

**EFFECTS OF GRAPHENE OXIDE AND  
COLLAGEN TYPE I ON MODULATING  
PROLIFERATION AND GENE EXPRESSION OF  
MESENCHYMAL STEM CELLS**



**BIOTECHNOLOGY RESEARCH INSTITUTE  
UNIVERSITI MALAYSIA SABAH  
2021**

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PROLIFERATION AND GENE EXPRESSION OF  
MESENCHYMAL STEM CELLS**

**HASELAMIRRAH BINTI MOHD AKHIR**



**UMS**

**THESIS SUBMITTED IN FULFILLMENT FOR THE  
DEGREE OF MASTER OF SCIENCE**

**BIOTECHNOLOGY RESEARCH INSTITUTE  
UNIVERSITI MALAYSIA SABAH  
2021**

**UNIVERSITI MALAYSIA SABAH**

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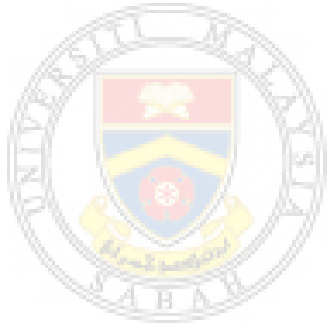
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## DECLARATION

I hereby declare that this thesis is based on my original work except for quotations, citation, equations and references which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UMS or other institutions.

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## CERTIFICATION

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DEGREE : **MASTER OF SCIENCE (BIOTECHNOLOGY)**

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## ABSTRACT

Mesenchymal stem cells derived from amnion (AM-MSCs) has the ability of self-renewal and multilineage differentiation capacity to generate adipocytes and osteoblasts. However, there are limitations for continuous stem cells culture in term of cell supply and the differentiation capacity of amnion-derived MSCs. Progresses in biomaterials engineering enable the study of MSCs in *in vitro* cell expansion system to overcome this limitation. Biomaterials create a physical environment which can manipulate cells without any requirement for chemical factors. This study aimed to investigate how graphene oxide (GO) and collagen (COL) modulate proliferation, stemness and differentiation potentials of AM-MSCs. AM-MSCs were isolated and cultured in DMEM/F12 media. AM-MSCs were grown in osteogenic and adipogenic condition with and without the presence of biomaterials. RT-PCR determined the expression of genes involved in self-renewal and differentiation genes. The roles of MAPK pathways in regulatin AM-MSCs were done using Western Blot. The results showed that AM-MSCs exhibited spindle-shaped like cells morphology. AM-MSCs cultured in both GO and COL plates showed comparable proliferation as compared to the controls. However, both biomaterials altered the stemness markers and differentiation genes differently. Results showed elevated expressions of stemness (*OCT3/4* & *NANOG*), osteogenic (*RUNX2* & *OCN*) and adipogenic (*CEBPA* & *CEBPB*) genes when cells cultured in the presence of COL for both basal and osteo-adipogenic condition. In the presence of GO without induction, the expression of stemness and osteogenic genes were maintained but not adipogenic genes. In AM-MSCs-GO sample, stemness genes were either suppressed or unaltered. Surprisingly, suppression of adipogenic genes (*CEBPA* & *CEBPB*) was observed in AM-MSCs-GO whereby osteogenic genes showed little effect. Besides that, the presence of collagen had some effect in upregulating the expression of the histone acetyltransferase (*hMOF*) and DNA methyltransferase (*DNMT3A* & *DNMT3B*), but little changes were found in GO samples. In addition, activation of ERK and p38 pathways were affected by COL but not GO. In conclusion, these results demonstrated that collagen enhanced both osteo-adipogenic differentiation in AM-MSCs, while graphene oxide has a much more reserved lineage-specific differentiation.

## **ABSTRAK**

### **MODULASI BIOMATERIAL TERHADAP PROLIFERASI DAN EKSPRESI GEN SEL MESENKIMAL BERASAL DARI AMNION**

*Sel stem mesenkima yang diperoleh dari amnion (AM-MSCs) mempunyai kemampuan pembaharuan diri dan kapasitas pembezaan sel untuk menjana adiposit dan osteoblas. Walau bagaimanapun, terdapat batasan bagi memperoleh sel stem dari segi bekalan sel dan kapasitas pembezaan sel stem. Kemajuan kejuruteraan biobahan membolehkan kajian MSC dalam pengembangan sel secara in vitro untuk mengatasi batasan tersebut. Biobahan mewujudkan persekitaran fizikal yang dapat memanipulasi sel tanpa sebarang keperluan untuk faktor kimia. Tujuan kajian ini adalah untuk mengkaji bagaimana graphene oksida (GO) dan kolagen (COL) memodulasi proliferasi, "stemness" dan potensi pembezaan AM-MSCs. AM-MSCs telah diasingkan dan ditumbuhkan dalam media DMEM/F12. AM-MSCs ditumbuh dalam keadaan osteogenik dan adipogenik dengan kehadiran atau tanpa biobahan. Ekspresi gen yang terlibat dalam pembaharuan diri dan pembezaan ditentukan oleh RT-PCR. Protein sasaran yang terlibat dalam modifikasi epigenetik dikesan dengan menggunakan Western Blot. Hasil kajian menunjukkan bahawa AM-MSCs berbentuk spindle serupa sel fibroblas dan mempunyai morfologi seperti sel epitelium. Pertumbuhan AM-MSCs bersama-sama dengan GO dan COL menunjukkan kadar proliferasi yang standing berbanding dengan kawalan. Walau bagaimanapun, kedua-dua biobahan mengubah penanda-penanda "stemness" (OCT3/4 & NANOG) dan gen osteogenik (RUNX2 & OCN) dan gen adipogenik (CEBPA & CEBPB) ketika sel dikultur dengan adanya COL di media normal dan media osteo-adipogenik. Dengan adanya GO tanpa induksi, ekspresi "stemness" dan gen osteogenik dipertahankan tetapi bukan gen adipogenik. Dalam sampel AM-MSCs-GO, gen "stemness" sama ada dikurangkan atau tidak berubah. Tambahan lagi, penyekatan gen adipogenik (CEBPA & CEBPB) dapat dilihat di AM-MSCs-GO di mana gen osteogenik menunjukkan sedikit kesan. Selain itu, kehadiran kolagen mempunyai beberapa pengaruh dalam mengatur ekspresi histone acetyltransferase (hMOF) dan DNA methyltransferase (DNMT3A & DNMT3B), tetapi sedikit perubahan ditemui pada sampel*



*GO. Di samping itu, pengaktifan laluan ERK dan p38 dipengaruhi oleh COL tetapi tidak pada GO. Kesimpulannya, hasil ini menunjukkan bahawa kolagen meningkatkan kedua-dua pembezaan osteo-adipogenik pada AM-MSC, sementara graphene oxide mempunyai pembezaan khusus garis diferensiasi sel yang jauh lebih tersimpan.*



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# TABLE OF CONTENTS

	Page
<b>TITLE</b>	i
<b>DECLARATION</b>	ii
<b>CERTIFICATION</b>	iii
<b>ACKNOWLEDGEMENT</b>	iv
<b>ABSTRACT</b>	v
<b><i>ABSTRAK</i></b>	vi
<b>TABLE OF CONTENTS</b>	viii
<b>LIST OF TABLES</b>	xi
<b>LIST OF FIGURES</b>	xii
<b>LIST OF SYMBOLS / ACRONYMS</b>	xiv
<b>LIST OF APPENDICES</b>	xvi
<b>CHAPTER 1: INTRODUCTION</b>	
1.1 Background of study	1
1.2 Significant of study	3
1.3 Hypothesis	3
1.4 Objectives	3
<b>CHAPTER 2: LITERATURE REVIEW</b>	
2.1 Mesenchymal stem cells	5
2.2 Amnion-derived Mesenchymal stem cell (AM-MSCs)	8
2.3 Isolation, expansion, and characterization of AM-MSCs	9
2.4 Lineage differentiation of MSCs	10
2.5 Cell proliferation of MSCs	10
2.5 AM-MSCs gene expression	12
2.5.1 Transcription factors involved in stemness, osteogenic and adipogenic differentiation of MSCs	13
2.6 Epigenetic modification	16
2.6.1 Histone modification and DNA methylation	17

2.7 Biomaterials	19
2.7.1 Collagen-based biomaterials	20
2.7.2 Graphene-based biomaterials	22
<b>CHAPTER 3: MATERIALS AND METHODS</b>	
3.1 Sample Collection	25
3.2 Isolation of AM-MSCs using Enzymatic digestion	25
3.3 Cell culture	26
3.4 Biomaterials	27
3.5 Proliferation Assay	27
3.6 Osteogenic and adipogenic differentiation assays	28
3.7 RNA extraction	29
3.8 Reverse Transcription PCR	29
3.9 Protein Extraction	30
3.10 Western Blotting	31
3.11 Statistical analysis	32
<b>CHAPTER 4: RESULTS</b>	
4.1 Morphology and proliferation of AM-MSCs	33
4.2 Characterisation of AM-MSCs	35
4.3 Effects of collagen type I on AM-MSCs	39
4.3.1 Cell morphology and proliferation	39
4.3.2 RNA Extraction	41
4.3.3 Effects of collagen on the Stemness of AM-MSCs	42
4.3.4 Effects of collagen on the lineage differentiation of AM-MSCs	43
4.3.5 Effects of collagen on epigenetic regulation	48
4.4 Effect of graphene oxide (GO) on the proliferation and morphology of AM-MSCs	52
4.4.1 RNA extraction	54
4.4.2 Effects of GO on the expression of stemness genes	55
4.4.3 Effects of GO on the differentiation of AM-MSCs	57
4.4.4 Effects of graphene oxide on epigenetic regulation	62

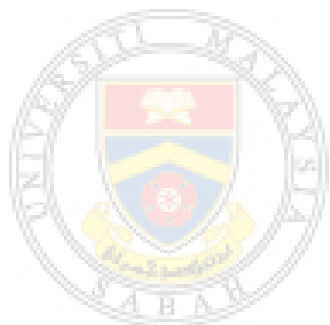
4.5 The effects of collagen and graphene oxide on the stemness and differentiation of AM- MSCs at the late passage	65
<b>CHAPTER 5: DISCUSSION</b>	69
<b>CHAPTER 6: CONCLUSION</b>	79
<b>REFERENCES</b>	81
<b>APPENDICES</b>	100



UMS  
UNIVERSITI MALAYSIA SABAH

## LIST OF TABLES

	Page
Table 3.1 : One-step RT-PCR cycling condition	30
Table 3.2 : Primary antibodies for western blot	31



UMS  
UNIVERSITI MALAYSIA SABAH

## LIST OF FIGURES

	Page
Figure 2.1 : Anatomy of human-term placenta isolated in this procedure	7
Figure 2.2 : Structure of Amniotic membrane	8
Figure 2.3 : Symmetry and asymmetry cell division	12
Figure 2.4 : Regulation of osteoblast by RUNX2	14
Figure 2.5 : Genes involved in adipogenic differentiation	15
Figure 2.6 : Histone acetylation and methylation in chromatin	18
Figure 2.7 : Graphene oxide nanomaterials	23
Figure 3.1 : Isolation of AM-MSc from newborn placenta	26
Figure 4.1 : Morphology and cell number of AM-MSCs	34
Figure 4.2 : Expression of surface markers in AM-MSCs	36
Figure 4.3 : Differentiation potential of AM-MSCs	38
Figure 4.4 : Effect of collagen on the cell morphology & proliferation	40
Figure 4.5 : RNA extracted from AM-MSCs with and without collagen	41
Figure 4.6 : Expression of stemness genes in AM-MSCs in the presence and absence of collagen	42
Figure 4.7 : Differentiation potential of MSC cultured with Collagen Type I	44
Figure 4.8 : Expression of osteogenic genes in AM-MSCs in the presence and absence of collagen at passage 6	46
Figure 4.9 : Expression of adipogenic genes in AM-MSCs in the presence and absence of collagen at passage 6	47
Figure 4.10 : Effect of collagen on mRNA expression of epigenetic marker	49
Figure 4.11 : SDS-PAGE of proteins extracted from AM-MSCs-COL after Comassie staining	51

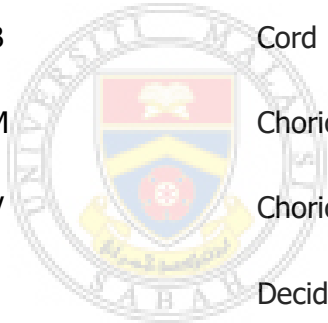
Figure 4.12	: Western blotting of the phosphorylation of ERK and p38 in MAPK pathway	52
Figure 4.13	: Effect of graphene oxide on the cell morphology and proliferation	53
Figure 4.14	: RNA extracted from AM-MSCs with and without graphene oxide	55
Figure 4.15	: Expression of stemness genes in the presence and absence of graphene oxide at passage 6	56
Figure 4.16	: Differentiation potential of AM-MSCs cultured with graphene oxide	58
Figure 4.17	: Expression of osteogenic genes in the presence and absence of graphene oxide at passage 6	60
Figure 4.18	: Expression of adipogenic genes in the presence and absence of graphene oxide at passage 6	61
Figure 4.19	: Expression of DNA methylation marker of AM-MSCs in the presence and absence of graphene oxide at passage 6	63
Figure 4.20	: SDS-PAGE of proteins extracted from AM-MSCs-GO after Comassie staining	64
Figure 4.21	: Western blotting of the phosphorylation of ERK and p38 in MAPK pathway	65
Figure 4.22	: Comparison of gene expression profile of AM-MSCs-COL at early and late passages in the presence of collagen	67
Figure 4.23	: Comparison of gene expression profile of AM-MSCs-GO at early and late passages in the presence of graphene oxide	68
Figure 5.1	: Illustrated diagram of signalling pathway ERK and p38 during osteogenesis when AM-MSCs were cultured in the presence of collagen	77
Figure 5.2	: Illustrated diagram of signalling pathway ERK and p38 during adipogenesis when AM-MSCs were cultured in the presence of collagen	77

## LIST OF SYMBOLS / ACRONYMS

MSCs	Mesenchymal stem cells
bFGF	Fibroblast growth factor
COL	Collagen
GO	Graphene oxide
ESCs	Embryonic stem cells
BM	Bone marrow
AT	Adipose tissue
DP	Dental pulp
AF	Amniotic fluid
AM	Amniotic membrane
AM-MSCs	Amniotic-derived mesenchymal stem cells
hAECs	Human epithelial cells
ISCT	International Society for Cellular Therapy
ECM	Extracellular matrix
iPSCs	Induced pluripotent stem cells
PBS	Phosphate buffered saline
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum



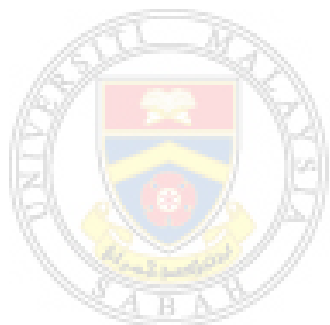
RFU	Relative fluorescence unit
CO <sub>2</sub>	Carbon dioxide
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
PCR	Polymerase Chain Reaction
RT-PCR	Reverse transcriptase polymerase chain reaction
WJ	Wharton's jelly
UC	Umbilical Cord
CB	Cord Blood
CM	Chorion membrane
CV	Chorion villi
D	Decidua
TF	Transcription factor
ECM	Extracellular membrane



UMS  
UNIVERSITI MALAYSIA SABAH

## LIST OF APPENDICES

	Page
Appendix A : Research ethics consent letter	100
Appendix B : List of sequence of forward and reverse primer used in PCR	101
Appendix C : SDS-PAGE Gel Preparation (10%)	104



UMS  
UNIVERSITI MALAYSIA SABAH

# CHAPTER 1

## INTRODUCTION

### 1.1 Background of study

Stem cell is categorized as unique cells due to their pluripotent, multipotent, and unipotent that enable the cells to develop into many cell types (Azandeh *et al.*, 2012). Mesenchymal Stem Cell (MSC) is an adult stem cell which has the ability of self-renewal and multilineage differentiation capacity (Han *et al.*, 2013). Okolicsanyi *et al.*, (2015) recently reported that MSCs isolated from other part of adult tissue showed significantly varying morphology, differentiation capabilities, and gene expression. MSCs can be grown for about 20 to 25 passages in the laboratory and still retain a stable morphology and normal chromosome complement.

Mesenchymal stem cells (MSCs) have created much enthusiasm as a potential hotspot for cell-based therapeutic research. These cells can be highly expanded and differentiated into a variety of cell types. The advantage of MSCs is that this cell can be directly obtained from an individual, therefore get rid of the complications related to the immune rejection of allogeneic tissue and infectious diseases (Katti, 2013). MSC therapies must be manipulated to be cultured *in vitro* and obtain a sufficient number of cells that can be used afterwards for treatment purposes.

Traditionally, adult MSCs have been isolated from the bone marrow. However, this is an invasive procedure and can be painful for the donor. Recent studies had implemented the study of MSCs by isolation of amniotic fluid and other parts of the placenta. MSCs obtained from these sources are more ethical and more accessible with the highest proliferation ability and lower immunogenicity as to compare with MSCs from bone marrow (Han *et al.*, 2013).

Most MSCs studies were investigated on MSCs isolated from the amniotic membrane (AM) of a fresh placenta, and this is because of an abundant source of MSCs can be found within the extracellular membrane and numbers of growth factor binding to extracellular matrix component of AM. One of the growth factors is an essential fibroblast growth factor (bFGF) known as a stimulator of self-renewal and cell survival (Yoon *et al.*, 2013). Enzymatic digestion method has been widely used to isolate MSCs from amniotic layer, but the sequential enzymatic treatments result in low yields of cells without any growth factors.

Further investigation is required to fully understand the characterisation of MSCs derived from various tissue sources for their ability of MSC self-renewal and multipotency. The molecular mechanisms that control MSC self-renewal, expansion and multilineage differentiation are still not well explored and remain an active area of investigation. The uses of biomaterial in stem cells also just recently becoming the area of interest in stem cell research. Biomaterials scaffolds combining with directed stem cell differentiation providing a strategy for tissue engineering and cellular delivery as a means of replacing diseased or damaged tissues.

*In vitro* culture may cause changes to cell biochemistry, topography or physical environment on cells (McMurray *et al.*, 2014). The role of mechanical signals *in vivo* can be assessed utilizing biomaterials *in vitro*. According to Chai and Leong (2007), mechano-sensitivity influence the differentiation of MSCs. However, the amount of cell that can be supplied during isolation is insufficient for clinical testing. These have been the major problem of *in vitro* expansion of stem cell study. MSCs losses their self-renewal and differentiation ability during subculturing. A major methodological approach in correlation to the study of biomaterials was found to be able to control cell maturity and homogenous differentiation of adult stem cells (Yoon *et al.*, 2018). However, the mechanisms that govern the MSCs cell-matrix interaction to the applied biomaterials during cell renewal and cell differentiation to specifically direct and maintain stem cell phenotype is not well explored. Two types of biomaterials were used in this study. They were a natural material, collagen type I (COL) and synthetically synthesized material,

graphene oxide (GO). This study suggested an interesting biomaterial approach to influence the differentiation of stem cells, gene expression and epigenetic of AM-MSCs. Both biomaterials provides structural and organizational cues for AM-MSCs and maintains cellular phenotype during cell fate detemination by creating the balance between their differentiation potential and self-renewal of MSCs.

## **1.2 Significant of study**

The limitation of sufficient cell number has hampered the clinical application of MSCs. Although collagen and graphene oxide has been shown to promote cell proliferation in bone marrow-derived MSCs, their implications on AM-MSCs is still not widely explored. The effect of each biomaterial will provide detailed of how such scaffolds can influence stem cell behavior. This study also provides information about the current knowledge of using biomaterials in combination with stem cells for tissue engineering applications.

## **1.3. Hypothesis**

The hypothesis of this study was biomaterials could improve cell proliferation and enhance the differentiation potential of AM-MSCs by regulating the expression of genes associated with stemness and differentiation and epigenetic events.

## **1.3 Objectives**

This study aimed to assess at the molecular level changes that occur before and after culturing AM-MSCs with biomaterials which were collagen type I (COL) and graphene oxide (GO).

The objectives of this research are:

- a) To isolate and characterize amniotic membrane mesenchymal stem cells.
- b) To compare differences in cell proliferation and differentiation potential affected by biomaterials.
- c) To examine how biomaterials regulate the expression genes related to stemness, differentiation and epigenetic modification.

## CHAPTER 2

### LITERATURE REVIEW

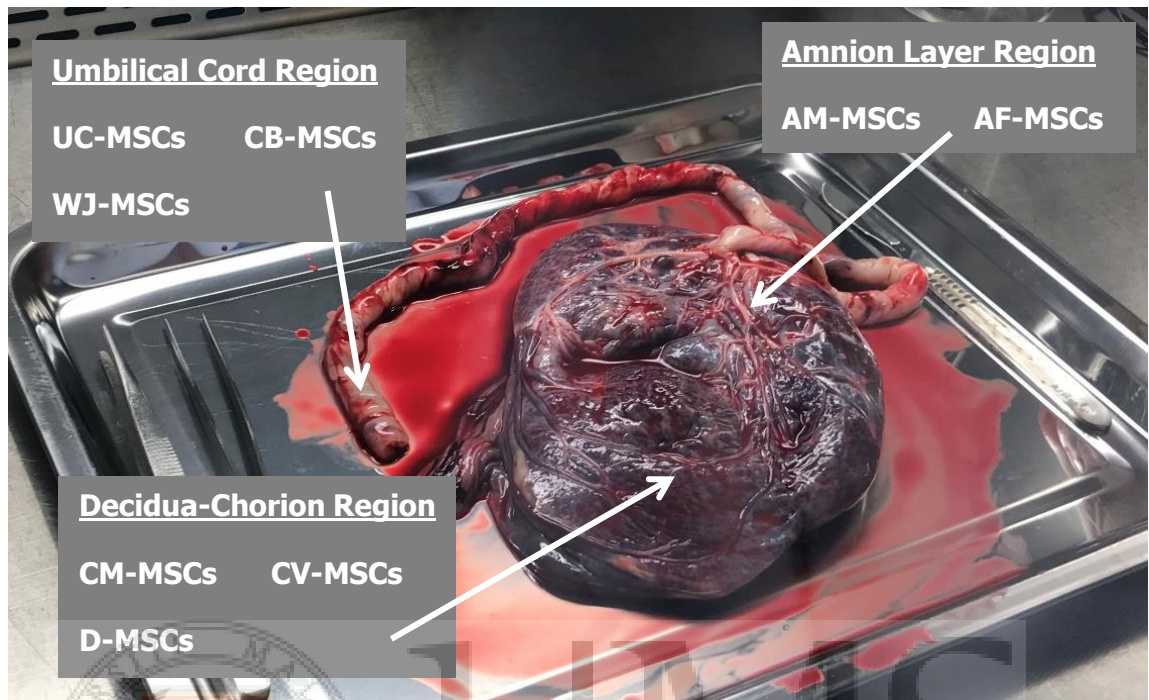
#### 2.1 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) have huge potential in cell-based therapies for tissue regeneration due to their proliferation, differentiation potential, immune-regulatory and anti-inflammatory potential (Pitterger *et al.*, 1999). MSCs are relatively lack ethical issues as compared with other types of stem cells. MSCs offer several advantages such as accessibility, multilineage differentiation potential, immunosuppressive effects and safe from the risk of malignant formation after infusion of allogeneic cells which is quite common in embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) (Kim & Park, 2017). Human mesenchymal stem cells can be isolated from a wide variety of tissues. Source of MSCs can be found in adult tissue such as in the bone marrow (BM), adipose tissue (AT) and dental pulp (DP) MSCs, also categorized as the adult MSCs. However, sources of MSCs derived from adult tissue required an invasive procedure. Previous studies had shown that these MSCs could gradually lose proliferation, differentiation, and immunomodulation potential during cell expansion (Wagner *et al.*, 2009). Recently, the human placenta has been an alternate source for non-invasive isolation of human MSCs as it is easily collected from the discarded placenta after labour. Stem cells from placentas sources have been investigated to constitute valuable sources of maternal and fetal cells that exhibit superior plasticity (Peister *et al.*, 2011; Rodrigues *et al.*, 2012).

Specific MSCs sources of placenta were studied either independently or simultaneously from three parts of placenta including MSCs from the umbilical cord, decidua-chorion plate and amnion region (Fig 2.1). Particular attention has been directed to human amnion region as both amniotic fluid (AF), and amniotic membrane (AM) represents rich sources of stem cells (Hass, Kasper, & Jacobs, 2011). AM-MSCs can be easily isolated and has a higher isolation efficacy as compared to other parts of placenta-

based MSCs (Bieback & Brinkmann, 2010). Mesenchymal stem cells also have the capacity to differentiate into various cell types, including adipocyte, osteocyte, chondrocyte, myocyte and neuron, make them an appealing hotspot for cell treatments (Ghorbani *et al.*, 2014).

Placenta contains MSCs of both maternal and fetal origin. Stem cells that were isolated from umbilical cord and amnion layer region contain fetal MSCs while in decidua-chorion region MSCs contain both fetal such as chorionic membrane derived MSCs and maternal, decidua MSCs. Zhu *et al.* (2014), studied both fetal and maternal MSCs with controlled experimental conditions and compared between cell populations pairs from the same donors. Their results showed that fetal origin have higher proliferative capacity and may be more favorable towards cell application and tissue engineering compared to maternal MSCs. In related to these study, fetal tissues Amniotic Membrane, Wharton's Jelly (WJ) and Villous Chorion (VC) can contribute equally as a good stem cell source of MSCs. Isolation and characterisation studied by Kannaiyan *et al.* (2019), showed that the biological properties of MSCs from these three sources were generally similar. WJ-MSCs and AM-MSCs proliferative potential were higher than VC-MSCs and WJ-MSCs can obtain a large number of cells in shorter time followed by AM-MSCS (Azarpira *et al.*, 2014; Pu *et al.*, 2017). However, AM-MSCs provide ease in term of isolation process while WJ-MSCs required extra dissection work to the umbilical cord region in order to obtain WJ. Our preliminary study showed that it is easier to obtain cell from amniotic tissue as compared to WJ tissue eventhough the sample came from the same individuals. There is no significant difference in the experimental result from both samples making AM as the preffered sample throughout these study.



**Figure 2.1: MSCs can be isolated from different compartments of the human-term placenta. Umbilical cord (UC), cord blood (CB), Wharton's jelly (WJ), chorion membrane (CM), chorion villi (CV), decidua (D), amnion-membrane (AM) and amniotic fluid (AF).**

The established minimal criteria for defining MSCs of the isolated cells: (i) adherence to plastic and spindle-shaped morphology, (ii) expressed specific surface markers CD73, CD90, CD105, and lack expression of CD14, CD34, CD45, and HLA-DR, and (iii) have the ability to differentiate into adipocyte, chondrocyte, and osteoblast (Dominici *et al.*, 2006). AM-MSC are a heterogeneous population that followed the minimum criteria proposed by the International Society for Cellular Therapy (ISCT).

However, Kim and Park (2017) also discussed the disadvantages of using human MSCs, in which there is a limitation on replicative lifespan, alteration of various functions including loss of multipotency might occur during cell expansion in *in vitro* culture. To apply cells for cell-based therapies, MSCs must be able to differentiate into specialised