GENERATION, PROPAGATION AND CHARACTERIZATION OF SECOND AND FULL-TERM AMNIOTIC FLUID-DERIVED MESENCHYMAL STEM CELL

By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

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DEDICATION

This thesis is dedicated to my dearest mother and brother for the understanding and encouragement they provided during all these years of the study

Abstract of the thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

GENERATION, PROPAGATION AND CHARACTERIZATION OF SECOND AND FULL-TERM AMNIOTIC FLUID-DERIVED MESENCHYMAL STEM CELL

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May 2016

Chair: Thilakavathy Karuppiah, PhD
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Mesenchymal stem cells (MSCs) are multipotent stem cells that are highly proliferative with the ability of self-renewal and the potential to differentiate into various cell lineages. The differentiation potential, self-renewability, immune-suppression properties and ease for genetic modification make them a frontier candidate for regenerative medicine, cell and gene therapy. To date, bone marrow (BM) is the most accessible source of MSCs for isolation, but BM aspiration is very invasive and a painful procedure. Although the success rate of stem cell retrieval and their expansion is high in adult bone marrow samples, certain conditions can limit their accessibilities. For instance, in some clinical cases such as bone marrow failure, aplastic anaemia, leukaemia or chemotherapy, patients often encounter complications of inadequate cellular fractions in their bone marrow aspiration, for these patients, the only alternative source of MSCs would be a second party donor. Most of the time, there are difficulties in finding the suitable donor. Moreover, the number and differentiation capacity of MSCs decrease significantly with ageing. Altogether, these necessitate a need to search for alternative sources of MSCs for research and therapeutics application. Human amniotic fluid cells (hAFCs) have been used as a diagnostic tool for prenatal diagnosis and they have been found to be a rich source of progenitor/stem cells. Human amniotic fluid (hAF) is usually considered as clinical wastes and inevitably discarded during amniocentesis, caesarean (c-section) and normal delivery. Two main populations of stem cells that can be isolated from human amniotic fluid, are amniotic fluid mesenchymal stem cells (hAF-MSCs) and amniotic fluid stem cells.

In current study hAF-MSCs are isolated from amniocentesis and caesarean samples and propagated in vitro using different growth medium. The study performed a comparative analysis for the effect of different media components on the properties of MSCs (cell morphology, cell growth, surface markers, colony forming unit assay and differentiating potential). The hAF-MSCs were the characterized using flow cytometry technique and screen a panel of cell surface markers. In conclusion, hAF-MSCs were successfully generated from the amniocentesis and cesarean amniotic fluid samples, without any significant characteristic difference between hAF-MSCs derived from the two gestation periods. XSFM was found to be the best medium for the generation and propagation of the hAF-MSCs in comparison to the DMEM-FBS and DMEM-HS. Therefore, this study suggests that the cesarean amniotic fluid, a waste product during delivery is feasible to generate xenogenic free hAF-MSCs for safe therapeutic application and possible hAF-MSCs\banking.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

PENJANAAN, PEMBIAKAN, DAN PENCIRIAN BAGI PERINGKAT KEDUA DAN FASA PENUH SEL INDUK MESENKIMA YANG DIPEROLEHI DARIPADA CECAIR AMNION

Oleh

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Sel induk mesenkima merupakan sel induk multipotensi yang mempunyai pembiakan yang aktif, memiliki keupayaan perubahan diri yang tinggi dan mampu membezakan dirinya kepada sel yang bercirikan mesoderma. Potensi membezakan diri, keupayaan pembaharuan diri, kegiatan immunotindasan dan kemudahan untuk modifikasi genetik oleh MSC menjadikan ia sebagai calon yang berpotensi dalam perubatan generasi semula dan sel atau gen terapi. Pada ketika ini, sumsum tulang belakang (BM) adalah sumber MSC yang paling mudah untuk isolasi, tetapi aspirasi BM adalah sangat invasif dan prosedur mendapatkan sumsum tulang adalah agak menyakitkan. Meskipun kejayaan pengambilan sel induk dan process memperbanyakkan kuantiti sel adalah tinggi daripada sampel sumsum tulang dewasa, tetapi pada kondisi tertentu keupayaan sel ini adalah terhad. Contohnya di dalam kes klinikal di mana terdapat kegagalan sumsum tulang, anemia aplastik, leukemia atau kemoterapi di mana pesakit sering mengalami komplikasi pecahan sel selular yang tidak memadai dengan aspirasi sumsum tulang mereka. Oleh itu satu-satunya sumber alternatif MSC daripada sumsum tulang dewasa, adalah penderma daripada pihak kedua. Kebiasaannya untuk mendapat penderma yang sesuai adalah sangat susah. Selain itu jumlah dan kapasiti untuk proses MSC membezakan dirinya akan menurun secara signifikan dengan pertambahan umur. Penyelesaian kepada permasalahan ini, adalah mencari sumber alternatif MSC yang baru untuk tujuan ujikaji dan aplikasi terapeutik. Penemuan sel cairan amnion manusia (hAFCs) telah digunakan sebagai sumber untuk diagnosis prenatal di mana terdapat penemuan yang mengatakan hAFCs ini kaya dari progenitor/sel induk. Cairan ketuban manusia (haf) biasanya dianggap sebagai sisa klinikal dan ia akan dibuang semasa amniosentesis, caesar (c-section) dan persalinan normal. Dua populasi utama sel induk yang dapat diisolasi dari cairan ketuban manusia,

adalah sel induk mesenkima cairan ketuban (hAF-MSCs) dan sel induk mesenkima cairan amnion.

Di dalam kajian ini, hAF-MSCs diisolasi dari amniosentesis dan sampel caesarean, di mana hAF-MSCs ditumbuhkan secara *in vitro* dengan menggunakan media pertumbuhan yang berbeza. Di dalam kajian ini analisis perbandingan telah dilakukan untuk mengetahui kesan komponen media yang berbeza pada sifat-sifat MSC (morfologi sel, pertumbuhan sel, penanda permukaan, uji unit pembentuk koloni dan potensi membezakan diri hAF-MSCs daripada sel yang lain). Analisis penanda permukaan sel hAF-MSCs adalah menggunakan teknik aliran sitometer dan penyaring panel penanda permukaan sel. Kesimpulannya, HAF-MSCs berjaya dihasilkan daripada sampel cairan amniosentesis dan caesarean amniotik, tanpa sebarang perbezaan ciri-ciri penting antara hAF-MSC yang diperoleh dari dua tempoh kehamilan. XSFM didapati medium terbaik untuk menghasilkan dan pertumbuhan hAF-MSCs berbanding dengan DMEM-FBS dan DMEM-HS. XSFM adalah penemuan yang terbaik untuk media penjanaan dan pertumbuhan hAF-MSC dibandingkan dengan DMEM-FBS dan DMEM-HS. Oleh itu , penelitian di dalam kajian ini menyarankan cairan ketuban caesarean yang dianggap sisa klinikal sepanjang proses kelahiran adalah dianggap paling layak untuk menghasilkan ,xenogenic hAF-MSCs untuk aplikasi terapeutik yang selamat dan kemungkinan juga untuk proses pengumpulan bank hAF-MSCs.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

AF	Amniotic Fluid
AFKL	Amniotic Fluid c-Kit, Lin
AF-MSCs	Amniotic Fluid Mesenchymal stem cells
bFGF	Basic Fibroblast Growth Factor
BM	Bone Marrow
BM-MSCs	Bone Marrow Mesenchymal Stem Cells
BD	BECTON DICKINSON
CD	Cluster of differentiation
CFU	Colony-forming units
CT	Culture Time
CBT	Cord Blood Transplant
CBF1	Core Binding Factor 1
DMEM	Dulbecco's Modified Eagle Medium
EAE	Experimental Autoimmune Encephalomyelitis
EDTA	Ethylene diaminetetra acetic acid
EGF	Epidermal Growth Factor
EPC	Endothelial Progenitor Cells
FACS	Fluorescence Activated Cell Storing
FBS	Fetal Bovine Serum
F2R	Coagulation Factor II Receptor
F2RL	Coagulation Factor II Receptor-Like
FITC	Fluorescein Isothiocyanate
GD2	Ganglioside 2
GFP	Green Fluorescence Protein
HAF	Human Amniotic Fluid
HAFC	Human Amniotic Fluid Cells
hAF-MSCs	Human Amniotic Fluid Mesenchymal Stem Cells
HLA	Human Leukocyte Antigen
HS	Human Serum
IFN-γ	Interferon Gamma
IL1-R	Interleukin Receptor 1
INRs	Immune non-responders
ICM	Inner Cell Mass
iPSCs	Induced Pluripotent Stem Cells
KIRs	Killer Immunoglobulin-like receptors
MACS	Magnetic Activated Cell Storing
MMP2	Matrix Metalloproteinase 2
MSCs	Mesenchymal stem cells
MS	Multiple Sclerosis
PDGF	Platelet Derived Growth Factor
PLA2G10	Phospholipase A2, group X
PDT	Population Doubling Time
PDN	Population Doubling Number
PRP	Platlet Rich Plasma

PE	Phycoerythrin
ssDNA	Single-Stranded DNA
TNF	Tumor Necrosis Factor
TGFB1	Transforming Growth Factor B1
VWF	Van – Willebrand factor
VEGF	Vascular Endothelial Growth Factor
XSFM	Xeno-and Serum-Free Media

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Mesenchymal Stem cells (MSCs) were isolated and described for the first time by Friedenstein and Petrakova from rat bone marrow (BM) (Friedenstein, Piatetzky-Shapiro, & Petrakova, 1966). MSCs are also referred to as BM stromal cells, BM stromal stem cells, colony-forming fibroblastic cells and mesenchymal progenitor cells (Baksh, Song, & Tuan, 2004) (Kemp, Hows, & Donaldson, 2005). MSCs are highly proliferative multipotent stem cells with the ability of self-renewal and the potential to differentiate into various cell lineages such as adipocytes, chondrocytes, osteoblasts, endothelial cells, cardiac myocytes, nerve cells, hepatocytes and pancreas cells (Kemp et al., 2005); A Alhadlaq & Mao, 2003; (Pittenger, 1999a) (Kim et al., 2014); (Jiang, Vaessen, et al., 2002)A Alhadlaq & Mao, 2003; Xiang et al., 2007; Kim et al., 2014). Differentiation potential of these cells was observed in *in-vivo*, *in-vitro* and *ex-vivo* cultures. Their differentiation potential, self-renewability, immunosuppresant properties (Le Blanc, Tammik, Rosendahl, Zetterberg, & Ringdén, 2003) and ease acceptive for genetic modification (Chan et al., 2005) makes them a frontier candidate for regenerative medicine, cell and gene therapy (Amara, Touati, Beaune, & de Waziers, 2014).

To date, BM is the most accessible source of MSCs for isolation. Indeed, BM contributes to most of the research and development on MSC biology. Most work concerning MSCs are done exclusively on adult bone marrow MSC (BM-MSCs), however, BM aspiration is an invasive and painful procedure which is at risk for infection, excessive bleeding and other complications (Duscher et al., 2014). Although the success rate of stem cell retrieval and their expansion is high in adult bone marrow samples, certain conditions can limit their accessibility. For instance, in some clinical cases such as bone marrow failure, aplastic anaemia, leukaemia or chemotherapy, patients often encounter complications of inadequate cellular fractions in their bone marrow aspiration. For these patients, the only alternative source of MSCs would be a second party donor. Most of the time, there are difficulties in finding the suitable donor. Moreover, the number and differentiation capacity of MSC decreased significantly with ageing (Makhluf, Mueller, Mizuno, & Glowacki, 2000; Mueller & Glowacki, 2001). Altogether, these necessitate a need to search for alternative sources of MSCs for research and therapeutic applications.

Mesenchymal stem cells have been isolated from other sources such as fat tissues (Ghorbani, Jalali, & Varedi, 2014), endometrium (Schüring et al., 2011), appendix (De Coppi et al., 2006) and synovium (D.-H. Lee et al., 2011); especially from tissue of fetal origin like cord blood (CB) (Divya et al., 2012), chorionic villi (Igura et al.,

2004) umbilical cord (UC) (Sarugaser, Lickorish, Baksh, Hosseini, & Davies, 2005) (Vellasamy, Sandrasaigaran, Vidyadaran, George, & Ramasamy, 2012) and amniotic fluid (AF) (De Coppi, Bartsch, et al., 2007; Peng, Wang, Chao, & Chang, 2007; Gosden, 1983).

In the last 7 years, human amniotic fluid cells (hAFCs) have been used as a diagnostic tool for prenatal diagnosis (Chadefaux-Vekemans et al., 2006) (Bossolasco et al., 2006) and they were found to be a rich source of progenitor/stem cells (De Coppi, Bartsch, et al., 2007) (Ditadi et al., 2009). Human amniotic fluid (hAF) is usually considered as clinical waste and is inevitably discarded during amniocentesis, caesarean and normal delivery. Two main populations of stem cells isolated from hAF are amniotic fluid mesenchymal stem cells (hAF-MSCs) and amniotic fluid stem cells (hAFSc) (M.-S. Tsai, Lee, Chang, & Hwang, 2004; De Coppi, Bartsch, et al., 2007). hAF-MSCs are ample and can be easily obtained from small volume (2-5 ml) of AF (Cananzi, Atala, & De Coppi, 2009), (Nadri & Soleimani, 2007). The percentage of their population is estimated at 0.9-1.5%, and can be easily cultured (Kunisaki et al., 2007). Transcriptome analysis of AFMSCSs show up-regulation involved in in uterine maturation and contraction such as PLA2G10 and in signal transduction pathways such as F2R, F2RL. These findings suggest an important role of AFMSCs in regulating the interactions between the uterus and the foetus during pregnancy (T.-H. Wang, Lee, & Hwang, 2011).

Thus, the early phase of this project focused on the generation of MSCs from hAF collected from different trimesters (second and third), where the generated cells were characterised using a panel of cell surface markers and their ability to differentiate the three lineages (adipocytes, osteoblasts and chondrocytes).

Propagation of human and animal cells in lab conditions requires appropriate culture media. The culture medium provides vital nutrients for cell growth, proliferation and metabolism. Basal media are mostly supplemented with fetal bovine serum (FBS), which enhance cell growth and proliferation (Price & Gregory, 1982), (Wessman & Levings, 1999). FBS contains rich content of growth factors and low gamma globulin content, these properties make it the standard supplement of cell culture media but it has some disadvantages, such as causes of the risk of transmission of animal pathogens and xenogeneic immuno reactions (Eloit, 1999) (Dormont, 1999). In this project, the effects of human serum (HS), FBS and xeno-and serum-free media (XSFM) on MSC properties (cell morphology, cell growth, surface markers and differentiating potential) were evaluated.

1.2 Hypotheses of the Study

The hypotheses of this study are:

i. MSCs can be generated from human amniotic fluid (hAF) obtained from amniocentesis and caesarean procedures.

ii. hAFMSCs can be generated using xeno- and serum-free medium and media containing human serum and FBS.

1.3 Objectives of the Study

The general objective of this project is:
To generate and characterize hAF-MSCs harvested from second and full-term hAF grown in different culture media.
The specific objectives of this study are:

i. To generate and propagate second trimester and full trimester hAF-derived hAF-MSCs in different culture media

ii. To characterize hAF-MSCs by immunophenotyping and trilineages differentiation assay.

iii. To compare the hAF-MSCs generated from second trimester hAF samples collected from amniocentesis and caesarean procedure.

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