CELLULAR INTERACTION BETWEEN HUMAN AMNION MESENCHYMAL STEM CELLS, HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS AND HUMAN DERMAL FIBROBLASTS IN 2D AND 3D *IN VITRO* TRICULTURE SYSTEMS

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JUDUL: **CELLULAR INTERACTIONS BETWEEN HUMAN AMNION MESENCHYMAL STEM CELLS, HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS AND HUMAN DERMAL FIBROBLASTS IN 2D AND 3D *IN VITRO* TRICULTURE SYSTEMS**

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DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, equations, summaries and references, which have been duly acknowledged.

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ABSTRACT

Human amnion mesenchymal stem cells (HAMCs) are capable of multilineage differentiation and have angiogenic potential. Tri-culture system is known to direct stem cell fate decisions. This study was carried out to determine the cellular interaction between HAMCs, human umbilical vein endothelial cells (HUVECs) and human dermal fibroblasts (HDFs) in 2-dimensional (2D) and 3-dimensional (3D) tri-culture systems *in vitro*. In the first part of the study, HAMCs, HUVECs and HDFs were cultured directly and indirectly in 2D tri-culture system at various seeding ratio (HAMCs:HUVECs:HDFs; 1:4.5:4.5, 5:2.5:2.5 and 9:0.5:0.5) until 7 days. Cell morphological observation, ELISA for vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) detection and immunofluorescence staining against von willebrand factor (vWF), stage specific embryonic antigen-4 (SSEA-4) and collagen Type I (colI) were performed. In the second part of the study, HAMCs, HUVECs and HDFs were embedded in fibrin scaffold at selected seeding ratio, 9:0.5:0.5 for 7 days. Cells morphological observation, ELISA and immunohistochemistry against SSEA-4, monoclonal mouse anti-human CD31 and alpha smooth muscle actin (SMA-α) were performed. The results in 2D study showed that HAMCs retained its spindle-shaped morphology with filopodia, and VEGF concentration in both direct and indirect tri-culture groups was significantly reduced at the end of culture whereas bFGF concentration remained constant. HAMCs in both direct and indirect 2D tri-culture positively expressed against SSEA-4 and ColI but negatively expressed vWF. Whereas in 3D study, HAMCs in tri-culture formed network- and capillary-like structures and showed significant reduction in VEGF and bFGF concentration on Day 7. They positively expressed SMA-α but negatively expressed SSEA-4 and CD31. The results in 2D study suggested that HAMCs do not differentiate into endothelial cell. However, VEGF molecules was activated to promote proliferation and migration. For 3D tri-culture study, HAMCs morphology demonstrated its involvement in angiogenesis, and differentiated into myofibroblasts cells. HAMCs in 2D tri-culture maintain its MSCs characteristic but not endothelial cell whereas HAMCs in 3D tri-culture do not possessed endothelial cell and MSCs characteristics. HAMCs in 2D do not influence and be influenced by other cell in direct and indirect contact. However, HAMCs differentiated and proliferated in the presence of fibrin. Therefore, the features of 3D tri-culture system model in this study have expanded the limitation of 2D tri-culture system. The 3D system has successfully provided fundamental knowledge of mimicking skin microenvironment for future work as well as future application towards *in vivo* study. Nevertheless, further fundamental studies are required to assess HAMCs differentiation and roles in angiogenesis in 2D and 3D *in vitro* tri-culture models.

ABSTRAK

Interaksi Selular antara Sel Stem Mesenkima Amnion Manusia, Sel Endotelial Vena Umbilikal Manusia dan Fibroblas Dermal Manusia Dalam Sistem Trikultur in vitro 2D dan 3D

Sel Stem Mesenkima Amnion Manusia (HAMCs) memiliki keupayaan untuk berubah menjadi pelbagai jenis sel dan mempunyai potensi angiogenik. Sistem tri-kultur diketahui mampu mengawal nasib sel stem. Kajian ini dijalankan untuk menentukan interaksi selular antara HAMCs, sel endotelial vena umbilikal manusia (HUVECs) dan fibroblas kulit manusia (HDFs) dalam sistem tri-kultur 2-dimensi (2D) dan 3-dimensi (3D) secara in vitro. Bahagian pertama kajian, HAMCs, HUVECs dan HDFs telah dikultur secara langsung dan tidak langsung dalam sistem tri-kultur 2D pada pelbagai nisbah pengkulturan (HAMCs:HUVECs:HDFs; 1:4.5:4.5, 5:2.5:2.5 dan 9:0.5:0.5) selama 7 hari. Pemerhation morfologi sel, ELISA untuk mengesan faktor pertumbuhan vaskular endothelial (VEGF) dan faktor pertumbuhan asas fibroblas (bFGF) dan immunofloresen terhadap von Willebrand factor (vWF), stage specific embryonic antigen-4 (SSEA-4) dan collagen Type I (ColI) telah dijalankan. Bahagian kedua dalam kajian, HAMCs, HUVECs dan HDFs dikultur di dalam perancah fibrin pada nisbah pengkulturan yang dipilih, 9:0.5:0.5 selama 7 hari. Pemerhatian morfologi sel, ELISA dan immunohistokimia terhadap SSEA-4, monoclonal mouse anti-human CD31 dan alpha smooth muscle actin (SMA-α) telah dijalankan. Dapatan kajian 2D menunjukkan bahawa HAMCs mengekalkan morfologi berbentuk gelendong serta filopodia, dan rembesan VEGF di dalam tri-kultur langsung dan tidak langsung berkurangan secara signifikan pada hari ke 7 manakala rembesan bFGF adalah malar. Manakala dalam kajian 3D, HAMCs membentuk struktur rangkaian dan kapilari, dan rembesan VEGF dan bFGF berkurangan secara signifikan pada kultur lewat. Ekspresi mereka pula positif terhadap SSEA-4 dan SMA-α tetapi negatif terhadap CD31. Dapatan kajian 2D mencadangkan bahawa HAMCs tidak berubah endotelial sel. Tetapi, molekul VEGF telah diaktifkan untuk mengalakkan percambahan dan migrasi. Dalam kajian tri-kultur 3D, morfologi HAMCs menunjukkan penglibatannya dalam angiogenesis, dan berubah kepada miofibroblas. HAMCs in tri-kultur 2D mengekalkan sifat MSC nya tetapi tidak terhadap sel endotelial, manakala tri-kultur 3D tidak lagi mempunyai sifat MSC dan sel endotelial. HAMCs dalam tri-kultur 2D tidak dipengaruhi dan mempengaruhi oleh sel lain dalam sentuhan langsung dan tidak langsung. Akan tetapi, HAMCs berubah dan bercambah di dalam fibrin. Oleh itu, ciri-ciri sistem tri-kultur 3D di dalam kajian ini telah mengembangkan had sistem tri-kultur 2D. Sistem tri-kultur 3D telah memberi pengetahuan yang asas dalam meniru persekitaran mikro kulit untuk kajian yang akan datang dan untuk aplikasi dalam klinikal pada masa depan yang menuju kepada kajian in vivo. Namun, kajian asas selanjutnya diperlukan untuk menilai perubahan HAMCs dan fungsinya dalam angiogenesis di dalam model in vitro tri-kultur 2D dan 3D.

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LIST OF ABBREVIATIONS

ABTS	-	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
Ang1	-	Angiopoietin-1
bFGF	-	basic Fibroblast Growth Factor
BM-MSCs	-	Bone Marrow-derived Mesenchymal Stem Cells
BMP-2	-	Bone Morphogenic Protein-2
ColI	-	Collagen Type I
CF	-	Cystic Fibrosis
CMAC	-	7-amino-4-chloromethylcoumarin
CTFR	-	Cystic Fibrosis Transmembrane Conductance Regulator
DMSO	-	Dimethyl sulfoxide
ECM	-	Extracellular Matrix
EDTA	-	Ethylenediaminetetraacetic acid
EGF	-	Epidermal Growth Factor
ELISA	-	Enzyme-Linked Immunosorbent Assay
ESCs	-	Embryonic Stem Cells
F12:DMEM	-	Ham's F12: Dulbecco's Modified Eagle Medium
FAK	-	Focal Adhesion Kinase
FBS	-	Fetal Bovine Serum
Flt4	-	Fms-related tyrosine kinase-4
HAECs	-	Human Amnion Epithelial Cells
HAM	-	Human Amnion Membrane
HAMCs	-	Human Amnion Mesenchymal Stem Cells
HA/TCP	-	Hydroxyl apatite/tricalcium phosphate
HBMSCs	-	Human Bone-Marrow Mesenchymal Stem Cells
hCDSC	-	Human Chorion-Derived Stem Cells
HDFs	-	Human Dermal Fibroblasts
hESCs	-	Human Embryonic Stem Cells
HGF	-	Hepatocyte Growth Factor
HIER	-	Heat Induced Epitope Retrieval
hMSCs	-	Human Mesenchymal Stem Cells
HRP	-	Horseradish Peroxidase
HSCs	-	Hematopoietic Stem Cells
HUVECs	-	Human Umbilical Vein Endothelial Cells
IL-1	-	Interleukin-1
IL-6	-	Interleukin-6
IL-8	-	Interleukin-8
IL-10	-	Interleukin-10
KDR	-	Kinase-insert Domain Receptor
KGF	-	Keratinocyte Growth Factor
MHC	-	Major Histocompatibility Complexes
MIF	-	Migration-Inhibitory Factor
MMPs	-	Metalloproteinases
MSCs	-	Mesenchymal Stem Cells
NGF	-	Nerve Growth Factor
nHA/CS/PLGA	-	Nano-hydroxyapatite/chitosan/poly(lactide-co-glycolide)
PBS	-	Phosphate Buffered Saline
PCL	-	ε-caprolactone
PDGF	-	Platelet-derived Growth Factor
PGE2	-	Prostaglandin E2

PGF	-	Placenta Growth Factor
PHBV/HA	-	Poly (3-hydroxybutyrate-co-3-hydroxyvalerate) and hydroxyl - apatite
PI3K	-	Phosphoinositide 3-kinase
PLGA	-	Poly(lactic-co-glycolic acid)
PLLA	-	poly-L-lactic acid
RA	-	Retinoic Acid
RAECs	-	Rat Aortic Endothelial Cells
SEM	-	Standard Error of the Mean
SMA-α	-	Alpha-Smooth Muscle Actin
SSEA-4	-	Stage Specific Embryonic Antigen-4
TCP-HA	-	Tricalcium phosphate/hydroxyapatite
TBS	-	Tris-Buffered Saline
TGF-α	-	Transforming Growth Factor-α
TGF-β	-	Transforming Growth Factor-β
Tie2	-	Tyrosin kinase-2
TIMPs	-	Tissue Inhibitors of Matrix Proteinases
TNF	-	Tumour Necrosis Factor
VEGF	-	Vascular Endothelial Growth Factor
VEGFR	-	Vascular Endothelial Growth Factor Receptor
vWF		Von Willebrand Factor

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CHAPTER 1

INTRODUCTION

1.1 Research background

Stem cells are defined as cells that have the capability to proliferate indefinitely and to differentiate into various cell lineages. Due to their self-renewal capacity and multilineage differentiation, stem cells have been extensively studied for their potential in skin regeneration (Burd, Ahmed, Lam, Ayyappan and Huang, 2007; Liu, Deng, Han, Liu, Wen, Lu, Geng, Huang and Jin, 2008). Stem cells can be further divided into two main groups: embryonic and adult stem cells. Embryonic stem cells (ESC) isolated from inner cell mass of a blastocyst are pluripotent (Bongso, Fong, Ng and Ratnam, 1994; Thomson, Itskovitz-Eldor, Shapiro, Waknitz, Swiergiel, Marshall and Jones, 1998), but their clinical applications are constrained by tendency to form tumours (Lawrenz, Schiller, Willbold, Ruediger, Muhs and Esser, 2004; Maitra, Arking, Shivapurkar, Ikeda, Stastny, Kassauei, Sui, Cutler, Liu and Brimble, 2005; Shih, Forman, Chu and Slovak, 2007), cellular immune rejection (Šarić, Frenzel and Hescheler, 2008) and ethical issue concerning human embryos as the sources (Daley, Ahrlund-Richter, Auerbach, Benvenisty, Charo, Chen, Deng, Goldstein, Hudson and Hyun, 2007; Edwards, 2007; Green, 2007). In contrast, the use of adult stem cells such as mesenchymal stem cells derived from bone marrow does not involve human embryos destruction and at the same time show similar functions as ESC (Takahashi and Yamanaka, 2006). However, the possibility of genetic alterations occurring with ageing could lead to loss of their functions (Mimeault, Hauke, Mehta and Batra, 2007).

Therefore, extensive investigations of human amnion-derived stem cells as a potential source of mesenchymal stem cells have been done. Human amnion mesenchymal stem cells (HAMCs) shared several remarkable features that make these

cells suitable as an alternative source of mesenchymal stem cells. HAMCs give high contribution in skin regeneration due to their high recovery of cells and potential in multilineage differentiation (Pan, Yang, Chiu, Lai, Wang, Chang and Cheng, 2006; Portmann-Lanz, Schoeberlein, Huber, Sager, Malek, Holzgreve and Surbek, 2006; Tamagawa, Oi, Ishiwata, Ishikawa and Nakamura, 2007), high capacity of proliferation and do not form tumour upon transplantation, do not elicit an immune reaction which may reduce the risks of rejection upon transplantation (Bailo, Soncini, Vertua, Signoroni, Sanzone, Lombardi, Arienti, Calamani, Zatti and Paul, 2004; Fatimah, Chua, Tan, Azmi, Tan and Abdul Rahman, 2013a; Kubo, Sonoda, Muramatsu and Usui, 2001), abundance and easy accessibility during pregnancy from healthy mothers as they are discarded after delivery (Marcus and Woodbury, 2008), easy isolation with high yield using enzymatic techniques and ethically free from controversial issues associated to non-invasive procedure during harvesting and in clinical use.

Angiogenesis is a process of capillaries formation branching from the pre-existing microvessels in the body system which is crucial in tissues regeneration such as in skin regeneration. Previous studies show that HAMCs have the angiogenic potential (Fatimah, Tan, Chua, Fariha, Tan and Hayati, 2013b) and they could spontaneously be differentiated into endothelial cells and form capillary-like structures in the *in vitro* matrigel assay (Alviano, Fossati, Marchionni, Arpinati, Bonsi, Franchina, Lanzoni, Cantoni, Cavallini, Bianchi, Tazzari, Pasquinelli, Foroni, Ventura, Grossi and Bagnara, 2007). Due to these beneficial properties, hCDSC was used in the construction of amnion composites and transplanted into the dorsal part of the athymic mice and successfully showed promising angiogenic and endogenic properties (Fariha, Chua, Tan, Lim and Hayati, 2013). While a few of MSCs properties in angiogenesis for tissue regeneration have been uncovered, HAMCs fate in a tissue microenvironment is still underexplored.

Stem cell niches are composed of numerous microenvironmental features, including soluble and insoluble factors, cues from other cells and the extracellular matrix, which coordinately regulate the stem cell fate decisions with precise spatiotemporal control (Moore and Lemischka, 2006). However, the complexity and integration of these various elements in HAMCs microenvironment remain poorly understood. MSCs is the central process in tissue regeneration such as in wound repair which requires a coordinated interplay among cells, growth factors and extracellular matrix proteins. Therefore, to better understand the HAMCs fate decisions *in vitro*, two-dimensional (2D) and three-dimensional (3D) tri-culture systems is usually implemented. Each system

models provide unique culture conditions that can be manipulated that allows us to further define the molecular milestone and the underlying mechanisms of differentiation and proliferation in HAMCs after interacting with human umbilical vein endothelial cells (HUVECs) and human dermal fibroblasts (HDFs).

The implementation of 2D and 3D *in vitro* model systems that could mimic the niche environments of a skin tissue as closely as possible by allowing dynamic cell-cell interactions are crucial as the relevant platforms for evaluating regenerative medicine therapies (Baraniak and McDevitt, 2010; Griffith and Swartz, 2006). The 2D system may provide a simple platform to conduct various cell-cell interaction dynamic such as allowing direct physical contact between cells and soluble factors as the mean of signalling mechanisms, and to prevent physical contact but permitted signalling mechanisms through soluble factors (Ball, Shuttleworth and Kielty, 2004). Whereas 3D system by using fibrin as biological scaffold may provide a way to recreate and closely mimic 3D environments in skin tissue which allows the study of more complex cellular interactions than in 2D system (Lesman, Koffler, Atlas, Blinder, Kam and Levenberg, 2011). Moreover, the use of primary human cells, tri-culture system containing three cell types to permit better simulation of interactions within realistic microenvironments, and biomaterials in 3D system have the potential to yield further progress toward tissue regeneration study. Three different cell types was chosen and implemented in this study due to their close proximity within the same skin tissue niche. HAMCs act as the alternative source of stem cells in tissue regeneration process, HUVECs represent the endothelial cells and HDFs represent the dermal fibroblast in skin microenvironment.

Therefore, in this study, we determined the direct and indirect cell-cell interaction between HAMCs, HUVECs and HDFs in 2D culture system *in vitro* at different time points via morphological changes of cells, protein expression changes analysis and growth factor quantification using Enzyme-linked immunosorbent assay (ELISA). We also determined the cell-cell interaction between HAMCs, HUVECs and HDFs in 3D-culture system *in vitro* at 9:0.5:0.5 seeding density at different time points via cell morphological analysis, end point differentiation marker analysis and growth factors quantification using ELISA. Seeding density of 9:0.5:0.5 was chosen due to its ability to balance the rapid growth of HUVECs and HDFs (by using smaller density) over slower growing HAMCs in tri-culture group. Fibrin gel is used as a biological scaffold in this study. The 3D fibrin based cell culture model is used to further explore the mechanisms by which HAMCs

promote or involve in the angiogenesis upon interaction with HUVECs and their respond to the presence of HDFs.

1.2 Significance of Study

Human amnion mesenchymal stem cells (HAMCs) as an alternative source of stem cells held promising future in skin regeneration to treat skin injury or diseases. Therefore, it is crucial to understand the stem cell niche (microenvironment) and its role in regulating the balance of self-renewal and differentiation through cell-cell interaction between HAMCs, HUVECs and HDFs. Thus, it is important to note that better understanding on the interactions between these cells could give survival chance and participation of HAMCs in skin regeneration *in vivo* that can be used in clinical application.

1.3 Objectives

1.3.1 General Objectives

The objectives of this study were to determine the direct and indirect cell-cell interaction in 2-dimensional (2D) culture system and to determine cell-cell interaction in 3-dimensional (3D) culture system between human amnion mesenchymal stem cells (HAMCs), human dermal fibroblasts (HDFs) and human umbilical vein endothelial cells (HUVECs).

1.3.2 Specific Objectives

a. To determine the direct cell-cell interaction between HAMCs, HUVECs and HDFs in 2D *in vitro* tri-culture system at different time-points via cell morphological observation, protein expression changes analysis and growth quantification using ELISA.
b. To determine the indirect cell-cell interaction between HAMCs, HUVECs and HDFs in 2D *in vitro* tri-culture system at different time-points via cell morphological observation, protein expression changes analysis and growth quantification using ELISA.
c. To determine the cell-cell interaction between HAMCs, HUVECs and HDFs in 3D *in vitro* tri-culture system with 9:0.5:0.5 seeding density at different time points via cell morphological observation, end point differentiation marker analysis and growth quantification using ELISA.

1.4 Hypothesis

HAMCs have dual characteristics of endothelial cells and mesenchymal cells. HAMCs influence and be influenced by other cells in direct and indirect contact. HAMCs differentiate and proliferate in the presence of fibrin.

CHAPTER 2

LITERATURE REVIEW

2.1 HAMCs and Their Differentiation Potential

2.1.1 HAMCs as The Alternative Source of Mesenchymal Stem Cells

It is believed that the discovery of therapeutics stem cells was initiated by 3 technological accomplishments that began in 1961 when early studies done using a revolutionary *in vivo* bioassay, unambiguously shows the existence of hematopoietic stem cells (HSCs) (Till and McCulloch, 1961). The second achievement was accomplished by Thomson, Itskovitz-Eldor, Shapiro, Waknitz, Swiergiel, Marshall and Jones (1998) on the isolation of human embryonic stem cells (hESCs) from blastocysts and the initiation of hESC lines for study. The most recent achievement was the successful production of induced formation of pluripotent stem cells from adult human dermal fibroblasts and other human somatic cells (Takahashi and Yamanaka, 2006).

ESCs can proliferate for long periods in *in vitro* and are able to differentiate into various types of cell lineages (Shamblott, Axelman, Littlefield, Blumenthal, Huggins, Cui, Cheng and Gearhart, 2001). This was demonstrated when the differentiation of ESCs into 3-dimensional, multicellular aggregates of differentiated and undifferentiated cells began after it was removed from feeder layers and transferred into suspension cultures. The cells were known as embryoid bodies which were composed of a random grouping of precursor and more fully differentiated cells from a wide variety of lineages and resembled early post-implantation embryos. However, the study on human ESCs is restricted due to ethical issue concerning the destruction of the embryo (Young, 2000).

The discovery of adult stem cells that can be isolated from several parts of the body, have opened up a new way to bring stem cell technology into action especially in regenerative medicine. Currently, adult stem cells were isolated from epidermis, dermis, hair follicle, adipose tissue, bone marrow and placental tissues. According to Brignier and Gewirtz (2010), the well-known example of adult stem cell is hematopoietic stem cells (HSCs) isolated from bone marrow. However, in recent years, extensive studies was done on mesenchymal stem cells (MSCs) isolated from bone marrow due to its excellent properties such as high proliferation potency and capability to differentiate into multiple cell lineages especially into osteogenic (Baglio, Devescovi, Granchi and Baldini, 2013; Hodgkiss-Geere, Argyle, Corcoran, Whitelaw, Milne, Bennett and Argyle, 2012; Liu, Konermann, Guo, Jager, Zhang and Jin, 2014).

Mesenchymal stem cells (MSCs) are mulitpotent non-hematopoietic that has the ability to differentiate into various mesoderm-type lineages, including osteoblasts, chondrocytes and adipocytes. MSCs were firstly described by Fridenshteĭn, Chaĭlakhin and Gerasimov (1986) as the clonal and plastic adherent cells. MSCs can be acquired from almost all organs, but extensive studies were done on bone marrow-derived mesenchymal cells (BM-MSCs) for therapeutic purposes such as in regenerative medicine. MSCs, which are mainly isolated from bone marrow, are capable to differentiate into various cell lineages and have high proliferative capability which makes it very significant in regenerative research. Recent studies had shown the differentiation and proliferation capability of BM-MSCs into endothelial cells, endometrial epithelial cells, cardiac cells, adipocytes, chondrocytes and osteoblasts *in vitro* (Baglio *et al.*, 2013; Liu *et al.*, 2014; Mathews, Bhonde, Gupta and Totey, 2012; Zhang, Cheng, Huang, Jiang, Cong, Zheng and Xu, 2012). However, to obtain BM-MSCs, it requires invasive as well as painful procedures. In addition, the cells could undergo genetic alterations and reduced functions depending on the age of the patient. Therefore, an alternative source is desirable and to obtain stem cells that have the same properties as BM-MSCs from abundance sources and easy access with better properties has been discovered namely MSCs isolated from placental tissues.

In recent years, extensive studies on human amnion mesenchymal stem cells (HAMCs) as the alternative source of stem cells have been done. Human amnion membrane (HAM) is located at the innermost layer of the placenta and harbours a source of mesenchymal stem cells (MSCs). There are two types of cells present in amnion membrane, both from different embryological origin: amnion epithelial cells

derived from embryonic ectoderm and amnion mesenchymal cells from embryonic mesoderm (Kmiecik, Niklinska, Kuc, Pancewicz-Wojtkiewicz, Fil, Karwowska, Karczewski and Mackiewicz, 2013). According to Kmiecik *et al.* (2013), HAMCs express low or moderate levels of the major histocompatibility complexes (MHC) class I and II antigens on its surfaces and indicates that HAMCs is immunoprivileged. Therefore, HAMCs have low immune rejection which is beneficial for therapeutic purposes or clinical application in the future (Lindenmair, Hatlapatka, Kollwig, Hennerbichler, Gabriel, Wolbank, Redl and Kasper, 2012). Other remarkable properties of HAMCs are anti-inflammatory activity, ability to differentiate into 3 germ layers: ectoderm, mesoderm and endoderm, and ease of harvesting in large numbers because placenta is usually discarded postpartum with the least ethical issues (Silini, Parolini, Huppertz and Lang, 2013; Tsuno, Yoshida, Nogami, Koike, Okabe, Noto, Arai, Noguchi and Nikaido, 2012). Furthermore, the isolation of the cells from placenta is very easy using enzymatic techniques (Parolini, Alviano, Bagnara, Bilic, Buhring, Evangelista, Hennerbichler, Liu, Magatti, Mao, Miki, Marongiu, Nakajima, Nikaido, Portmann-Lanz, Sankar, Soncini, Stadler, Surbek, Takahashi, Redl, Sakuragawa, Wolbank, Zeisberger, Zisch and Strom, 2008). The minimal criteria of HAMCs is according to the characteristic of stem cell as stated by the International Society for Cellular Therapy, such as plastic adherent, fibroblast-like or spindle-like appearances, forming clonal colonies, able to express typical range of MSCs markers as listed in Table 2.1, and able to differentiate into mature cell lineages *in vitro* (Dominici, Le Blanc, Mueller, Slaper-Cortenbach, Marini, Krause, Deans, Keating, Prockop and Horwitz, 2006).

It was shown that HAMCs have the same phenotype as BM-MSCs and higher capacity of proliferation and multilineage differentiation potential than the latter (Zhao, Ise, Hongo, Ota, Konishi and Nikaido, 2005). In addition, amnion membrane transplantation promotes re-epithelialization, reduces inflammation and fibrosis as well as modulates angiogenesis (Dua, Gomes, King and Maharajan, 2004; Solomon, Wajngarten, Alviano, Anteby, Elchalal, Pe'er and Levi-Schaffer, 2005). According to Shimazaki, Yang and Tsubota (1997), several growth factors produced from amniotic membrane are engaged in these processes such as Transforming Growth Factor-β (TGF-β) and basic Fibroblast Growth Factor (bFGF). In addition, another growth factors discovered were Epidermal Growth Factor (EGF), Transforming Growth Factor-α (TGF-α), Keratinocyte Growth Factor and Hepatocyte Growth Factor (Koizumi, Inatomi, Sotozono, Fullwood, Quantock and Kinoshita, 2000). Recent study by Warrier, Haridas