DISCRIMINATION OF BIOCHEMICAL CHANGES IN HUMAN WHARTON'S JELLY DERIVED MESENCHYMAL STEM CELLS CULTURED UNDER XENO-FREE SYSTEMS BY FT-IR MICROSPECTROSCOPY

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การวินิจฉัยการเปลี่ยนแปลงทางชีวเคมีในเซลล์ต้นกำเนิดชนิดมีเซ็นไคม์ที่คัดแยกได้จากเนื้อเยื่อวาร์ตันเจลลีของมนุษย์ที่เพาะเลี้ยงในระบบที่ปราศจากส่วนประกอบที่ได้จากสัตว์ด้วยเทคนิค FT-IR MICROSPECTROSCOPY

นางสาวหทัยวรรณ โชคชูวัฒนาเลิศ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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หทัยวรรณ โชคชูวัฒนาเลิศ : การวินิจฉัยการเปลี่ยนแปลงทางชีวเคมีในเซลล์ต้นกำเนิดชนิดมีเซ็นไคม์ที่คัดแยกได้จากเนื้อเยื่อวาร์ตันเจลลีของมนุษย์ที่เพาะเลี้ยงในระบบที่ปราศจากส่วนประกอบที่ได้จากสัตว์ด้วยวิธี FT-IR MICROSPECTROSCOPY (DISCRIMINATION OF BIOCHEMICAL CHANGES IN HUMAN WHARTON'S JELLY DERIVED MESENCHYMAL STEM CELLS CULTURED UNDER XENO-FREE SYSTEMS BY FT-IR MICROSPECTROSCOPY) อาจารย์ที่ปรึกษา: รองศาสตราจารย์ ดร.รังสรรค์ พาลพ่าย, 66 หน้า

เซลล์ต้นกำเนิดมีเซนไคม์ (mesenchymal stem cells; MSCs) คือเซลล์ต้นกำเนิดที่เก็บได้จากจากเนื้อเยื่อโตเต็มวัยที่มีความน่าสนใจเป็นอย่างยิ่งในการนำไปใช้ในการรักษาด้วยวิธีสเต็มเซลล์บำบัด อย่างไรก็ตามการใช้ซีรัมที่ได้จากตัวอ่อนโค (fetal bovine serum; FBS) ในระบบเพาะเลี้ยงเซลล์แบบดั้งเดิมนั้นก่อให้เกิดความกังวลด้านความปลอดภัยในการรักษาเมื่อนำเซลล์ดังกล่าวไปใช้กับผู้ป่วย นอกจากนั้นพบว่าคุณลักษณะสำคัญของเซลล์ต้นกำเนิดมีเซนไคม์ เช่น อัตราการเพิ่มจำนวน การแสดงออกของสารพันธุกรรมและศักยภาพในการเปลี่ยนแปลงไปเป็นกลุ่มเนื้อเยื่อชั้นกลาง (mesoderm) มีการเปลี่ยนแปลงภายใต้ระบบการเพาะเลี้ยงที่แตกต่างกัน ดังนั้นเพื่อส่งเสริมให้เกิดความปลอดภัยในการนำไปใช้ในการรักษาทางคลินิกการศึกษานี้จึงมีเป้าหมายในการสร้างระบบการเพาะเลี้ยงที่ปราศจากการใช้สารที่มาจากสัตว์โดยใช้ซีรัมที่ได้จากสายสะดือมนุษย์ (hCBS) ในการเพาะเลี้ยงเซลล์ต้นกำเนิดมีเซนไคม์ที่ได้จากวาร์ตันเจลลีมนุษย์ (hWJ-MSCs) ผลของระบบการเพาะเลี้ยงที่แตกต่างกันต่อคุณลักษณะของเซลล์ต้นกำเนิดมีเซนไคม์ได้รับการศึกษาโดยใช้กระบวนการการตรวจสอบคุณลักษณะที่เป็นมาตรฐานเปรียบเทียบกับผลที่ได้จากการศึกษาด้วยเทคนิค Fourier transform infrared (FT-IR) microspectroscopy ในการศึกษานี้สายสะดือแต่ละเส้นจะถูกแบ่งและเพาะเลี้ยงในระบบการเพาะเลี้ยงเซลล์แบบดั้งเดิมที่มีส่วนประกอบที่มาจากสัตว์โดยใช้ FBS และระบบการเพาะเลี้ยงที่ปราศจากส่วนประกอบที่มาจากสัตว์โดยใช้ hCBS โดย hWJ-MSCs ที่เพาะเลี้ยงด้วย hCBS (hWJ-MSCs-hCBS) แสดงการแบ่งตัวสะสมที่สูงกว่าและใช้เวลาในการแบ่งตัวน้อยกว่าอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับ hWJ-MSCs ที่เพาะเลี้ยงด้วย FBS (hWJ-MSCs-FBS) อย่างไรก็ตามไม่พบความแตกต่างของรูปร่างของเซลล์และคุณลักษณะของเซลล์ต้นกำเนิดมีเซนไคม์เมื่อทดสอบด้วยกระบวนการการตรวจสอบคุณลักษณะที่เป็นมาตรฐาน ในทางตรงกันข้ามจากการวิเคราะห์องค์ประกอบหลัก (Principal component analysis; PCA) ของ FT-IR สเปกตรัมของ hWJ-MSCs ที่ได้จากระบบการเพาะเลี้ยงทั้งสองแสดงให้เห็นว่าสเปกตรัมของ hWJ-MSCs-FBS และ hWJ-MSCs-hCBS สามารถแบ่งแยกออกจากกันด้วยองค์ประกอบหลักที่ 1 โดยสามารถอธิบายความแปรปรวนทั้งหมดในชุดข้อมูลได้ถึงร้อยละ 63 โดย hWJ-MSCs-FBS แสดงค่า

การดูดกลืนแสงสูงกว่าในช่วงไขมัน (สเปกตรัมการยืดหดของพันธะ C-H ในช่วง 3,000-2,800 เซ็นติเมตร$^{-1}$) ตำแหน่งสูงสุดของการยืดหดของพันธะลิปิดเอสเทอร์ C=O (1,743 เซ็นติเมตร$^{-1}$) และช่วงของกรดนิวคลีอิก (1,261-900 เซ็นติเมตร$^{-1}$) แต่ทว่า hWJ-MSCs-hCBS มีการเพิ่มขึ้นของโปรตีนที่มี β-sheet สูง (การเพิ่มการดูดกลืนแสงที่1,639 เซ็นติเมตร$^{-1}$)

โดยสรุปการศึกษานี้แสดงให้เห็นว่าชนิดของซีรัมที่ใช้ส่งผลกระทบโดยตรงต่อคุณลักษณะของ hWJ-MSCs ที่เพาะเลี้ยง การค้นพบนี้เป็นหลักฐานแสดงให้เห็นว่าระบบการเพาะเลี้ยงที่ปราศจากส่วนประกอบที่ได้จากสัตว์มีความเหมาะสมสำหรับใช้ในการเพิ่มจำนวนเซลล์ต้นกำเนิดมีเซนไคม์เพื่อวัตถุประสงค์ในการรักษาทางคลินิก นอกจากนี้การศึกษานี้เป็นการศึกษาแรกที่แสดงให้เห็นว่าเทคนิค FT-IR microspectroscopy ร่วมกับการวิเคราะห์องค์ประกอบหลักสามารถแยกเซลล์ที่ได้จากกระบวนการเพาะเลี้ยงที่แตกต่างกันได้ในขณะที่กระบวนการการตรวจสอบคุณลักษณะที่เป็นมาตรฐานไม่สามารถแยก เทคนิคนี้แสดงหมู่ฟังก์ชันของสารอินทรีย์ของ hWJ-MSCs ได้ด้วยการวัดการดูดกลืนแสงอินฟราเรดเพียงครั้งเดียวโดยปราศจากกระบวนการการติดฉลากที่ซับซ้อน ดังนั้นจากข้อมูลข้างต้นแสดงให้เห็นว่าเทคนิค FT-IR microspectroscopy เป็นเทคนิคทางเลือกที่โดดเด่นในการตรวจสอบคุณลักษณะของเซลล์ต้นกำเนิดมีเซนไคม์

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2555 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

HATAIWAN CHOKECHUWATTANALERT : DISCRIMINATION OF BIOCHEMICAL CHANGES IN HUMAN WHARTON'S JELLY DERIVED MESENCHYMAL STEM CELLS CULTURED UNDER XENO-FREE SYSTEMS BY FT-IR MICROSPECTROSCOPY. THESIS ADVISOR : ASSOC. PROF. RANGSUN PARNPAI, Ph.D., 66 PP.

XENO-FREE CULTURE SYSTEM/MESENCHYMAL STEM CELLS PROPERTIES/FT-IR MICROSPECTROSCOPY

Mesenchymal stem cells (MSCs) are adult stem cells of particularly interest for stem cell-based therapy. However, the uses of fetal bovine serum (FBS) in conventional culture systems have raised concerns about the therapeutic safety for use with patients. Moreover, key properties of MSCs such as proliferation rate, transcriptional profiles, and mesodermal differentiation potential have been shown to be altered under different culture systems. Therefore, in order to facilitate the safety of clinical applications, this study aimes to establish a xeno-free culture system using human cord blood serum (hCBS) for human Wharton's Jelly derived MSCs (hWJ-MSCs) derivation. The effects of different culture systems on hWJ-MSCs properties were studied by standard characterization methods compared with results obtained using Fourier transform infrared (FT-IR) microspectroscopy. In this study, individual umbilical cords were divided and cultured by conventional xeno- (fetal bovine serum: FBS) and xeno-free (human cord blood serum: hCBS) culture systems. hWJ-MSCs cultured in hCBS (hWJ-MSCs-hCBS) exhibited a significantly higher cumulative population doubling with shorter population doubling time when compared with hWJ-

MSCs cultured in FBS (hWJ-MSCs-FBS). However, there were no differences in cell morphology and MSCs properties detected by standard characterization methods. In contrast, Principal component analysis (PCA) of FT-IR spectra of hWJ-MSCs derived from both culture systems revealed that the spectra of hWJ-MSCs-FBS and hWJ-MSCs-hCBS could be discriminated in scores plots along PC1, which can be explain by the 63% of the total variance in the dataset. hWJ-MSCs-FBS revealed higher absorption in lipid bands (C-H stretching spectral region (3,000-2,800 cm^{-1})), lipid ester C=O stretching peak (1,743 cm^{-1}), and nucleic acids region (1,261-900 cm^{-1}) whereas hWJ-MSCs-hCBS have higher β-sheet rich proteins (increase in absorbance at 1,639 cm^{-1}).

In summary, this study demonstrated that the type of serum supplement directly influences the properties of the cultivated hWJ-MSCs. These findings provided the evidence that the xeno-free culture system is suitable for the *ex vivo* expansion of clinical grade MSCs. Moreover, this study was the first to demonstrate that FT-IR microspectroscopy coupled with PCA analysis, but the standard characterization methods could not, could discriminate the cells derived from different culture systems. This technique reveals the organic functional groups of hWJ-MSCs via a single measurement of an infrared absorption, without any requirement of complicated labeling process. Therefore, the data presented here shows that FT-IR microspectroscopy is an alternative outstanding technique for MSCs characterization.

School of Biotechnology Student's Signature_____

Academic Year 2012 Advisor's Signature_____

Co-advisor's Signature _____

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Hataiwan Chokechuwattanalert

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LIST OF ABBREVIATIONS

MSCs	=	mesenchymal stem cells
hWJ-MSCs	=	human wharton's jelly-derived mesenchymal stem cells
hBM-MSCs	=	human bone marrow-derived mesenchymal stem cells
AT-MSCs	=	adipose tissue-derived mesenchymal stem cells
FBS	=	fetal bovine serum
HS	=	human serum
hCBS	=	human cord blood serum
CBP	=	cord blood plasma
hWJ-MSCs-F	=	human wharton's jelly-derived mesenchymal stem cells cultured in conventional xeno (FBS) system
hWJ-MSCs-hCBS	=	human wharton's jelly-derived mesenchymal stem cells cultured in xeno-free (hCBS) system
FT-IR	=	fourier transform infrared
IR	=	infrared
PD	=	population doubling
CPD	=	cumulative population doubling
PDt	=	population doubling time
CFU-F	=	colony forming unit fibroblast
PCA	=	principle component analysis

CHAPTER I

INTRODUCTION

1.1 Background

Mesenchymal stromal cells or Mesenchymal stem cells (MSCs) are multipotent adult stem cells that are of particular interest for stem cell-based therapies because these cells are readily available in patient, capable of expand *in vitro* and can be induced to differentiate into various specific cell types; including bone, cartilage, cardiomyocyte, neuron, and insulin producing cells (Chao et al., 2008; H. S. Wang et al., 2004; Zhang et al., 2010). Despite of their differentiation capacities, MSCs were found to have low immunogenicity and immunomodulatory properties (Aggarwal and Pittenger, 2005; Zhou et al., 2011). Currently, the presence of MSCs is demonstrated virtually throughout the body. For example, MSCs could be isolated from bone marrow (Pittenger et al., 1999), adipose tissue (Fraser et al., 2008), dental pulp (Liu et al., 2009), umbilical cord blood (Phuc et al., 2011) and umbilical cord tissue called Wharton's jelly (H. S. Wang et al., 2004). Wharton's jelly (WJ) is the mucoid connective tissue found inside the umbilical cord. It has been demonstrated that WJ of the human umbilical cord is the abundant source of primitive mesenchymal stem cells, as human Wharton's jelly-derived mesenchymal stem cells (hWJ-MSCs) posses the greater *in vitro* proliferation capability, higher frequency of colony forming units and shorter doubling times when compared to human bone marrow-derived mesenchymal stem cells (hBM-MSCs) (Troyer and Weiss, 2008).

Several publications reported the successful *in vitro* differentiation of hWJ-MSCs into many specific cell types such as adipocyte, osteocyte, chondrocyte (Majore et al., 2011), glial cells and neurons (Mitchell et al., 2003), dopaminergic neurons (Fu et al., 2006), cardiomyocytes (H. S. Wang et al., 2004), and insulin-producing cells (Chao et al., 2008; H.-S. Wang et al., 2011; Wu et al., 2009). Without dealing with embryos and invasive collection procedures, hWJ-MSCs are certainly attractive and controversial-free adult stem cells used for regenerative medicine.

Culture systems used to isolate and expand MSCs is still an important issue. The use of fetal bovine serum (FBS) in conventional culture system raises concern about the therapeutic safety, including the contamination of xenogenic proteins as well as the risk of pathogen transmission (Bieback et al., 2009; Jung et al., 2009; Ma et al., 2012). To overcome these limitations, many of the studies attempt to established xeno-free culture system using a variety of human supplement (autologous- and allologous human serum, human blood derived-platelet derivatives, human cord blood serum, etc.) and commercial serum-free medium (Bieback et al., 2009; Hartmann et al., 2010; Ma et al., 2012; Meuleman et al., 2006; Phadnis et al., 2006; Shetty et al., 2007; Stute et al., 2004). Of these, allogenic human umbilical cord blood serums (hCBS) are of particular interest for FBS replacement, as they are enriched with nutrients, growth factors and readily available after birth (Ma et al., 2012). Moreover, hCBS has been reported to support the better growth of bone marrow- and placenta-derived MSCs (Ma et al., 2012; Phadnis et al., 2006; Shetty et al., 2007). However, until now, there is no report on the use of hCBS in the isolation and expansion of hWJ-MSCs.

Interestingly, there were the evidences that different culture system can alter MSCs properties such as proliferation rate, transcriptional profiles, and mesodermal

differentiation potential. Jung and co-workers (2009) reported the enhancement of proliferative activity and osteogenic potential of hBM-MSCs cultured in the medium supplemented with allogenic human CBS compared to those cultured in FBS. Notably, these properties were reversed when the cultures conditions were switched. By Microarray-based screening, Lindroos *et al*. (2010) found that when expanded human adipose tissue-derived MSCs (AT-MSCs) in the medium containing allogenic human serum (HS), genes involved in the cell cycle pathway were overexpressed, compared to those cultured in FBS condition. Therefore, in order to facilitate the safety clinically translational applications, the effects of different culture system influence on MSCs characteristics need to be clarified. To discriminate the effects of culture system influence on MSCs at biochemical level, Fourier transform infrared (FT-IR) Microscopy is a novel analytical tool that provides unique optical markers, like a molecular fingerprint, for stem cells characterization. Molecular and structural information of whole cells will be obtained, as spectral information, via a single measuring the absorption of light in the mid-infrared region (from 4,000 to 300 wavenumbers, cm^{-1}) (Chan and Lieu, 2009). When combined with multivariate analysis, FT-IR microscopy can distinguish the difference of murine embryonic stem cells (mESCs) during spontaneous differentiation (Ami et al., 2008). This study aims to establish the xeno-free culture system using hCBS for hWJ-MSCs derivation. Conventional xeno (fetal bovine serum: FBS) system will be used as reference. MSCs properties of hWJ-MSCs obtained from both conditions will be analyzed and compared by standard characterization methods coupled with FT-IR Microscopy.

1.2 Research objectives

1.2.1 To isolate and expand human Wharton's jelly-derived mesenchymal stem cells (hWJ-MSCs) by conventional xeno (fetal bovine serum: FBS) and xeno-free (human cord blood serum: hCBS) culture systems.

1.2.2 To characterize and compare the properties of hWJ-MSCs obtained from conventional xeno- and xeno-free (hWJ-MSCs-F and hWJ-MSCs-hCBS, respectively) culture system by standard characterization methods.

1.2.3 To identify and compare FT-IR spectrum of hWJ-MSCs-F and hWJ-MSCs-hCBS by FT-IR Microspectroscopy.

1.3 Research hypotheses

1.3.1 Under the conventional xeno- and xeno-free culture system, MSCs could be isolated and expanded from Wharton's jelly tissue of human umbilical cords.

1.3.2 By standard characterization methods, cells obtained from both culture systems should exhibit the typical properties of mesenchymal stem cells, which are self-renewal, clonal expansion, multipotent differentiation as well as ability to maintain normal karyotype *in vitro***.**

1.3.3 FT-IR spectrum of hWJ-MSCs-F and hWJ-MSCs-hCBS could be obtained. These spectral signatures could be used as a tool for hWJ-MSCs characterization.

1.4 Scope of the study

1.4.1 hWJ-MSCs were isolated under conventional xeno- and xeno-free culture

conditions. In order to compare the efficiency between these conditions, the single umbilical cord was divided into 2 groups for different culture systems; fetal bovine serum (FBS) and human cord blood serum (hCBS). Properties of hWJ-MSCs derived from these systems were examined and compared by standard characterization methods. Examined properties include colony forming units-fibroblast (CFU-F), cumulative population doubling (CPD), Population doubling time (PDt), expression profile of surface antigens (e.g. *CD45*, *CD73*, *CD90* and *CD105*) and karyotype analysis. Moreover, multipotent property were verified by the differentiation of hWJ-MSCs into mesodermal lineages; chondrocytes, adipocytes and osteocytes.

1.4.2 hWJ-MSCs derived from conventional xeno- and xeno-free culture conditions were analyzed by FT-IR Microspectroscopy.

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CHAPTER II

LITERATURE REVIEW

2.1 Mesenchymal Stem Cells (MSCs)

Stem cells are the cells that have 2 main properties: self-renewal and multipotency. Self renewal refers to ability to undergo numerous cell divisions while retaining their stemness, whereas multipotency means ability to differentiate into multiple mature functional cell types (Nombela-Arrieta et al., 2011). In theory, under the appropriate signals, endogenous stem cells residing in a wide variety of tissues play role in tissue repair and maintenance (Barry and Murphy, 2004). As a result, the biology of stem cells and their therapeutic potential have gained wide interest for tissue engineering and regenerative medicine. Based on their origin, stem cells can simply be classified as embryonic stem cells (ESCs), adult stem cells and, very recently, induced pluripotent stem cells (iPSC). ESCs and iPSC are pluripotent stem cells. Although, they are able to differentiate into derivatives of all 3 germ layers, translating ESCs and iPSC into clinical application has been hampered by issues such as an ethic controversies, safety concerns in reprogramming, as well as the possibility of tumorigenicity. Hence, many attempts have been focusing on another kind of stem cells.

Human bone marrow-derived Mesenchymal Stem Cells (hBM-MSCs) are adult stem cells that first identified, as a minor population, in bone marrow stroma (Friedenstein, 1961). It is now clear that hBM-MSCs are multipotent adult stem cells

capable of *ex vivo* expansion and induced to differentiate into cells of connective tissue lineages including osteoblasts, adipocytes and chondrocytes.(Pittenger et al., 1999). Currently, the presence of MSCs is demonstrated virtually throughout the body. For example, MSCs could be isolated from bone marrow (Pittenger et al., 1999), adipose tissue (Fraser et al., 2008), dental pulp (Liu et al., 2009), umbilical cord blood (Phuc et al., 2011) and umbilical cord tissue called Wharton's jelly (H. S. Wang et al., 2004). Of note, relevant to their anatomical location, isolated MSCs show the variation in their properties such as growth kinetic, immunophenotype, and differentiation potentials. Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) prescribed three minimal criteria used to identify MSCs: i) plastic adherence in standard culture conditions, ii) display a phenotype of $CD105^+$, $CD73^+$, $CD90^+$, $CD34^-$, $CD45^-$, $CD11b^-$, $CD14b^-$, $CD19^-$, $CD79a^-$, and HLA-DR^- and iii) multipotency differentiation, ability to differentiate into osteoblasts, adipocytes and chondrocytes, *in vitro* (Figure 2.1) (Dominici et al., 2006).

Among stem cell, MSCs are considered as an outstanding candidate because of their availability, home and engraft ability, immunosuppressive properties as well as ability to differentiate into various specific cell types (reviewed by Brooke et al., 2007). As evidenced by a number of clinical studies, transplantation of MSCs resulted in therapeutic effects of many diseases (Table 2.1) (reviewed in García-Gómez et al., 2012).

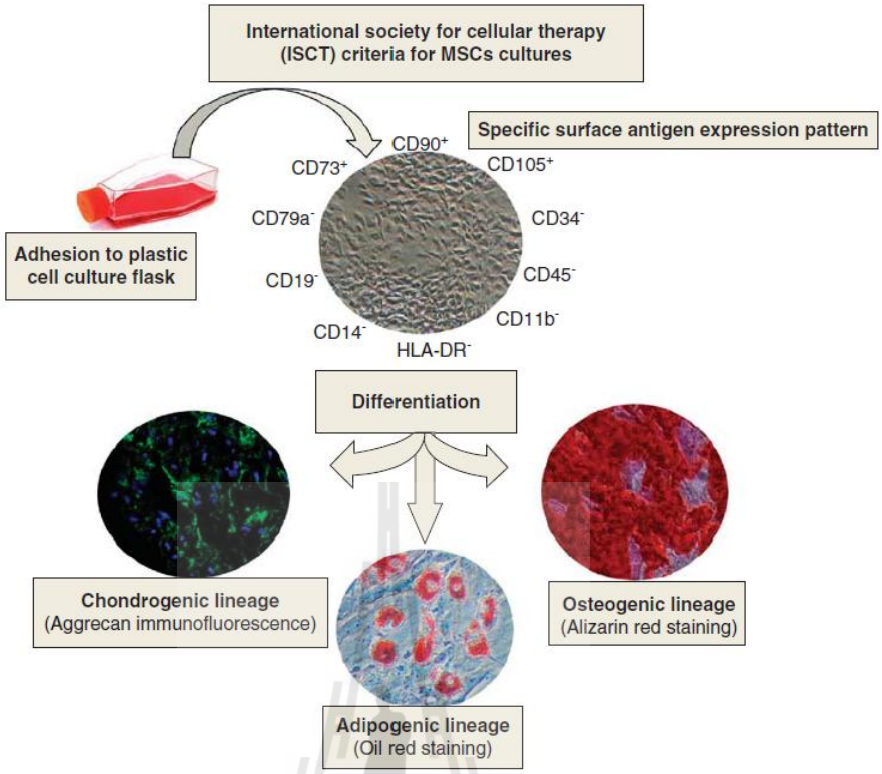

Figure 2.1 Three minimal criteria recommended for identification of MSCs. (García-Gómez et al., 2010).

2.2 Human umbilical cord and human Wharton's jelly derived MSCs

Although showing an impressive therapeutic response in clinical studies, only 0.001-0.01% of MSCs presented in isolated bone marrow cells (Pittenger et al., 1999). Moreover, the numbers of hBM-MSCs obtained by marrow aspiration were proven to decline with donor age (Stolzing et al., 2008). As results, many researchers have searched for the alternative sources of MSCs. Human umbilical cord is an extraembryonic tissue that comprised of two arteries and one vein surrounded by proteoglycans and mucopolysaccharides-rich connective tissue named Wharton's jelly

Table 2.1 Completed clinical studies of MSCs transplantation (reviewed in García-Gómez et al., 2012).

Condition	Phase	Cell source	Study	Sponsor
Crohn's disease	II	Allogeneic BM-MSCs	Prochymal adult human mesenchymal stem cells for treatment of moderate-to-severe Crohn's disease (NCT00294112)	Osiris Therapeutics (USA)
Liver cirrhosis	I/II	Autologous BM-MSCs differentiated to progenitor hepatocytes	Improvement of liver function in liver cirrhosis patients after autologous mesenchymal stem cell injection: a Phase I - II clinical trial (NCT00420134)	Shaheed Beheshti Medical University (Iran)
Graft versus host disease	II	Allogeneic BM-MSCs	Safety and efficacy study of adult human mesenchymal stem cells to treat acute GVHD. (NCT00136903)	Osiris Therapeutics (USA)
Crohn's disease	III	Allogeneic BM-MSCs	Extended evaluation of Prochymal adult human stem cells for treatment-resistant moderate-to-severe Crohn's disease (NCT00543374)	Osiris Therapeutics (USA)
Graft versus host disease	III	Allogeneic BM-MSCs	Efficacy and safety of adult human mesenchymal stem cells to treat patients who have failed to respond to steroid treatment for acute graft versus host disease (GVHD) (NCT00366145)	Osiris Therapeutics (USA)
Familial hypercholesterolemia	I	Allogeneic BM-MSCs differentiated to hepatocytes	Bone marrow stem cells as a source of allogenic hepatocyte transplantation in homozygous familial hypercholesterolemia (NCT00515307)	University of Tehran (Iran)
Recovery following partial medial meniscectomy	I/II	Allogeneic BM-MSCs	A Phase I/II study of chondrogen delivered by intra-articular injection following meniscectomy (NCT00225095)	Osiris Therapeutics (USA)

Table 2.1 (Continued).

Condition	Phase	Cell source	Study	Sponsor
Adult periodontitis	I/II	BM-MSCs* + BM-MSCs* differentiated to osteoblasts	Clinical trials of regeneration for periodontal tissue (NCT00221130)	Translational Research Informatics Center, Kobe, Hyogo, (Japan)
Graft versus host disease	II	Allogeneic BM-MSCs	Safety and efficacy of Prochymal for the salvage of treatment-refractory acute GVHD patients (NCT00284986)	Osiris Therapeutics (USA)
Myocardial ischemia	I/II	Autologous BM-MSCs	Stem cell therapy for vasculogenesis in patients with severe myocardial ischemia (NCT00260338)	Rigshospitalet (Denmark)
Diabetic foot	II	Autologous BM-MSCs versus Autologous BM-MNCs	Induced wound healing by application of expanded bone marrow stem cells in diabetic patients with critical limb ischemia (NCT01065337)	Ruhr University of Bochum (Germany)
Anal fistula	III	Autologous AT-MSCs	Efficacy and safety of adipose stem cells to treat complex perianal fistulas not associated to Crohn's disease (NCT00475410)	Cellerix (Spain)
Depressed scar	II/III	Autologous AT-MSCs differentiated to adipocytes	Safety and efficacy of autologous cultured adipocytes in patient with depressed scar (NCT00992147)	Anterogen Co., Ltd (Republic of Korea)

*Undefined autologous or allogenic cell source.

Abbreviation: AT-MSCs: Adipose tissue derived MSCs, BM-MNCs: Bone marrow derived mononuclear cells, BM-MSCs: Bone marrow derived MSCs.

(Figure 2.2) (Karahuseyinoglu et al., 2007). Physically, Wharton's jelly (WJ) supports the structure of umbilical blood vessels during pregnancy. Interestingly, it has been demonstrated that WJ of the umbilical cord is an abundant source of primitive MSCs (Mitchell et al., 2003). Human Wharton's jelly-derived MSCs (hWJ-MSCs) meet all criteria used to describe MSCs, as mentioned previously. By immunophenotype analysis, hWJ-MSCs share a high similar expression profiles with hBM-MSCs (Table 2.2) (Anzalone et al., 2010). Moreover, several publications reported the successful *in vitro* differentiation of hWJ-MSCs into various specific cell types beyond their mesodermal lineage, such as glial cells and neurons (Mitchell et al., 2003), dopaminergic neurons (Fu et al., 2006) as well as insulin-producing cells (Chao et al., 2008; H.-S. Wang et al., 2011). When compared with hBM-MSCs, hWJ-MSCs exhibited a higher frequency of colony forming units and greater expansion capability with faster population doubling times, suggesting by the high expression of telomerase activity (Troyer and Weiss, 2008).

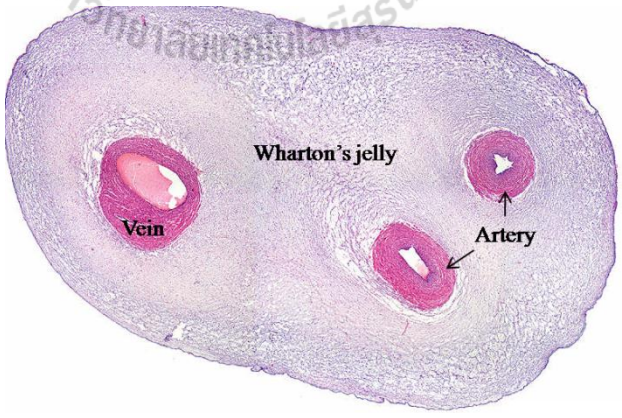

Figure 2.2 Cross section of normal human umbilical cord. Haematoxylin and Eosin (H&E) staining (University of Oslo, 2012).

Table 2.2 Similarity of marker expression pattern (Anzalone et al., 2010).

Markers	hBM-MSCs	hWJ-MSCs
CD10	+	+
CD13	+	+
CD14	−	−
CD29	+	+
CD31	−	−
CD33	−	−
CD34	−	−
CD44	+	+
CD45	−	−
CD49e	+	+
CD51	+	+
CD54	+	NA
CD56	+	−
CD59	+	NA
CD68	NA	+
CD71	+	NA
CD73	+	+
CD79	−	NA
CD80	−	+
CD86	−	−
CD90	+	+
CD105	+	+
CD117	−	+
CD163	NA	−
CD166	+	+
CD235a	−	NA
CK-7	NA	−
CK-8	NA	+
CK-18	+	+
CK-19	+	+
Connexin-43	+	+
GATA-4	+	+
GATA-5	NA	+
GATA-6	NA	+
GFAP	+	+
HLA-A	+	+
HLA-B	+	+
HLA-C	+	+
HLA-DR	−	−
HLA-G	+	+
HNF-4α	NA	+
Nanog	+	+
Nestin	+	+
NSE	+	+
Oct3⁄4A	+	+
α-SMA	+	+
Vimentin	+	+

Abbreviation: NA, not applicable.

Without involving the use of embryos and invasive collection procedures, hWJ-MSCs are certainly attractive and controversy-free adult stem cells with potential for use in regenerative medicine practice.

2.3 Effects of culture systems on human MSCs properties

Now a day, stem cell technologies have gained acceptance from medical profession. As review by García-Gómez et al. (2012), MSCs are regards as Advanced Therapy Medicinal Product (ATMP) and Human Cell, Tissue, and Cellular and Tissue-based product (HCT/P) in Europe and USA, respectively. However, the use of this technology to clinical practice is still hindered by awareness of culture systems used to produce (isolate and expand) clinical grade human MSCs. Based on the source of supplement, culture systems used to isolated and expanded MSCs can be divided into 2 groups: i) Conventional xeno- and ii) Xeno-free systems. Because this study aimed to setting up a Good Manufacturing Practice (GMP) criteria used for isolation and expansion of clinical grade human MSCs, conventional xeno system refers to the *in vitro* culture systems that use animal serum or animal serum component in the culture media used for culturing human cells. Generally, fetal bovine serum (FBS) or fetal calf serum (FCS) is a popular supplement used for culturing mammalian cells (Phadnis et al., 2006). However, the use of FBS in conventional culture system raises concern about the therapeutic safety, including the contamination of xenogenic proteins as well as the risk of pathogen transmission (Bieback et al., 2009; Jung et al., 2009; Ma et al., 2012). In addition, there were evidences that culture conditions contributed to the profound alteration of cultivated cells. Change in lipid content of cultured cells has been proven to correlate with fatty acid profiles of supplemented

serum, varying among species of animal of serum origin (Stoll and Spector, 1984). A study on murine keratinocytes demonstrated that levels of linoleic acid (LA) were 4-fold decrease in 5 days cultured cells, suggesting the low level of this essential fatty acid in FBS compared with normal mouse serum (Isseroff et al., 1985). Moreover, various studies indicated that different culture conditions resulted in different gene expression profile (Bieback et al., 2010; Lindroos et al., 2010) as well as osteogenic and adipogenic differentiation potential of cultivated MSCs (Jung et al., 2009; Lindroos et al., 2010; Tekkatte et al., 2012).

To overcome these limitations, many of the studies attempted to establish xeno-free culture system using a variety of human supplements including autologous- and allologous human serum (HS) (Shahdadfar et al., 2005), human blood derived-platelet derivatives (Bieback et al., 2009) and human cord blood serum (hCBS) (Shetty et al., 2007). In 2009, Bieback and colleagues demonstrated that pooled human platelet collected from buffy coat units of blood group O-type donors can be used as a FBS replacement. They reported that expansion kinetics of hBM-MSCs expanded by medium supplemented with 10% pooled human platelet lysate (pHPL) were significantly enhanced compared with cells cultured in 10% FBS, 10% HS and 10% thrombin-activated platelet releasate in plasma (tPRP). Regarding with MSCs properties, they reported that hBM-MSCs derived from aforementioned culture condition were similar in cell surface maker expression, immunosuppressive action and multipotency (adipogenic- and osteogenic-) differentiation. However, in order to obtain 1 batch of pHPL, approximately 40-50 donations were required. In the same year, Jung and colleagues reported that 10% allogenic hCBS supplement is a unique humanized culture condition used for hBM-MSCs derivation. By comparing growth

kinetics of hBM-MSCs cultured in medium containing either 10% FBS, hCBS, HS, they found that hBM-MSCs cultured in hCBS exhibited a significant higher proliferation rate than other conditions (Figure 2.3 A). Interestingly, these accelerated effects were observed only in hCBS culture system, but not cord blood plasma (CBP) at various concentrations tested (5-15%) (Figure 2.3 B). Consistent with superior growth kinetics, results from RT-PCR demonstrated that hBM-MSCs cultured in hCBS expressed higher *Oct*-4 than in FBS (Figure 2.3 C). Notably, the proliferation potentials were reversed when culture conditions (hCBS, FBS) were exchanged (Figure 2.3 D) Moreover, as indicated by early onset of visible calcification, higher level of mineralized nodules and higher Alkeline phosphatase (ALP) activity, osteogenic differentiation potential was enhanced in hBM-MSCs cultured in hCBS than in FBS. Converse to osteogenic potential, adipogenic differentiation was lower in hBM-MSCs cultured in hCBS than in FBS, determined by 3-fold decrease in number of Oil Red O stained lipid droplets.

Very recently, Ma and colleagues (2012) demonstrated that culture media supplemented with 10% cord blood serum derived from 4 blood groups (A-, B-, O- and AB-CBS) contributed to the significant higher proliferation rate of human Placenta derived MSCs (hPDMSCs) compared with those cultured in FBS supplement. In addition, these accelerated effects were reported in all cord blood serum (A-, B-, O- and AB-CBS), irrespective of MSCs donors and blood groups matching. Of these, allogenic human umbilical cord blood serums (hCBS) are of particular interest for isolation and expansion of clinical grade human MSCs. Moreover, until now, there is no report on the use of hCBS in the isolation and the expansion of hWJ-MSCs.

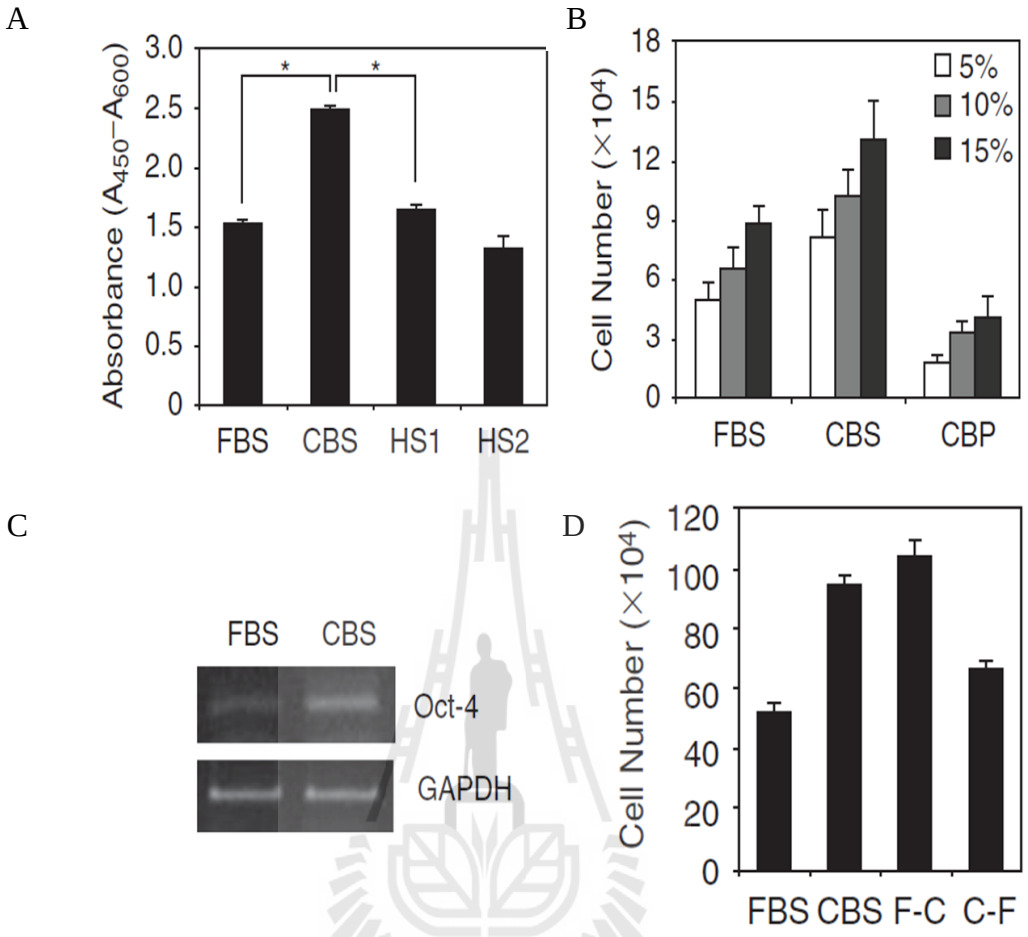

Figure 2.3 Effects of different serum supplement on hBM-MSCs (Jung et al., 2009). (A) Comparison of growth kinetic of hBM-MSCs grown in medium containing FBS, hCBS and HS. The proliferative activity was determined by WST-1 assay. (B) Effects of various CBP concentration on hBM-MSCs proliferation. (C) Higher *Oct*-4 expression were detected in hBM-MSCs cultured in hCBS. (D) Phenotypic reversion of hBM-MSCs when culture conditions were switched. F-C means switching from FBS to hCBS supplement whereas C-F means switching from hCBS to FBS.

2.4 Biochemical study of human stem cells characteristics by FT-IR microspectroscopy

As mentioned earlier, different serum supplement resulted in distinct MSCs properties. Therefore, in order to facilitate the safety clinically translational applications, the effects of different culture system influence on MSCs characteristics need to be precisely clarified. To discriminate the effects of culture system influence on MSCs at biochemical level, Fourier transform infrared (FT-IR) Microspectroscopy is a novel analytical tool that provides unique optical markers for stem cells characterization. This technique based on the selective absorption of mid-infrared radiation frequency, range between 4,000-400 cm^{-1}, of the chemical bonds of the sample. Basically, a molecule can be looked like two masses linked by a spring-like chemical bond. By passing IR through a sample, infrared-active molecules will absorb energy from the radiation, then dipole moment of molecules change via vibrations (Figure 2.4). Determination of infrared (IR) absorptions caused by molecular vibration resulted in IR spectrum of a molecule.

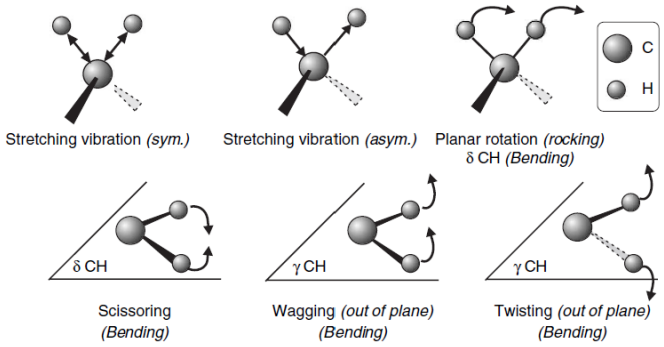

Figure 2.4 Example of molecular vibrational modes of CH_2 group (Rouessac and Rouessac, 2007a).

Of note, absorption of radiation occurs only when the frequency of incoming IR is the same frequency as one of the fundamental modes of vibration of the molecule. Table 2.3 demonstrated correlation between organic functional groups and IR absorption bands (Rouessac and Rouessac, 2007b). Therefore, in a large assembly of molecules, coupling of vibrations contributed to the unique spectrum pattern, like a molecular fingerprint of the entire molecule (Stuart, 2004b). When matching with a well established spectrum, this peak-by-peak correlation can be used for molecular identification (Silverstein et al., 2005). Practically, IR spectrum coupled with other analytical methods has been used in a large number of studies of biological molecule such as lipids, proteins and nucleic acids. The most intense IR spectrum of these biological molecules were observed in the following regions: lipids (3,000-2,800 cm^{-1}), proteins (1,700-1,300 cm^{-1}) and nucleic acids (1,300-900 cm^{-1}) (Machana et al., 2012). Main spectral vibrations of lipids region are caused by C-H stretching (CH_2- and CH_3-) vibration of hydrocarbon tail of lipid. Generally, asymmetric (at 2,920 cm^{-1}) and symmetric (at 2,851 cm^{-1}) stretching of CH_2- are the strongest bands in the lipid spectra (Stuart, 2004a). In addition, around 1,800-1,700 cm^{-1}, there are characteristic bands of ester group caused by C=O stretching of the lipid head-group (Machana et al., 2012). Table 2.4 is the list of major IR bands of lipids.

Characterization of proteins mostly depend on absorption bands associated with amides I and amide II. Amide I band (1,700-1,500 cm^{-1}) caused by the stretching vibration of C=O (80%) and C-N (10%) groups coupled with in-plane N-H bending (10%) (Stuart, 2004a). It is a useful band used for identification of secondary structure of protein (Table 2.5)

Table 2.3 Correlation between organic functional groups and IR absorption bands (Rouessac and Rouessac, 2007b).

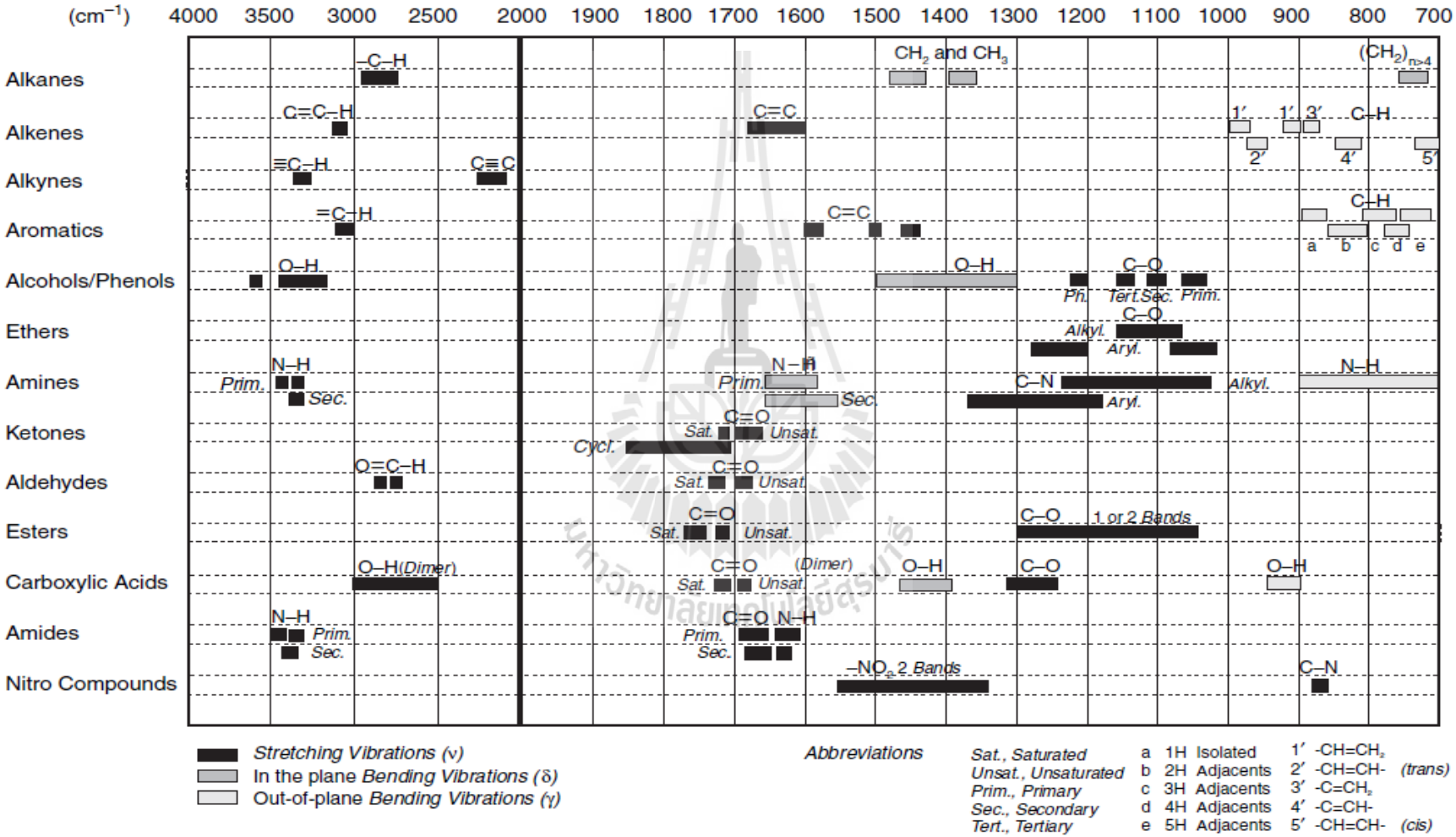

Table 2.4 Major IR bands of lipids (Modified from Stuart, 2004a).

Wavenumber (cm^{-1})	**Assignment**
3,010	=C–H stretching
2,956	CH_3 asymmetric stretching
2,920	CH_2 asymmetric stretching
2,870	CH_3 symmetric stretching
2,850	CH_2 symmetric stretching
1,730	C=O stretching
1,485	$(CH_3)_3N^+$ asymmetric bending
1,473, 1,472, 1,468, 1,463	CH_2 scissoring

Table 2.5 Secondary structure of protein indicated by amide I band (Stuart, 2004a).

Wavenumber (cm^{-1})	**Assignment**
1,695 – 1,670	Intermolecular β-structure
1,690 – 1,680	Intramolecular β-structure
1,666 – 1,659	'3-turn' helix
1,657 – 1,648	α-helix
1,645 – 1,640	Random coil
1,640 – 1,630	Intramolecular β-structure
1,625 – 1,610	Intermolecular β-structure

Herein, FT-IR Microspectroscopy has been proven to be a powerful tool for studying of macromolecular events occurred in whole cells (Ami et al., 2008). Molecular and structural information of the whole cells will be obtained as spectral information via a single measuring the absorption of mid-infrared radiation (Chan and Lieu, 2009). When combined with multivariate analysis, FT-IR microspectroscopy can probe the change of biomolecular events occurred during spontaneous differentiation of murine embryonic stem cells (Ami et al., 2008), chondrogenic differentiation of human MSCs (Chonanant et al., 2011), and hepatocyte differentiation of rat BM-MSCs (Ye et al., 2012). Therefore, in order to provide a beneficial evidence for setting up of Good Manufacturing Practice (GMP) criteria used for isolation and expansion of clinical grade hWJ-MSCs, the suitable xeno-free system need to be established. In this

study, effects of xeno-free (hCBS) system on hWJ-MSCs were clarified by a precise analytical technique named FT-IR microspectroscopy. Changes in MSCs properties as well as the macromolecular events of cultivated hWJ-MSCs were investigated using standard criteria prescribed for identification of MSCs and FT-IR microspectroscopy, respectively. In addition, derivation of hWJ-MSCs spectral signature will be beneficial in establishment of FT-IR spectral database used for hWJ-MSCs characterization.

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CHAPTER III

DISCRIMINATION OF BIOCHEMICAL CHANGES IN HUMAN WHARTON'S JELLY DERIVED MESENCHYMAL STEM CELLS CULTURED UNDER XENO-FREE SYSTEMS BY FT-IR MICROSPECTROSCOPY

3.1 Abstract

Recently, Mesenchymal Stem Cells (MSCs) biology and their therapeutic potential have gained widely interest for tissue engineering and regenerative medicine. Interestingly, although sharing similar phenotype, there has been evidenced that MSCs derived from different culture system altered their key properties such as proliferation rate, transcriptional profiles, and mesodermal differentiation potential. This study aimes to establish a xeno-free culture system using allogenic human cord blood serum (hCBS) for human Wharton's jelly-derived mesenchymal stem cells (hWJ-MSCs) derivation. Effects of different culture system on hWJ-MSCs properties were studied by standard characterization methods which were compared with results obtained using Fourier transform infrared (FT-IR) microspectroscopy. Individual umbilical cords was divided and cultured by conventional xeno- (10% fetal bovine serum: FBS) and xeno-free (10% human cord blood serum: hCBS) culture systems.

Interestingly, hWJ-MSCs cultured in hCBS (hWJ-MSCs-hCBS) exhibited a significantly higher cumulative population doubling with shorter population doubling time. Moreover, Principal component analysis (PCA) revealed the spectra of hWJ-MSCs derived from both culture systems could be discriminated in scores plots along PC1, which explained the 63% of total variance in the dataset. hWJ-MSCs cultured in FBS (hWJ-MSCs-F) revealed higher absorbance in lipid bands (C-H stretching spectral region (3,000-2,800 cm^{-1}), lipid ester C=O stretching peak (1,743 cm^{-1}), and nucleic acids region (1,261-900 cm^{-1}) when compared with hWJ-MSCs-hCBS. In contrast, hWJ-MSCs-hCBS increased β-sheet rich proteins (increase in absorbance at 1,639 cm^{-1}), suggesting the expression of functional proteins related to the higher cell proliferation rates observed under the xeno-free culture system.

In summary, this study demonstrated that the type of serum supplement directly affected the properties of cultivated hWJ-MSCs. Moreover, this study first demonstrated that FT-IR microspectroscopy coupled with PCA analysis, but not standard characterization methods, could discriminate the cells derived from different culture systems. This technique enables an insight on organic functional groups of hWJ-MSCs via a single measurement of an infrared absorption, without any requirement of complicated labeling process. Therefore, the data presented here shows that FT-IR microspectroscopy is an alternative outstanding technique for MSCs characterization.

3.2 Introduction

Mesenchymal stem cells (MSCs) are multipotent adult stem cells that are of particular interest for stem cell-based therapies because these cells are readily

available in patients, capable of expansion *in vitro* and differentiating into a diverse range of specific cell types (Brooke et al., 2007). Moreover, apart from their differentiation capacities, MSCs were found to have low immunogenicity and immunomodulatory properties (Aggarwal and Pittenger, 2005). Recently, Wharton's Jelly (WJ) tissues were proven to harbor primitive mesenchymal stem cells compared to human bone marrow-derived mesenchymal stem cells (hBM-MSCs) (Troyer and Weiss, 2008). Several publications reported successful *in vitro* differentiation of human Wharton's jelly-derived mesenchymal stem cells (hWJ-MSCs) into many specific cell types beyond their mesodermal lineage, such as glial cells and neurons (Mitchell et al., 2003), dopaminergic neurons and insulin-producing cells (Chao et al., 2008). Without involving the use of embryos and invasive collection procedures, hWJ-MSCs are certainly attractive and controversy-free adult stem cells with potential for use in regenerative medicine practice.

Culture system used to isolate and expand MSCs is still an important issue. The use of fetal bovine serum (FBS) in conventional culture system raises concern about the therapeutic safety, including the contamination of xenogenic proteins as well as the risk of pathogen transmission (Bieback et al., 2009; Jung et al., 2009; Ma et al., 2012). To overcome these limitations, many of the studies attempted to establish a xeno-free culture system using a variety of human supplement (autologous- and allologous human serum, human blood derived-platelet derivatives, human cord blood serum, etc.) and commercial serum-free medium (Bieback et al., 2009; Hartmann et al., 2010; Ma et al., 2012; Meuleman et al., 2006; Phadnis et al., 2006; Shetty et al., 2007; Stute et al., 2004). Of these, allogenic human umbilical cord blood serums (hCBS) are of particular interest for FBS replacement, as they are enriched with

nutrients, growth factors and readily available after birth (Ma et al., 2012). However, until now, there is no report on the use of hCBS in the isolation and the expansion of hWJ-MSCs. Moreover, there has been evidence that different culture system can alter key properties of MSCs such as proliferation rate, transcriptional profiles, and mesodermal differentiation potential (Jung et al., 2009; Lindroos et al., 2010). Therefore, in order to facilitate the safety clinically translational applications, the effects of different culture system influence on hWJ-MSCs characteristics need to be clarified.

To discriminate the effects of culture system influence on hWJ-MSCs at biochemical level, Fourier transform infrared (FT-IR) Microspectroscopy is a novel analytical tool that provides unique optical markers, like a molecular fingerprint, for stem cells characterization. Molecular and structural information of the whole cells will be obtained as spectral information via a single measuring the absorption of light in the mid-infrared region (from 4,000 to 300 wavenumbers, cm^{-1}) (Chan and Lieu, 2009). For example, spectral vibrations of lipids region caused by C-H stretching of hydrocarbon tail of lipid were observed in spectral regions from 3,000-2,800 cm^{-1}. In addition, around 1,800-1,700 cm^{-1}, there are characteristic bands of ester group caused by C=O stretching of the lipid head-group (Machana et al., 2012). Absorbance bands observed between 1,700 and 1,500 cm^{-1} are characteristic bands of amides I and II of protein (Machana et al., 2012). Amide I band (1,700-1,500 cm^{-1}), the most useful infrared band used for identification of secondary structure of proteins, caused by the stretching vibration of C=O (80%) and C-N (10%) groups coupled with in-plane N-H bending (10%) (Stuart, 2004). When combined with multivariate analysis, FT-IR microspectroscopy can probe the change of biomolecular events occurred during

spontaneous differentiation of murine embryonic stem cells (Ami et al., 2008), chondrogenic differentiation of human MSCs (Chonanant et al., 2011), and hepatocyte differentiation of rat BM-MSCs (Ye et al., 2012).

This study aimes to establish a xeno-free culture system using allogenic human cord blood serum (hCBS) for hWJ-MSCs derivation. The effects of different culture system on hWJ-MSCs properties were studied by standard characterization methods which were compared with results obtained from Fourier transform infrared (FT-IR) microspectroscopy.

3.3 Materials and Methods

3.3.1 Isolation and expansion of hWJ-MSCs

Umbilical cords (n=2) with mother's informed consent were collected and preserved immediately after full-term delivery of caesarian or normal labor at Maharat Hospital, Nakhon Ratchasima. This work has been approved by Ethics Committee for Researches Involving Haman Subjects, Suranaree University of Technology and Maharat Nakorn Ratchasima Hospital Institutional Review Board (MNRH IRB). Umbilical cords were stored in 4°C sterile phosphate buffered saline without calcium and magnesium (PBS). Then, umbilical cords were transferred to Embryo Technology and Stem Cell Research Center (ESRC), Suranaree University of Technology. Isolation of hWJ-MSCs was carried out using tissue explants method as previously described by Petsa *et al*. (2009) with some modifications. Briefly, the umbilical cords were immersed in 75% ethanol for 30 seconds. Then, the umbilical cord were washed in sterile PBS and cut into 2 cm length pieces. Each piece of umbilical cord was cut open lengthwise and gelatinous (Wharton's jelly) tissue

surrounding the vessels were excised and diced into small fragments (about 3x3 mm). Next, WJ tissues were plated onto 6-well tissue culture plate (TPP, *Trasadingen*, Switzerland) and left for 1-2 minutes at room temperature. The tissues were then carefully covered with 1 ml of growth medium comprised of alpha modification of Eagle's medium (α-MEM; Sigma-Aldrich, USA) supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin (Invitrogen, *California, USA*) and 10% of one of the following sera: fetal bovine serum (FBS; Hyclone, Utah, USA) and allogenic human cord blood serum (hCBS; In house made). The samples were then incubated at 37ºC in a humidified atmosphere of 5% CO_2 in air for 7-10 days. Mediums were replaced every 2 days and when the visible colonies of hWJ-MSCs were observed, the tissue explants were removed. When the cells reaching approximately 70% confluence, cells were harvested by synthetic enzyme, TrypLE™ Express (Gibco®, Invitrogen) and transferred to T25 flask (SPL life sciences, Gyeonggi-do, Korea), seeding at a density of 4,000 cells/cm^2. The cells were expanded until passage 3 (P3), then either experimented directly or cryopreserved with 10% Dimethyl sulfoxide (DMSO; Sigma-Aldrich) and stored in liquid nitrogen.

3.3.2 Preparation of allogenic human umbilical cord blood serum

Human umbilical cord bloods used in this study were screened from Department of Obstetrics and Gynaecology of Maharat Hospital, Nakhon Ratchasima, for the free of serious blood-borne disease (HIV and Hepatitis B) and sexually transmitted infectious disease (Syphilis). For the isolation of hCBS, human umbillical cord blood were collected from the umbilical vein after full-term delivery of caesarian labor, at Maharat Hospital, Nakhon Ratchasima, and kept in a sterile 50-mL conical

tube (Biologix, Kansas, USA) without any anticoagulant preservation. Cord blood sera were isolated by centrifugation at 1,700×g for 30 minutes at room temperature. Then, hCBS were gently collected and transferred to a new sterile 50-mL conical tube. Next, hCBS were heat inactivated by placing in a 56°C water bath for 30 minutes. Afterward, hCBS were quickly cooled down and filtered through 0.45 and 0.2 µm sterile membrane filter, consequently (Supor® 200 PES Membrane Disc Filter; Pall corporation, Michigan, USA). Sterile hCBS were used directly for further investigation or stored at -20 °C.

3.3.3 Characterization of hWJ-MSCs by standard methods

3.3.3.1 Population doubling time of hWJ-MSCs

In order to examine the growth kinetic of hWJ-MSCs, passage 4 of hWJ-MSCs were seeded at a density of 4,000 cells/cm^2 in 35 mm diameter tissue culture dish (SPL life sciences). Cells were cultured under their originated condition for 72 hours. Later, cells from each group were harvested, stained with 0.4% trypan blue (Sigma), counted and replated at the same density. Each condition was performed in triplicate and cells were cultivated over seven passages (P4-P10). Mean values of cell counts were calculated. Then, population doubling (PD) and population doubling time (PDt) were determined by the following formulation (Nekanti et al., 2010). NI is inoculums cell number. NH is harvest cell number and t is time (in hours).

$$PD = (\lg NH - \lg NI)/ \lg 2$$

$$PDt = t*\lg 2/ (\lg NH - \lg NI)$$

3.3.3.2 Colony forming unit-fibroblast (CFU-F) assay.

Pssage 4 of hWJ-MSCs were seeded at a density of 10 cells/cm^2 in 60 mm diameter tissue culture dishes (SPL life sciences). Cells were cultured under their originated system for 14 days. Mediums were changed every 2 days. After 14 days post-seeding, the cells were fixed in 4% paraformaldehyde (PFA, Sigma) for 20 minutes. Fixed cell were washed 3 times with PBS followed by 0.5% crystal violet (Sigma) staining. After 30 minutes of staining, cells were washed 3 more times with PBS. Then, stained cells were observed under inverted microscope (Olympus, model BH2-RFL-T3) at 40 magnifications. An aggregate of ≥ 50 cells were counted as a colony. Numbers of the colony were then calculated by the following equation. Each condition was performed in triplicate.

$$\%\text{CFU-F} = \frac{\text{Total number of colony}}{\text{Initial cells seeded}} \times 100$$

3.3.3.3 Immunophenotype analysis

Passage 4 of hWJ-MSCs were plated onto 4-well tissue culture dish (Nunc, Roskilde, *Denmark*) and cultured under their originate condition until reaching 70% confluence. The cells were then washed with PBS for 3 times and fixed by 4% PFA (Sigma) at room temperature for 15 minutes. The fixed cell were then washed 3 more times with PBS and incubated with blocking buffer consisting of 10% normal goat serum at room temperature for 2 hours. After that, the cells were incubated at 4°C overnight with primary antibodies raised against CD45, CD73, CD90 and CD105 (all from *Santa Cruz* Biotechnology, *California, USA*). Next, the samples were washed 6 times with PBS and incubated with secondary antibody, Alexa fluor®

488 goat anti rabbit IgG (1:1000; Invitrogen), for 2 hours at room temperature. After 6 times washing with PBS, cells were then counterstained with 1 mg/ml 40, 6-diamino-2-phenylindole (DAPI; Sigma-Aldrich) for 10 minutes before 2 times washed with PBS. Finally, the cells were observed and photographed under a fluorescent microscope (Olympus, model BH2-RFL-T3).

3.3.3.4 Multipotency analysis

In order to test multipotency, isolated cells from each condition were induced to differentiate into mesodermal lineages which are osteoblasts, adipocytes and chondrocytes.

i) Osteogenic differentiation

hWJ-MSCs at the density of 2×10^4 cells/cm^2 were plated onto 0.1% gelatin (Sigma) coated 35 mm diameter tissue culture dish (SPL life sciences) and cultured in their originated condition until reaching 80% confluence. Next, cells were induced under osteogenic differentiation by cultivated in growth medium with reduced 5% serum content, supplemented with 100 nM dexamethasone, 0.2 mM L-ascorbate-2-phosphate, 2 mM L-glutamine, 10 mM β-glycerophosphate, 100 units/ml penicillin and 100 µg/ml streptomycin (all from Sigma-Aldrich). Medium were subsequently changed every 2 days for 3 weeks. After that, cells were fixed in 4% PFA (Sigma) for 30 minutes and washed 3 times by deionized (DI) water. Calcium depositions of the cells were visualized by Alizarin Red S (Sigma) staining for 30 minutes. Then, cells were washed with DI water until their backgrounds were cleared. Finally, stained cells were observed under inverted microscope (Olympus, model BH2-RFL-T3).

ii) Adipogenic differentiation

Cells were prepared as described for osteogenic differentiation until reaching 80% confluence. Afterwards, the medium contains 5% serum content and was supplemented with 10 µg/ml insulin, 100 µM indomethacin, 1 µM dexamethasone, 0.5 mM isobutyl methylxanthine (IBMX), 100 units/ml penicillin and 100 µg/ml streptomycin. Medium were subsequently replaced every 2 days for 3 weeks. Then, cells were fixed in 4% PFA (Sigma) for 30 minutes and washed 3 times by PBS. The evidence of fat globules formation was confirmed by Oil Red O (Sigma) staining for 30 minutes. Then, cells were washed 3 times by PBS and stained cells were observed under inverted microscope (Olympus, model BH2-RFL-T3).

iii) Chondrogenic differentiation

To induce chondrogenic differentiation, the cells were prepared as described for osteogenic differentiation until reaching 80% confluence. Then, the medium contains 5% serum content and was supplemented with 0.05 mM ascorbate 2-phosphate, 40 µg/ml L-proline, 1 mM sodium pyruvate, 100 nM dexamethasone, 100 units/ml penicillin and 100 µg/ml streptomycin (all from Sigma-Aldrich). Additionally, 10 ng/ml TGF-β3 (R&D Systems, Minnesota, USA) and 1% ITS-X (Gibco®, Invitrogen) were also supplemented. Medium were replaced every 2 days for 3 weeks. After that, cells were fixed with 4% PFA (Sigma) for 30 minutes and washed 3 times by PBS. In order to detecte extracellular matrix produced by chondrocyte, cells were stained by Alcian blue 8GX (Sigma) for 30 minutes. Then, cells were washed 3 times by PBS and stained cells were observed under inverted microscope (Olympus, model BH2-RFL-T3).

3.3.3.5 Karyotype analysis

Karyotyping of hWJ-MSCs were carried out using a standard Giemsa staining procedure (Bayani and Squire, 2004) with slightly modification. Briefly, early (P2) and late (P12) passages of hWJ-MSCs were cultured in T25 flask under their originated condition. After the cells reaching at about 80% confluence, cells were directly added with 0.1µg/ml colcemid solution (Gibco®, Invitrogen) and cultured for another 2 hours. Then, cells were harvested, transferred to a 15-ml conical tube (Biologix, Kansas, USA) and centrifuged at 300×g for 5 minutes. After that, supernatant were discarded and cell pellet were treated with 10 ml pre-warmed hypotonic solution, 0.075 M KCl, for 15 minutes at 37°C. Cells were then pre-fixed by adding with 3 ml cold methanol/acetic acid (3:1). Next, the samples were centrifuged at 300×g for 5 minutes and supernatant were discarded. For fixation, cell pellets were added with 10 ml cold methanol/acetic acid and then centrifuged at 300×g for 5 minutes. The fixation steps were repeated 2 times. After that, the samples were dropped on chilled glass slides, allowed to dry and stained with 5% KaryoMax® Giemsa Stain (Gibco®, Invitrogen) at room temperature for 5 minutes. Then, slides were washed with tap water and blotted dry using tissue paper. After that, slides were mounted with Permount (Sigma) and covered by cover glass. Finally, slides were examined under a light microscope. Images of the individual metaphase spreads were captured by Jenoptik digital camera and karyotyped using the Ikaros software (Metasystem, Carl Zeiss, Germany). Each sample were examined at least 20 metaphase spread.

3.3.4 Characterization of hWJ-MSCs by FT-IR microspectroscopy

3.3.4.1 FT-IR microspectroscopy of hWJ-MSCs

Passage 2 of hWJ-MSCs cultured under the conventional xeno (FBS) and the xeno-free (hCBS) system were analyzed by FT-IR microspectroscopy. Briefly, at 80% confluence, cells were washed 3 times by PBS and harvested by enzymatic treatment. Then, cell pellets were washed thoroughly by resuspended in 0.9% NaCl for 3 times. After that, 5 µl of cell suspension were dropped onto Low-e slides (Kevley Technologies, Chesterland, OH, USA) then vacuum dried in a desiccator. Next, cells on the slide were rinsed with deionized (DI) water 2-3 times and dried in the desiccator prior to analyze. The experiment was performed in triplicate.

FT-IR analysis was carried out at the Synchrotron Light Research Institute (Public Organization), Thailand, using a Bruker Hyperion 2000 microscope (Bruker Optik GmbH, Ettlingen, Germany) with a globar source of IR radiation. This system was equipped with a nitrogen cooled MCT (HgCdTe) detector with a 36 × IR objective. FT-IR spectra were obtained from 68 µm × 68 µm aperture size, reflection mode, collecting 64 scans at spectral resolution of 6 cm^{-1} over a measurement range of 4,000-600 cm^{-1}. Background spectra were collected from sample-free-areas, every 10 spectra acquired. Spectral data sets were processed by OPUS 6.5 software (Bruker Optics Ltd, Ettlingen, Germany). High-quality FT-IR spectra, maximum absorbance between 0.5 and 0.8 absorbance units, were selected for further analysis.

3.3.4.2 Multivariate data analysis

Principle Component Analysis (PCA) was performed by Unscrambler software (version 9.7, CAMO Software AS, Oslo, Norway) for determination of a significant variation between data sets. Data manipulation processes included taking the second derivative using Savitzky-Golay algorithm with nine points smoothing, normalization with Extended Multiplicative Signal Correction (EMSC) and six principle components (PCs) analysis were performed.

Integrated peak areas were analyzed by OPUS 6.5 software. Unsupervised hierarchical cluster analysis (UHCA) of the FT-IR spectra was performed using Ward's algorithm, OPUS 6.5 software.

3.3.5 Statistical analysis

All experiments were repeated three times. Statistical analysis was performed using SPSS 12.0 (SPSS, Inc., USA). Data are represented as mean ± standard error. Statistical differences were calculated using independent paired *t*-test. Difference were considered significant at *$p < 0.05$ and statistically significant at **$p < 0.01$.

3.4 Results and discussion

Recently, advance in biotechnology have enabled stem cell to become feasible for regenerative medicine practice. Among different types of stem cell, MSCs are considered as an outstanding candidate because of their availability, home and engraft ability, immunosuppressive properties as well as ability to differentiate into various specific cell types (Brooke et al., 2007). As evidenced by a number of pre-

clinical studies, transplantation of MSCs resulted in therapeutic effects of many diseases (reviewed in García-Gómez et al., 2012). However, the use of fetal bovine serum (FBS) in conventional culture system raises concern about the therapeutic safety. Therefore, in order to provide a beneficial evidence for setting up of Good Manufacturing Practice (GMP) criteria used for isolation and expansion of clinical grade MSCs, we studied the use of xeno-free (hCBS) system for hWJ-MSCs derivation. Comparative analysis of hWJ-MSCs properties were investigated using standard criteria prescribed for identification of MSCs compared with results obtained using Fourier transform infrared (FT-IR) microspectroscopy.

3.4.1 Xeno-free (hCBS) system supported a superior expansion of hWJ-MSCs

Two umbilical cords were used in this study. Hence, four hWJ-MSCs cell lines were successfully established under conventional xeno (FBS; named hWJ-MSCs-F) and the xeno-free (hCBS; named hWJ-MSCs-hCBS) systems. At primary passage, outgrowths of cells were observed during 5-7 days of culture. As shown in Fig. 3.1 A and B, outgrowth cells derived from both systems had the fibroblast-like morphology. After harvested and continue to expanded in their originated condition, hWJ-MSCs-F and hWJ-MSCs-hCBS grew as monolayer of adherent fibroblast like cells (Fig. 3.2 C and D). In this study, there were no remarkable differences in cell morphology observed among different culture systems. hWJ-MSCs from both conditions displayed a typical fibroblast-like morphology.

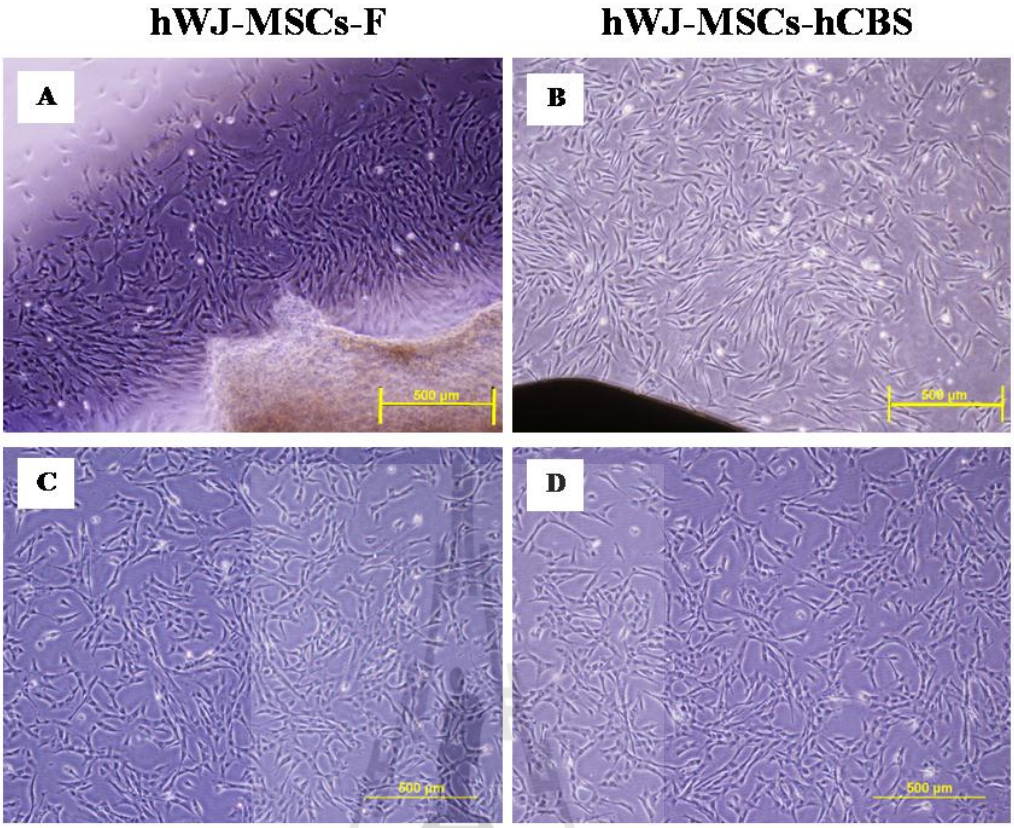


Figure 3.1 Morphology of hWJ-MSCs derived from conventional xeno (FBS) and xeno-free (hCBS) culture systems. Representative phase contrast images of hWJ-MSCs-F (A) and hWJ-MSCs-hCBS (B), at primary passage. hWJ-MSCs derived from both conditions were plastic adherent with a typical fibroblast-like morphology (C and D). Scale bar: 500 µm.

In order to compare the expansion potentials between hWJ-MSCs-F and hWJ-MSCs-hCBS, cumulative population doubling (CPD) and population doubling time (PDt) were determined over a period of seven passages as described (Hatlapatka et al., 2011). We found that hWJ-MSCs-hCBS exhibited a significantly higher CPD with shorter PDt at all passage tested (Fig 3.2). These were in consistent with previous reports that human allogenic cord blood serum contributed to the accelerated

expansion of hBM-MSCs as compared to FBS (Shetty et al., 2007) and allogenic human adult serum (Jung et al., 2009) cultivated cells. Moreover, Jung and colleagues also found that, these accelerated effects were observed only in hCBS culture system, but not cord blood plasma (CBP) at various concentrations tested (5-15%). Interestingly, these accelerated effects were reported in cord blood serum of all blood groups (A-, B-, O- and AB-CBS), irrespective of MSCs donors and blood groups matching (Ma et al., 2012).

3.4.2 Evaluation of hWJ-MSCs properties by standard methods

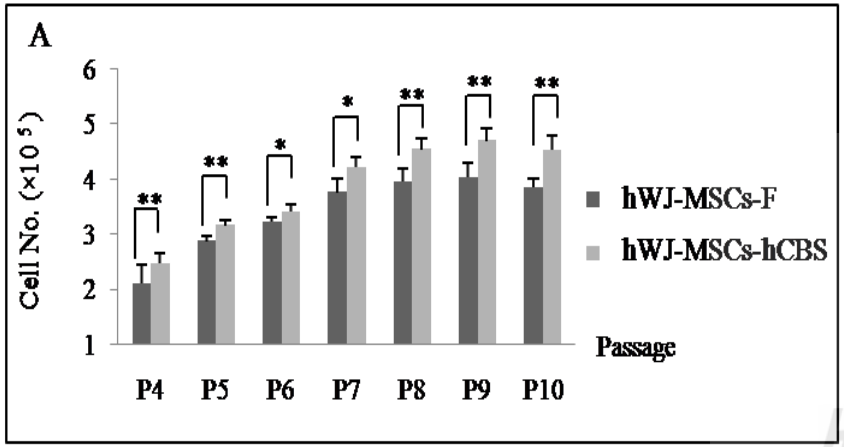
Based on the difference observed in growth kinetics, we next investigated whether differences in culture systems have any effect on hWJ-MSCs properties using standard criteria prescribed for identification of MSCs (Dominici et al., 2006).

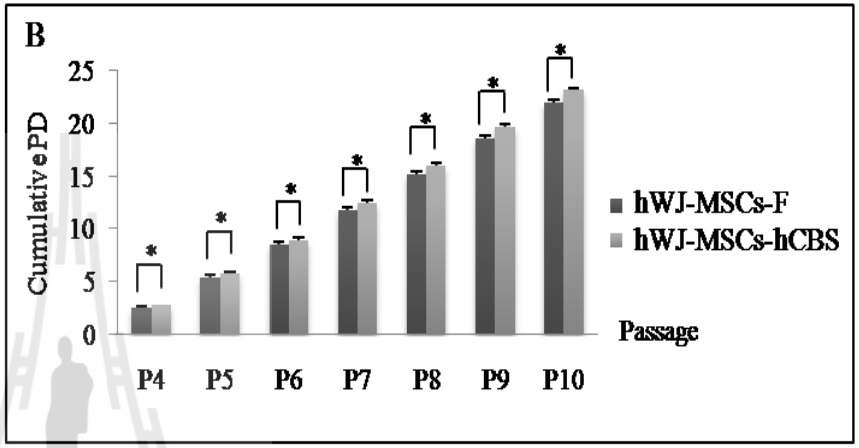
Regarding with cells clonality, hWJ-MSCs derived from both systems were evaluated by CFU-F assay and results were displayed as average percentage ± SEM. hWJ-MSCs-hCBS displayed a CFU-F potential of 20.39 ± 6.43%, whereas hWJ-MSCs-F performed 21.16 ± 4.67%. However, average percentages of CFU-F frequency were not statistical different ($p > 0.05$). For immunocytochemical staining, we found that hWJ-MSCs derived from both culture systems were positive for CD73, CD90 and CD105, but negative for CD45 (Fig 3.3). There were no differences observed in surface antigen expression pattern of MSCs. In addition, multipotency of hWJ-MSCs was evaluated by the induction of mesodermal lineage differentiation. After 21 days of induction, hWJ-MSCs derived from both conditions were able to differentiate toward osteoblasts, adipocytes, and chondrocytes, as evidenced by

calcium minerization, lipid droplets formation and extracellular matrix proteoglycan accumulation, respectively (Fig 3.4).

Therefore, there were no significant differences observed between hWJ-MSCs-F and hWJ-MSCs-hCBS analysis by standard characterization methods. The isolated cells displayed the similar CFU-F frequency, immunophenotype and mesodermal differentiation potentials. These findings were in conformity with previous reports that MSCs derived from difference culture conditions exhibited no differences in surface marker expressions and mesodermal differentiation potential (Bieback et al., 2009; Hartmann et al., 2010; Shetty et al., 2007; Venugopal et al., 2011). However, contradictory results showed that differences in culture conditions resulted in different osteogenic and adipogenic differentiation potential of MSCs (Jung et al., 2009; Lindroos et al., 2010; Tekkatte et al., 2012).

Furthermore, although not including in standard characterization criteria, genetic stability is an important characteristic of MSCs used for therapeutic purpose. To achieve this aim, we evaluated karyotype of hWJ-MSCs-F and hWJ-MSCs-hCBS at early (P2) and late (P12) passages. From karyotype analysis, hWJ-MSCs used in this study showed normal female (46, XX) and male (46, XY) karyograms, matched with the sex of the umbilical donors. We found that hWJ-MSCs-F and hWJ-MSCs-hCBS were able to maintained normal karyotype over several passages (Fig 3.5).





C

Population doubling time (hrs.)

Group	P4	P6	P8	P10
hWJ-MSCs-F	29.38 ± 2.65	23.25 ± 0.25	21.24 ± 0.53	21.46 ± 0.36
hWJ-MSCs-hCBS	26.59 ± 1.03	22.68 ± 0.43	20.06 ± 0.33	20.10 ± 0.49

Figure 3.2 Influence of difference culture systems on proliferation potentials of hWJ-MSCs. Comparison of total cells yield (A), Cumulative population doubling (B) and Population doubling time (C) of hWJ-MSCs-F and hWJ-MSCs-hCBS over several passages. Mean ± SEM from three experiments are shown. Statistical significance is indicated (independent paired *t*-test, *p<0.05, **p<0.01).

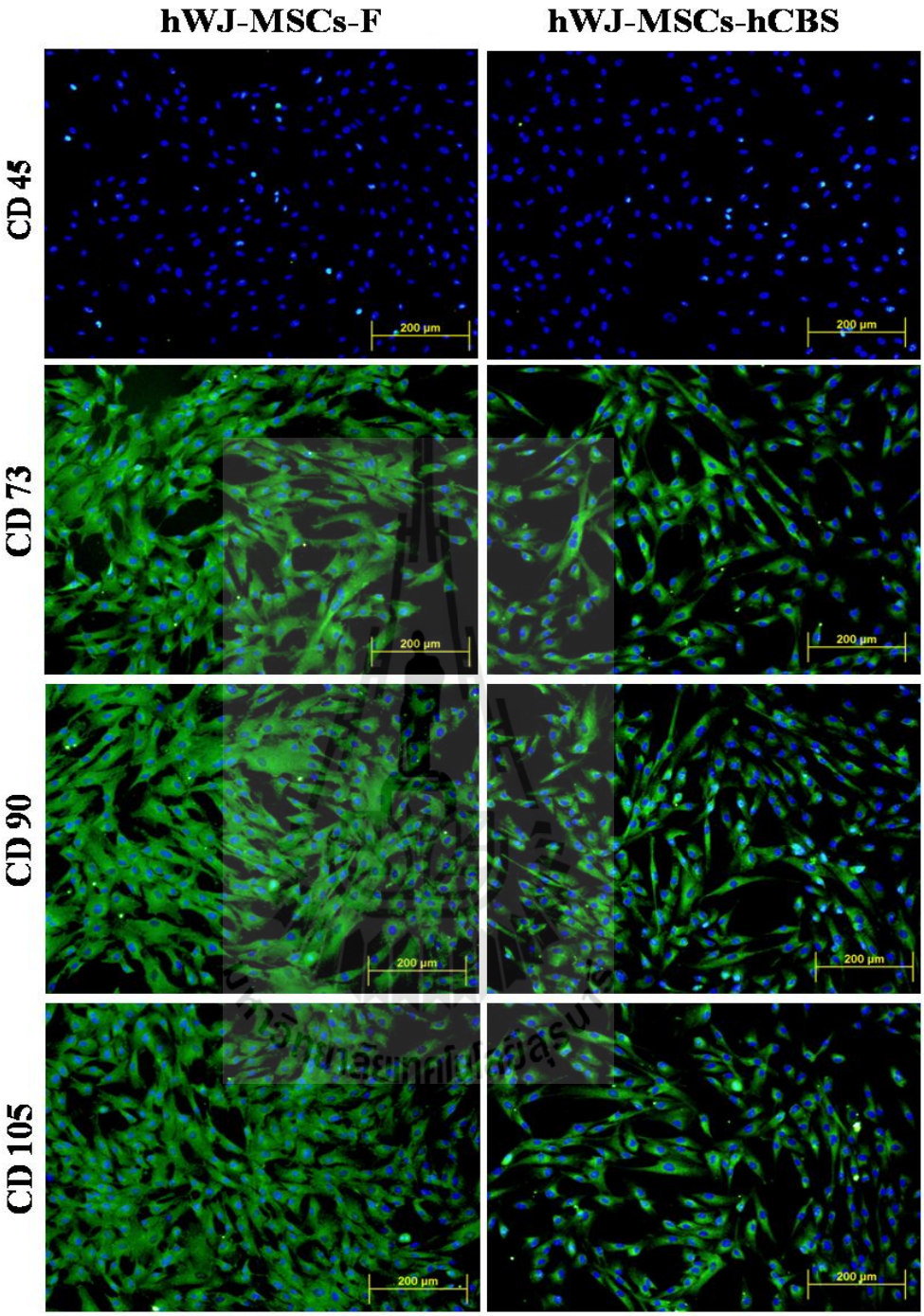


Figure 3.3 Immunocytochemical staining of surface antigen expression pattern. Representative images of immunophenotype of hWJ-MSCs-F and hWJ-MSCs-hCBS. Cells were positive for CD73, CD90 and CD 105 but negative for CD 45. Scale bar: 200 µm.

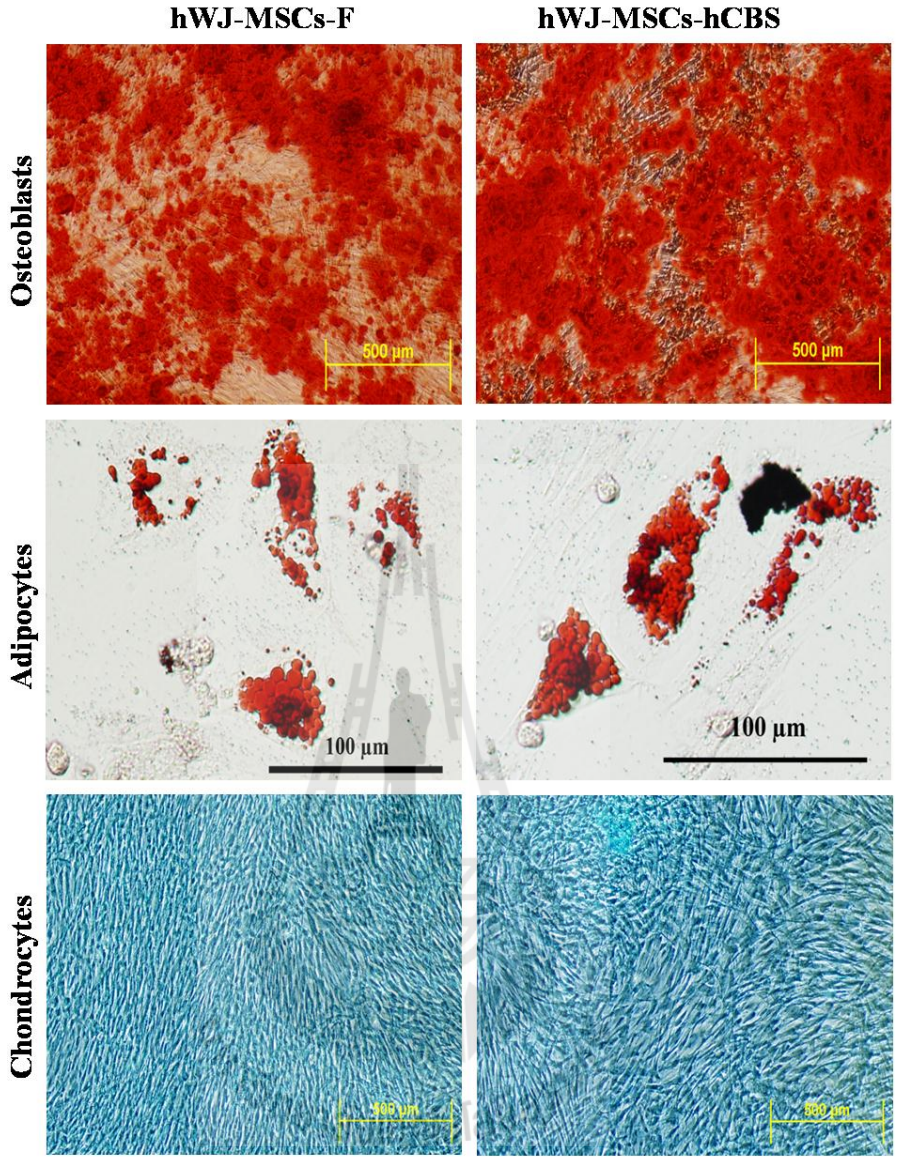


Figure 3.4 **Mesodermal lineage differentiation of hWJ-MSCs.** Passage 4 of hWJ-MSCs-F and hWJ-MSCs-hCBS were subjected to differentiate toward osteoblasts, adipocytes and chondrocytes. Representative images showed the detectable of calcium minerization (scale bar: 500 µm), lipid droplets formation (scale bar: 100 µm) and extracellular matrix proteoglycan accumulation (scale bar: 500 µm), stained with Alizarin Red, Oil red O and Alcian blue, respectively.

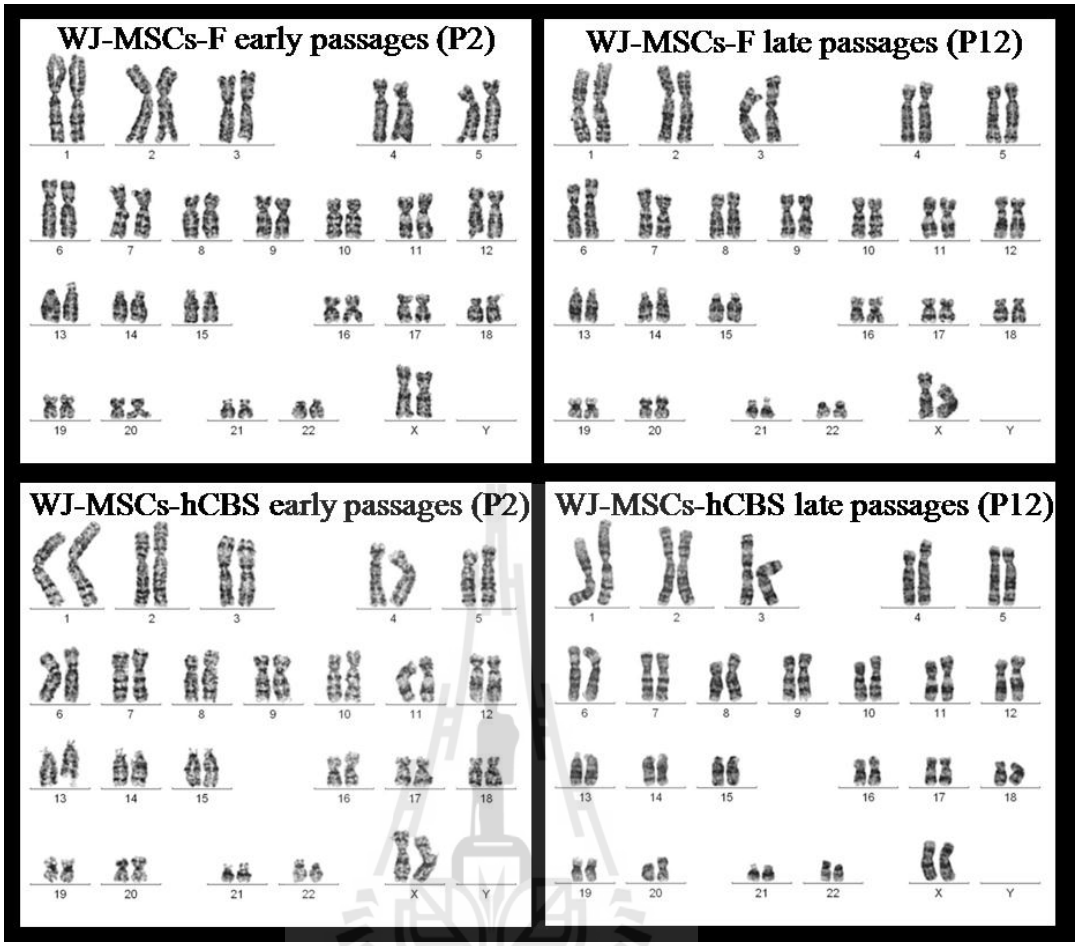


Figure 3.5 **Karyotype analysis**. Stability of karyotype were observed in hWJ-MSCs-F and hWJ-MSCs-hCBS. Representative images showed normal G-band karyotype (46, XX) of the same cell line at early (P2) and late (P12) passages.

3.4.3 Evaluation of hWJ-MSCs properties by FT-IR microspectroscopy

In order to verify the observable difference in proliferation potential at biomolecular level, hWJ-MSCs derived from both systems were analyzed by FT-IR microspectroscopy. In this study, a total of 433 FT-IR spectra from 3 replicate experiments of hWJ-MSCs-F (hWJ-MSCs-F1, 2 and 3) and hWJ-MSCs-hCBS (hWJ-

MSCs-hCBS1, 2 and 3) were analyzed. The average preprocessed FT-IR spectra were showed in Fig. 3.6 A. In order to enhance the differences between hWJ-MSCs-F and hWJ-MSCs-hCBS, a 2nd derivative analysis of the spectra was performed. Remarkable changed were observed in a range of 3,000-2800 cm^{-1} and 1,750-900 cm^{-1} (Fig. 3.6 B and C). We found that hWJ-MSCs-F revealed higher absorbance in lipid bands (C-H stretching of the CH_2 and CH_3 groups in the lipid acyl chain spectral region (3,000-2,800 cm^{-1})), lipid ester C=O stretching peak (1,743 cm^{-1}), and nucleic acids region (1,261-900 cm^{-1}) (Stuart, 2004) when compared with spectral from hWJ-MSCs-hCBS group. These findings were consistent with the previous report that culture conditions contributed to the profound alteration in fatty acid composition and gene expression profiles of cultivated cells. Change in lipid content of cultured cells has been proven to correlate with fatty acid profiles of supplemented serum, varying among species of animal of serum origin (Stoll and Spector, 1984). Moreover, a study of fatty acid composition of murine keratinocytes demonstrated that levels of linoleic acid (LA) were 4-fold decrease in 5 days cultured cells as compared to noncultured epidermis cells and whole epidermis (percentage ± SD of total fatty acids: 3.87 ± 0.69 for 5 days cultured cells, 15.78 ± 3.18 for noncultured epidermis cells, and 14.91 ± 1.43 for whole epidermis), suggesting the low level of this essential fatty acid in fetal calf serum (FCS) used in the cultured medium compared with normal mouse serum (MS) (percentage ± SD of total fatty acids: 4.61 ± 0.26 for FCS vs. 26.66 ± 2.07 for MS) (Isseroff et al., 1985). However, until now, there is no report of fatty acid composition of hCBS. Therefore, in order to clarify the missing information, a study of fatty acid composition of hCBS is necessary to be set up further.

Regarding to the absorbance differences observed in nucleic acids region (1,261-900 cm^{-1}), this finding may imply to the difference in nuclear metabolism between hWJ-MSCs-F and hWJ-MSCs-hCBS. This assumption is based on the previous report that difference cultured conditions resulted in difference transcriptome of adipose tissue derived MSCs (ASCs) (Bieback et al., 2010; Lindroos et al., 2010). Data from microarray analysis revealed that from a total of 34,039 genes, 90 genes (out of the 102 differentially expressed gene) involved in development and differentiation, extracellular matrix, adhesion and migration and signal transduction/cell signaling were highly upregulated (≥2-fold change) in ASCs cultured under conventional xeno (FBS) condition compared to those cultured in xeno-free (10% human serum and 10% thrombin activated platelet released plasma) system (Bieback et al., 2010).

It was also shown that the absorbance of β-sheet rich protein (Stuart, 2004), observed at 1,639 cm^{-1}, was higher in hWJ-MSCs-hCBS group than those of hWJ-MSCs-F group (Fig. 3.6 C). A possible explanation is that the superior proliferation potential might possibly have occurred as a result of the expression of functional proteins central to the cell cycle pathway. Supporting this assumption, results from comparative microarray analysis revealed that genes categorized in cell cycle pathway, such as *cell division cycle (CDC)* and *cyclins (CCNs) genes*, were overexpressed in ASCs cultured under allogenic human serum when compared with those cultured in FBS (Lindroos et al., 2010). In agreement with 2^{nd} derivative spectra, the results of mean integrated areas of average spectra confirmed that the difference observed in lipid (3,000-2,800 cm^{-1}), lipid ester (1,743 cm^{-1}), amide I (1,639 cm^{-1}), and

nucleic acids (1,261-900 cm^{-1}) region were significant difference (*p<0.05, **p<0.01) (Fig. 3.6 D).

To determine whether FT-IR spectroscopy could be used to discriminate between hWJ-MSCs-F and hWJ-MSCs-hCBS, Principal component analysis (PCA) was carried out to identify the majority of the variances within the spectral data sets that contribute to the clustering (Chan and Lieu, 2009). In this study, PCA was performed on the respective 2^{nd} derivative spectra following with EMSC of hWJ-MSCs-F and hWJ-MSCs-hCBS. When focused on the spectral range: 3,000-2,800 cm^{-1}, 1,750-1,670 cm^{-1}, and 1,645-850 cm^{-1}, spectra were clearly separated corresponding to their cultivated systems. As shown in PCA score plots, the spectra of hWJ-MSCs-F and hWJ-MSCs-hCBS could be discriminated along PC1, which explained 63% of total variance in the dataset (Fig. 3.7 A). In agreement with the difference of absorbance observed on 2^{nd} derivative spectra, PCA loading plots (Fig. 3.7 B) showed that peak positions that contributed most to the clustering were concordant with the spectral regions/peaks observed on 2^{nd} derivative spectra. hWJ-MSCs-F spectra can be distinguished from hWJ-MSCs-hCBS spectra by having negative PC1 scores (Fig. 3.7A). This can be explained by these spectra having highest positive value for PC1 loading (Fig. 3.7B) with variable of 1,663 cm^{-1}. The spectra from hWJ-MSCs-hCBS are clearly separated by positive correlation of PC1 score (Fig 3.7A), which have heavily load on negative PC1 (Fig. 3.7B) at 2,923 cm^{-1}, 2,852 cm^{-1}, 1,743 cm^{-1}, 1,467 cm^{-1}, 1,220 cm^{-1}, 1,085 cm^{-1}, and 966 cm^{-1}. These data indicated that the absorbance of β-sheet rich protein (1,633 cm^{-1}) of hWJ-MSCs-hCBS was higher than the spectra of hWJ-MSCs-F, whereas the absorbance of lipid (2,923 cm^{-1} and

2,852 cm^{-1}), lipid ester (1,743 cm^{-1}), and nucleic acids region (1,220 cm^{-1}, 1,085 cm^{-1}, and 966 cm^{-1}) of hWJ-MSCs-F were higher than the spectra of hWJ-MSCs-hCBS.

In addition, hierarchical cluster analysis was employed by Ward's algorithm using 2nd derivative and then vector normalized over the spectra region from 3,000-2,800 cm^{-1} and 1,800-900 cm^{-1}. As shown in dendrogram, spectrum from hWJ-MSCs-F and hWJ-MSCs-hCBS were clearly separated (Fig. 3.8).

3.5 Conclusion

In order to obtain the clinical grade MSCs, the use of xeno-free (hCBS) system is more permissive when compared with conventional xeno (FBS) system. Herein, this study demonstrated that type of serum supplement directly affected the properties of cultivated hWJ-MSCs. hWJ-MSCs-hCBS exhibited a superior expansion potential when compared with hWJ-MSCs-F. This result may imply that xeno-free culture system is suitable for the *ex vivo* expansion of human MSCs. Moreover, this study first demonstrated that FT-IR microspectroscopy coupled with PCA analysis, but not standard characterization methods, could discriminate the cells derived from different culture systems. A possible explanation is that the standard characterization techniques (CFU-F frequency, immunophenotype, and mesodermal differentiation potentials) are not sensitive and specific enough to clarify the difference between hWJ-MSCs-F and hWJ-MSCs-hCBS. By providing an insight on organic functional groups of biological molecule, FT-IR microspectroscopy revealed the difference of hWJ-MSCs-F and hWJ-MSCs-hCBS at biochemical levels. Spectral differences between hWJ-MSCs-F and hWJ-MSCs-hCBS are due to differences in the relative levels of lipid (3,000-2,800 cm^{-1}), lipid ester (1,743 cm^{-1}), amide I (1,639 cm^{-1}), and

nucleic acids (1,261-900 cm^{-1}) in those sample. Importantly, the complex biological feature, like a cell identity, of hWJ-MSCs could be obtained via a single measurement of an infrared absorption, without any requirement of complicated labeling process. Therefore, the data presented here showed that FT-IR microspectroscopy is an alternative outstanding technique for MSCs characterization. In order to translate this technique into routine setting, future work should be done on the establishment of spectral data base of hWJ-MSCs.

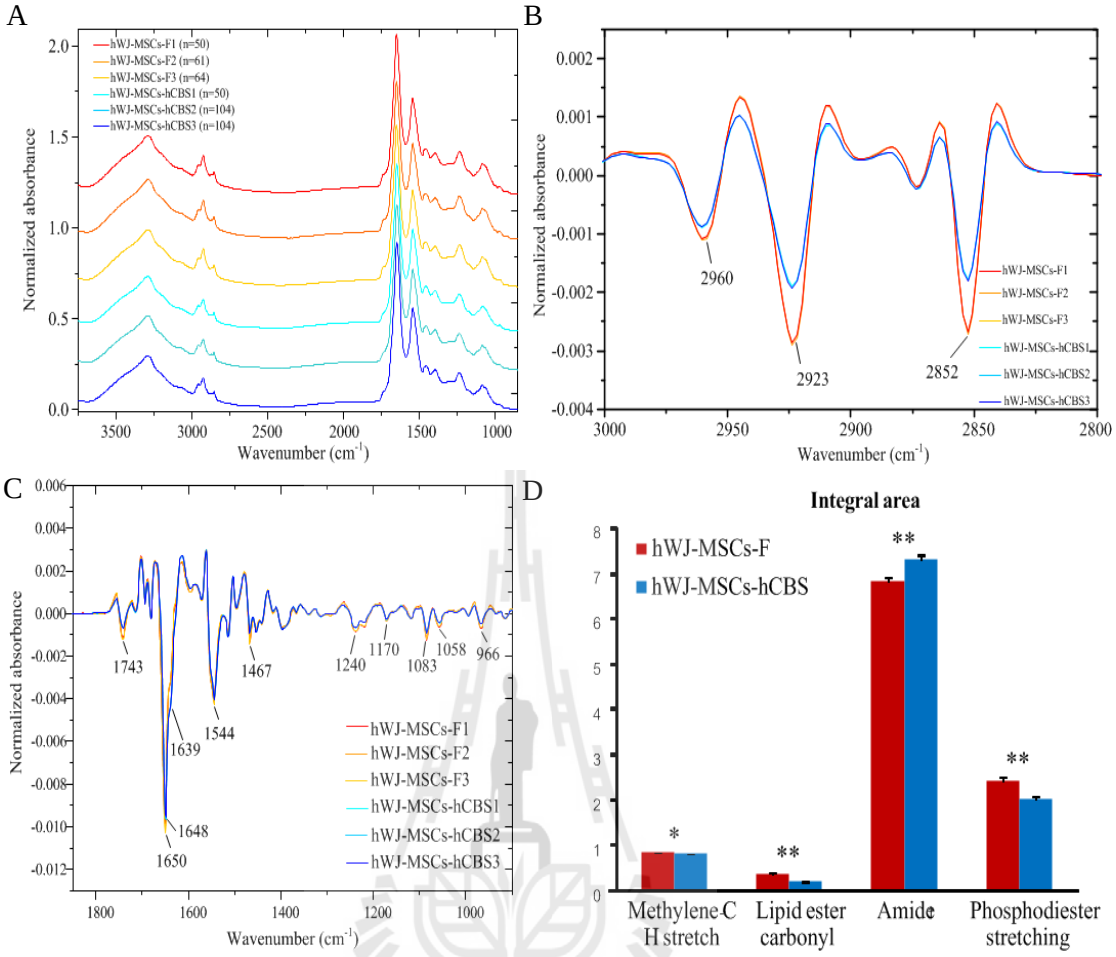


Figure 3.6 **hWJ-MSCs properties probed by FT-IR microspectroscopy.** Average original FT-IR spectra of hWJ-MSCs-F (hWJ-MSCs-F1, 2 and 3) and hWJ-MSCs-hCBS (hWJ-MSCs- hCBS1, 2 and 3) after combined and done vector normalization (A). Average 2nd derivative spectra were displayed over the range of 3,000-2,800 cm^{-1} (B) and 1,800-800cm^{-1} (C). Histogram of mean integrated areas calculated on lipid (methylene C-H stretch; 3,000-2,800 cm^{-1}), lipid ester (lipid ester carbonyl; 1,743 cm^{-1}), amide I (1,639 cm^{-1}), and nucleic acids (phosphodiester stretching; 1,261-900 cm^{-1}) region (D). Statistical significance is indicated (independent paired *t*-test, *p<0.05, **p<0.01).

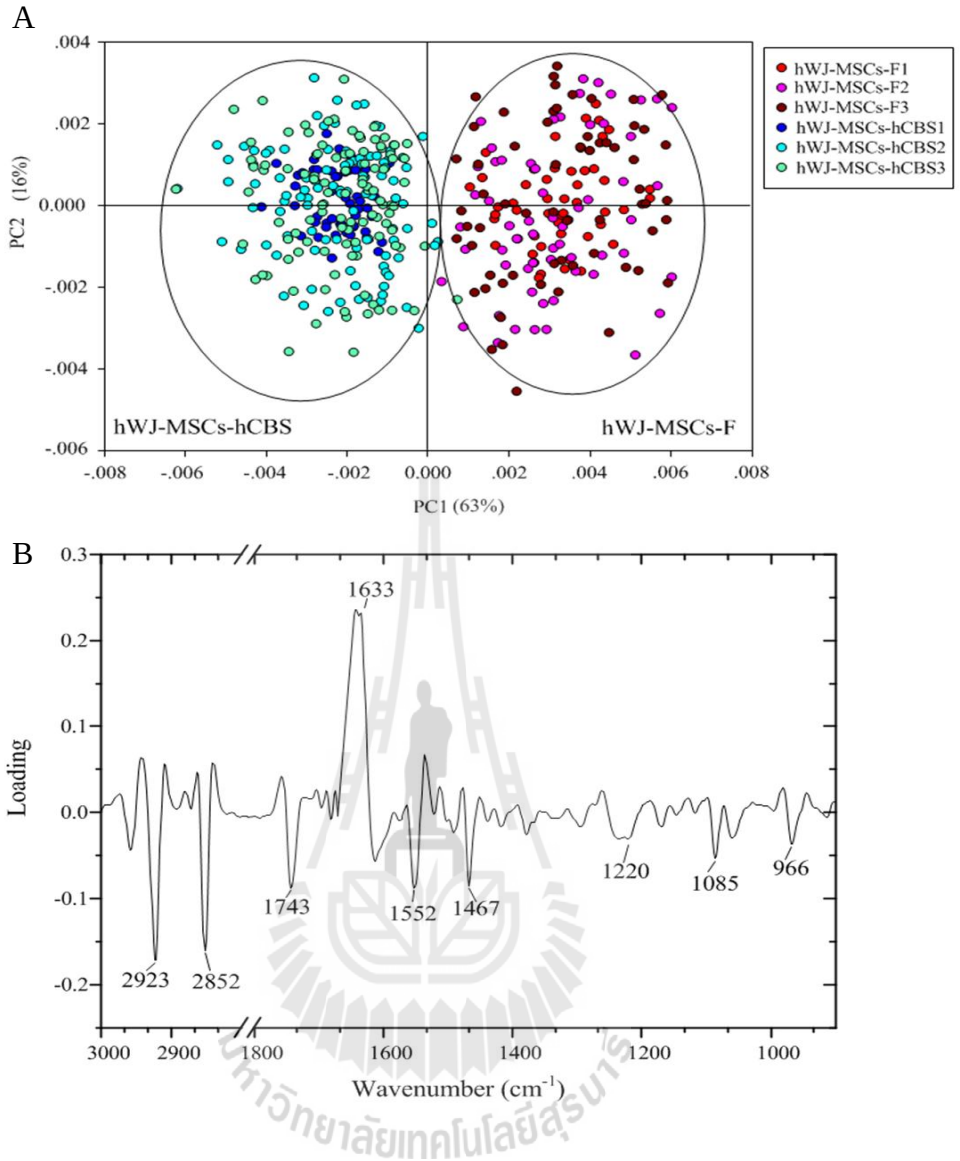


Figure 3.7 **PCA analysis.** PCA of hWJ-MSCs-F (hWJ-MSCs-F1, 2 and 3) and hWJ-MSCs-hCBS (hWJ-MSCs- hCBS1, 2 and 3). PCA score plots (A) and PC1 loading plots (B). Spectra used for PCA were performed 2nd derivative and EMSC at the region of 3,000-2,800 cm^{-1}, 1,750-1,670 cm^{-1}, and 1,645-850 cm^{-1}. The chemical composition of the two groups was classified with PC1 score plot which explained 63 % of the total variance.

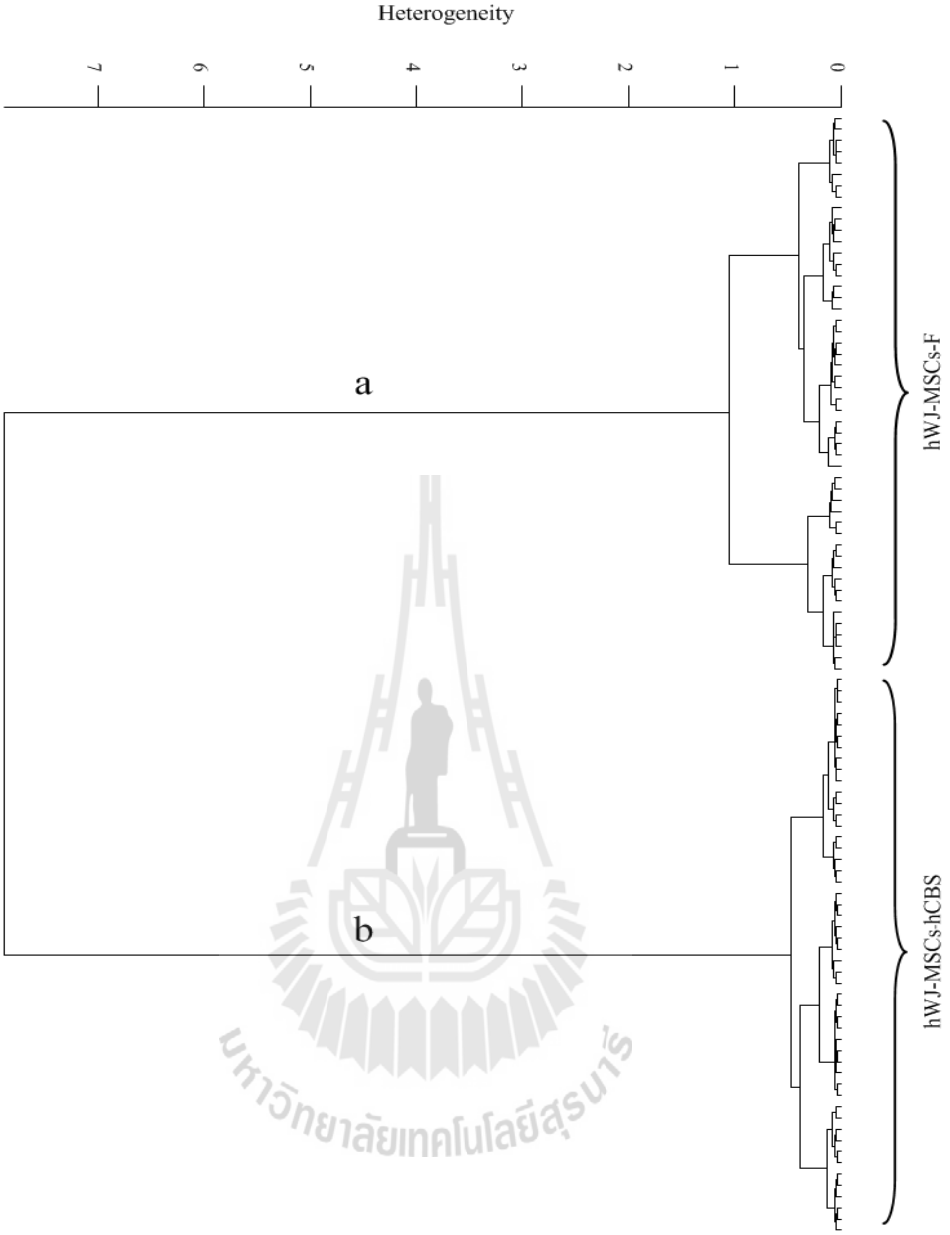


Figure 3.8 Hierarchical cluster analysis of the FT-IR spectra. Dendrogram obtained by cluster analysis of hWJ-MSCs-F and hWJ-MSCs-hCBS. Cluster analysis was employed by Ward's algorithm using second derivative and then vector normalized over the spectra region from 3,000-2,800 cm^{-1} and 1,800-900 cm^{-1}.

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BIOGRAPHY

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