived mesenchymal stem cells (hDPMSCs) differentiation potential to generate neuron like cell. The method of these studies have been promoted by using several small growth factors as well as epidermal growth factor (EGF), fibroblast growth factor (FGF), PDGF, Forskolin, then has been characterized phenotype, proliferation assay, differentiation, through immunocytochemistry, flowcytometry by using CD105, CD90, Oct4, Sox2, GFAP, β-Tubuline Nestine, The results have shown significantly advanced efforts of membrane amnion and dental pulp derive mesenchymal stem cell can generated into neuron. The both resources mesenchymal stem cells contributed possibility from the initial stages of definitive ectoderm formation to the differentiated stages of maturation of neuron cells.

Keywords: hAMSCs, hDPSCs, proliferation, differentiation, neuron like cell.

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1 INTRODUCTION

Neuron disease like Parkinsons, Alzheimer's are the most common neuron disorder with increasing incidence worldwide, predicted to increase every year. Currently, conventional therapies are not widely successful because neuron degeneration leads to several associated ailments and system disorders. In future, stem cell therapy is expected to be more powerful than existing treatments for this pervasive and debilitating disease. Naturally, much attention has been directed to the generation of neuron without tumor formation or immune rejection from human adult stem cells in the last few years. A new generation of research has recently focused on multipotent, pluripotent stem cells and induce pluripotent stem cells (iPSCs) through inserting gene such as *OCT3/4, SOX2, c-MYC,* and *KLF-4*. The approach the human iPSCs can be derived from various non-pluripotent cells, such as adipose cells, amniotic fluid cells, hepatocytes, blood cells, fibroblasts, and bone marrow cells (Takahashi,et al., 2006), although embryonic stem cell more easy to develop into different type cell, but their problems are still in ethical issue (Lo and Parham, 2009).

In recent years there has been increased interest in mesenchymal stem cells and their potential utility in both tissue engineering and repair (Rantam, et al., 2015). Animal studies have provided a useful tool for defining a number of diverse potential applications for MSCs. Kamandjaja et al (2015). Demonstrated that cultured mesenchymal cells could home and repair bone jaw in New Zealand with rabbit. The ability of cultureexpanded human mesenchymal cells to contribute to the functional repair of an injured pancreas has also been examined using a variety of animal models (Rantam, et al., 2015). MSCs can repair an 8 mm defect in a rat jaw when placed into a porous cylinder which was then implanted into the bone jaw. After 6 weeks, the defect containing the MSC-loaded implant completely healed, while the defects containing cylinders filled with control cell populations failed to heal successfully (Hardijantini, et al., 2015). These studies indicate that culture-expanded MSCs are able to both persist and contribute to de novo bone formation in vivo.

Studies in sheep have addressed the possibility of using mesenchymal cells to enhance engraftment of transplanted hematopoietic cells (Kamadjaja, et al., 2015), and transplantations of defected neuro femoralis New Zealand rabbit with bone marrow derived MSCs enhanced function the both rabbit back extremitas (Christijogo, 2015). Transplantation of unprocessed whole bone marrow cells has been shown to restore microenvironmental function, suggesting that unprocessed bone marrow contains stromal precursors as well as hematopoietic precursors that contribute to hematopoietic regeneration following transplantation. It has been interpret this linear correlation to imply a relationship between vascularity and local MSCs population (Kubis et al., 2006; Meirelles et al., 2006).

The apparent ability of MSCs to give rise to cells of multiple germ layers, however, must be examined cautiously, as undifferentiated mesenchymal cells have been shown to spontaneously express neural markers, In these research focus of the potential MSCs differentiation ability with different resources MSCs from human amnion membrane and human dental pulp.

2 MATERIALS AND METHODS

2.1 Collection and Preparation of Human Membrane Amnion

Cesarean section was performed in the Central Operating Theatre of Dr. Soetomo General Hospital Surabaya on Healthy full term neonate. After the baby delivered, amnion membrane was cut out, the placenta together with the remaining amnion membrane was

evacuated and placed in the sterile kidney-shape stainless steel receptacle. About 10 cm length was obtained and washed in Phosphate Buffer Saline (PBS) three times to remove the excess blood and blood clot. Next, amnion membrane was soaked in Ringer Lactate solution containing 2.5 µg/mL gentamycin and 1000 U/mL amphotericin B for 20 minutes. The medical ethical review board of Dr. Soetomo General Hospital has already approved this protocol.

2.2 Isolation and Culture of hAMMSCs

Amnion membran was dissected into small pieces about 1 cm with knife into fine pieces of 1 mm3 and was used to isolate and culture primary hAMMSCs. Then was transferred to 0.25% trypsin and digested in 37°C for 40 minutes, then centrifuged, have the supernatant removed, this processed was then repeated twice. The crushed and digested sample, then subjected to PBS containing 0.75 mg/ml collagenase IV (Sigma-Aldrich, St. Louis, MO, USA) and 0.075 mg/mL DNAse I (Takara Bio, Shiga, Japan), incubated at 37°C for 60 minutes. This was followed by filtration with cell strainer and pellet collection upon centrifugation for 10 minutes to finally obtain the cells. The single cells collected were then cultured on collagen-coated dishes using alpha modification of minimum essential medium eagle (α-MEM) (Gibco BRL, Gaithersburg, MD, USA), supplemented with human leukemia inhibitory factor (10 ng/mL) and fetal bovine serum (FBS) (Gibco BRL). Primary cells growth was observed under the microscope. The timing of cell confluence was recorded. The medium was changed once every three days. When the confluence reached 90%, the cell splitting was done using trypsin. Half to two thirds of the cells were then re-plated onto a new dish of the same medium. The isolation procedure used was according to the Laboratory of Stem Cell, Institute of Tropical Disease, Airlangga University protocol (Rantam et al., 2015).

2.3 The hAMMSCs Phenotypic Characterization

2.3.1 Immunocytochemistry

Single cell of cultured hAMMSCs were plated onto coverslips. The cells were rinsed five times with PBS tween. 20, drying and fixed with formaldehyde 10% for 15 minutes, and then they washed four times with PBS tween.20 and let to dry for few minutes. The cells were blocked by bovine serum albumin (BSA) for 30 minutes, incubated with fluorescein isiothiocyanate (FITC)-labeled monoclonal antibody anti-human Cellular Differentiation (CD)105, CD90 and CD45 for 60 minutes. Cells were rinsed with PBS twice and ready for analysis. Immunostained cells with CD105, CD90 and CD45 expression were analyzed using fluorescence microscope, and other label like.

2.3.2 Flow Cytometry

Cultured hAMMSCs were trypsinized and suspended in α-MEM, washed with PBS, and fixated in formaldehyde 10% for 10 minutes, and finally were closed in 10% BSA for one hour. Cells were incubated using Human MSCs Analysis Kit (BD StemflowTM, BD Biosciences) with primary antibodies mouse anti-human CD90, CD105, and negative cocktail containing CD45, for 40 minutes. The unbound antibodies, then removed by washing with PBS. The bound primary antibodies were labeled using FITC-conjugated anti-mouse antibody by incubation for 30 minutes. The cells were then visualized and analyzed using FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA.

2.4 Proliferation and Differentiation Assay

Proliferation assay was analyzed by using markers labeled DAPI, and for the plasticity analysis of stem cells were used markers Oct4, Sox2. Then have been done analysis of

differentiation cells using some markers GFAP, β-Tubulin and nestin, after have been added growth factors (EGF, FGF, PDGF, and Forskolin) in the medium complete. The whole methods have been done according to Rantam, et al., (2015) and differentiation assay according to Chamberlain (2007).

3 RESULTS

The biological and molecular crosstalk among FGFs, EGF, PDGF, and Forskolin and others molecular like retinoic acid indicates important plays an essential role in neuron specifications. They have a significant effect on the differentiation of MSCs into neuron. The result of these research like below;

3.1 Isolation and Culture Stem Cells

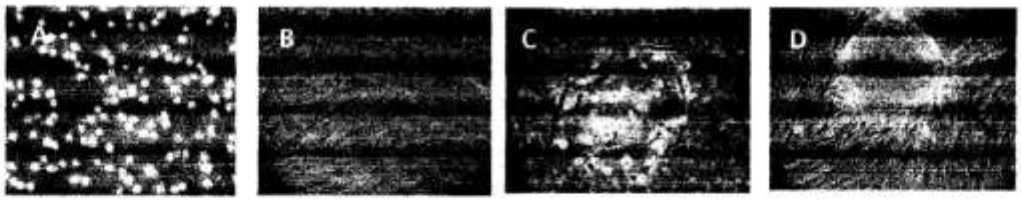

Fig.1. Isolation and culture of mesenchymal stem cell. A. Isolated stem cell from dental pulp, B. cultured stem cell of dental pulp 7 days, C. isolated stem cell from amniotic membrane and incubated at 37°C 2 days, D. cultured stem cell of amniotic membrane 7 days. Arrow in Fig. C and D. are colony stem cell.

Base on the results in Fig.1. have been shown that the both stem cell have relative different as well as by their growing, and the both stem cell can growth monolayer 7 days after cultured in petridish. To determine of stem cells diferensiasi became mesenchymal stem cell have been done characterization by using markers CD90, CD105 and CD45. The results like in Fig.2.

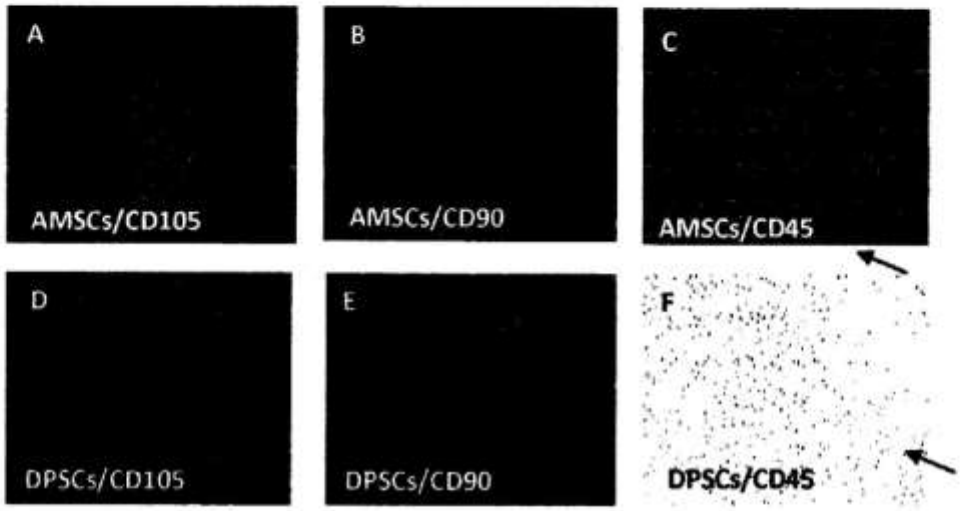

Fig.2. Phenotyping of stem cells using markers superficial membrane protein to determine of mesenchymal stem cell (MSCs). **A&B**. amnion membrane derived MSCs expressed CD105 and CD90, **C**. amnion membrane derived MSCs expressed CD45-. **D&E**. Dental pulp derived MSCs expressed CD105 and CD90. **F**. Dental pulp derived MSCs expressed a lack CD45 like at arrow. In these cases are actually stem cell still not purify. perhaps were caused system purify using Ficoll sometime, and the best purification of stem cell are using cell sorter.

The both resources of stem cells have been derived MSCs shown that expression CD105, CD90, but negative expression of CD45. Although these MSCs have expressed too CD73, but data is not shown. To analyze of the plasticity like pluripotent stem cells have been used markers Oct4 and Sox2.

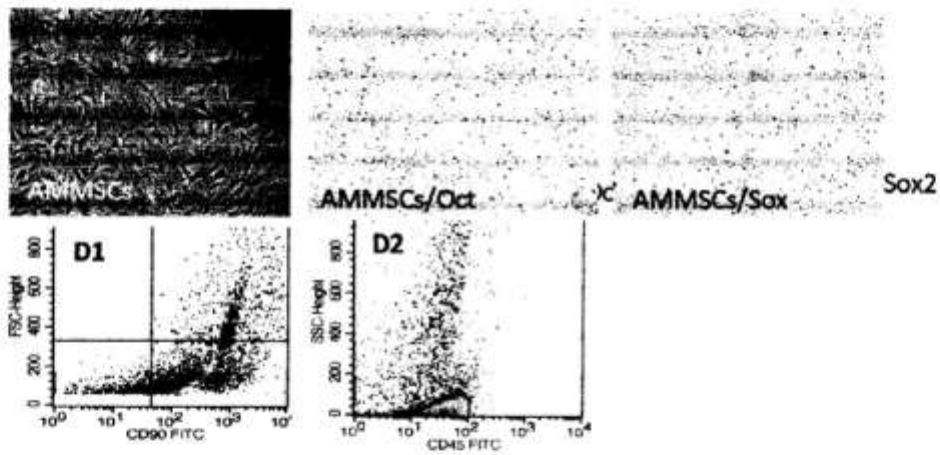

Fig. 3. Analysis pluripotent isolated stem cell from amniotic membrane and dental pulp using markers Oct4 and Sox2. Imaging microscope (20x). Green fluorescence staining are stem cells expressed Oct4 and Sox2, but without colour are negative stem cells expressed Oct4 and Sox2. And D1 and D2 flow cytometri analysis showed expression of CD90 and there are some CD45 expressed on superficial membrane cells (*h*AMMSCs) derived MSCs. But *h*DPMSCs data not shown.

In these studies showed that *h*AMSCs and *h*DPMSCs expression Oct4 and Sox2, but data of *h*DPMSCs not shown. Base on proliferation assay by using MTT in Fig.4

showed little distinct between hAMSCs and hDPMSCs. Staining using DAPI were documented under fluorescence microscope without and with filter showed similar no different.

3.2 Proliferation Assay

Proliferation assay in these research were monitored by using MTT, and staining using DAPI. The both stem cell between hAMMSCs and hDPMSCs have view different while their proliferation as well aas in Fig.4. showed that explored stem cell from hAMMSCs have rapid growth then from hDPMSCs. And also like in Fig 4.B1 and C1 are similar like B2 and C2 the both cell stained using DAPI.

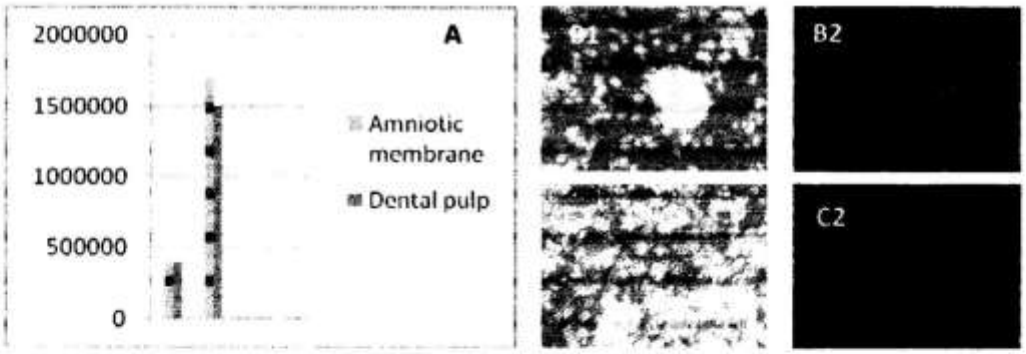

Fig.4. Proliferation of MSCs were analyzed by using MTT, and DAPI. **A.** Total cell count of cultured MSCs in 5cm Petridis 7 days after cultured MSCs, **B.**1&2 Amniotic derived MSCs have been stained by using DAPI marker. C. 1&2. Dental pulp derived MSCs have been stained DAPI marker.

3.3 Differentiation MSCs Into Neuron Cells

In these chapter has been shown that all bioactive like growth factor have an important role to induce of stem cell to begin of differentiation. Growth factors were added in medium culture of stem cell. After 7 days, medium were collected, and cells were analyzed using some kit like in Fig.5. A2, A3, B2.

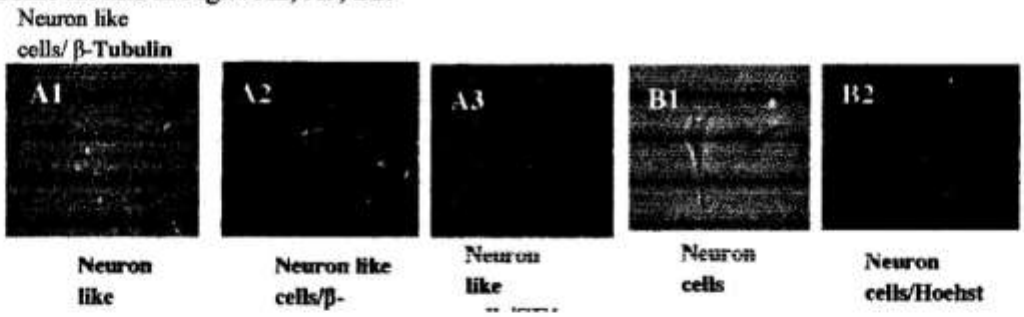

Fig.5. Differentiation analysis at 21 days after induced growth factors of amnion membrane and dental pulp derived MSCs 3rd passage. A. Isolated stem cell from Dental Pulp, B. Isolated stem cell from amnion membrane. The both hAMMSCs and hDPMSCs were grown in medium complete using α-MEM, and antibiotic 1/1000, FCS 20%.

Stem cells differentiations have some stage 1 until 5 with different properties or capacity into specific cells as well as desire cell type like neuron cell type. In our

studies have been used growth factor EGF, FGF, PDGF and Forskolin. Through addition of growth factors to the complete growth medium have shown that the both cell have ability to generate into neuron cells, like in Fig.5. A2, A3, B2 above. Simple strategy of stem cell differentiation as below;

4 DISCUSION AND FINDING

The fifth different stages during the differentiation of neuron cells from MSC stem cells are specification, expansion, and differentiation. Small and large molecules potentially play an important role in the formation of definitive ectoderm and further differentiation into neuron.

The results of our studies in stage isolation and characterization Fig. 1. and Fig. 2 & 3 have been shown that human amnion membrane and human dental pulp have high potential as stem cell resources, although the both of stem cell have view different especially in growing properties. *h*AMMSC more rapid then *h*DPMSCs. But the both cells have found not only proliferation became multipotent but followed too pluripotent markers Oct4 and Sox2 although not dominant. These finding slowly remove after three time passage. Because stem cell differentiated into progenitor cells (Steward, et al., 2014). But recently found that *OCT4* repressed WNT pathway signaling during the self-renewal process (Davidson, et al., 2012)

Some different receptors play important roles in the FGF, EGF, PDGF and Forskolin signaling pathway mediated by the four main tyrosine kinase receptors FGFR1, FGFR2, FGFR3, and FGFR4 especially of FGF induction (Ornitz, et al., 1996). Therefore, growth factors promotes a close developmental relationship between the neuron and other organs such as the liver.

Furthermore, the effect of growth factors alone is insufficient, but the addition of optimal growth factors along with a liver inhibitor results in others differentiation of MSCs. However, although FGF, EGF, PDGF and Forskolin plays a vital role in tissue and cells formation, the function of the growth factors signaling pathway is not yet fully understood. Eighteen different growth factors influence expression of the various growth factors involved in the regulation of some cell or tissue expansion. It can caused transdifferentiation of MSCs in to many kinds of cell type (Keilhoff, et al., 2008)

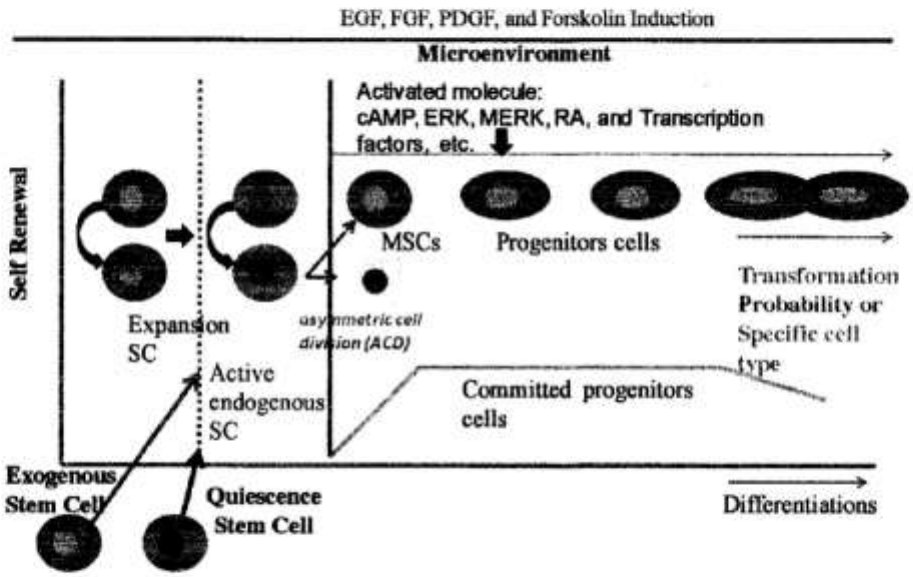

Fig.6.Simplify of stem cell strategy differentiation. After induced using EGF, FGF, PDGF, and Forskolin, through microenvironment were MSCs actived to internalize via some molecule like receptors followed actived endogenous stem cells, and gen transcription through promoted cAMP, ERK, MERK, RA, and active transcription factors like CREB, C-fos, C-Jun, c-myc, ELK-1 data not shown, and others. Finally stem cell differentiated into progenitors cells or transformation probability became specific cell type.

Also,Transforming growth factor signaling, Fibroblast growth factor signaling, WNT signaling, bone morphogenetic protein (BMP) signaling, and retinoic acid receptor signaling. A comprehensive understanding of neuron development must distinguish extracellular signals at each stage and also recognize the fundamental molecular mechanisms of each molecule and factors that activate its respective signal to trigger MSCs to differentiate into neuron cells (Marson, et al., 2010).

Base on the data in Fig.5. shown that *h*AMMSCs and *h*DPMSCs can differentiate into neuron cells. Because these cells can be typical induced to proliferate in vitro in response to some signaling like EGF, FGF, PDGF, and forskolin and also retinoic acid. This way MSCs can be induced too using mitogen, neural stem cells futher differentiate into the major cell types of the nervus system, including neurons, astrocytes and oligodendrocytes (Steward, et al., 2014). These experiment and base on the expression of GFAP, β-Tubulin, Nestin, Hoehst, suggested that adult stem cell from different resources can be developed to generate into neurons cells, The second finding is mechanism of differentiation through induced of growth factor like EGF, FGF, PDGF, and Forskolin. Activated MSCs through growth factors and some receptors depend on kinds of receptors. Induced growth factor to target MSCs like FGF, EGF, PDGF and forskolin active differentiation factors through cAMP, RA, MERK, SHH, then active transcription factor. Finally expressed product protein pathway in superficial membrane, although in these

mechanism WNT/beta catenin have promoted in transcription factors (Marson , et al., 2008; Wagner, et al., 2010).

5 CONCLUSION

*h*AMMSCs and *h*DPSCs derive mesenchymal stem cells (MSCs) are multipotent cells that are able to generate a wide range of cell types, including neural cells, which makes them incredibly interesting in restorative therapies in the future for patients suffering from neurological diseases. A lot of induction protocols indicate that many signaling pathways may be involved in the neural. *h*AMMSCs and hDPMSCs, indeed, the signalization pathways of Hedgehog, cAMP, Retinoic acid, MERK and the neurotrophins-activated pathways have been implicated into the maturation of adult *h*AMMSCs and *h*DPMCSCs into neural-like cells. After an induction process consisting in various activators, lengths and conditions of culture, treated cells adopt a neural morphology express markers (at the transcriptome level as well as at the protein level) that are usually described to characterize neurons at different developmental stages in *h*AMMSCs as well as in *h*DPFMSCs. These ways are *h*AMMSCs and *h*DPMSCs can develop and have suggested as neural restorative in the future.

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