การพัฒนาวิธีการใหม่ในการเพิ่มจำนวนเซลล์ต้นกำเนิดเมสเซนไคม์มอลจากวาร์ตันเจลลี่และการเปลี่ยนแปลงให้เป็นเซลล์ตับ

นางสาวพัชรี ปราศจาก

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาจุลชีววิทยา
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2555

DEVELOPMENT OF NEW METHODS FOR WHARTON'S JELLY MESENCHYMAL STEM CELLS EXPANSION AND HEPATOCYTE DIFFERENTIATION

Patcharee Prasajak

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Microbiology

Suranaree University of Technology

Academic Year 2012

DEVELOPMENT OF NEW METHODS FOR WHARTON'S JELLY MESENCHYMAL STEM CELLS EXPANSION AND HEPATOCYTE DIFFERENTIATION

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

Thesis Examining Committee

N. Muangsan
(Asst. Prof. Dr. Nooduan Muangsan)
Chairperson

(Asst. Prof. Dr. Wilairat Leeanansaksiri)
Member (Thesis Advisor)

Chavaboon Dechsukhum
(Dr. Chavaboon Dechsukhum)
Member

Tassanee Saovana
(Assoc. Prof. Dr. Tassanee Saovana)
Member

(Prof. Dr. Sittisak Honsawek)
Member

(Prof. Dr. Sukit Limpijumnong)
Vice Rector for Academic Affairs

P. Manyum
(Assoc. Prof. Dr. Prapun Manyum)
Dean of Institute of Science

พัชรี ปราศจาก : การพัฒนาวิธีการใหม่ในการเพิ่มจำนวนเซลล์ต้นกำเนิดเมสเซนไคม์มอลจากวาร์ตันเจลลี่และการเปลี่ยนแปลงให้เป็นเซลล์ตับ (DEVELOPMENT OF NEW METHODS FOR WHARTON'S JELLY MESENCHYMAL STEM CELLS EXPANSION AND HEPATOCYTE DIFFERENTIATION) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ทนพญ. ดร.วิไลรัตน์ ลี้อนันต์ศักดิ์ศิริ, 168 หน้า.

ปัจจุบันนี้เซลล์ต้นกำเนิดเมสเซนไคม์มอลของมนุษย์ที่แยกได้จากวาร์ตันเจลลี่ (WJ-MSCs) จัดว่าเป็นเครื่องมือที่มีประสิทธิภาพสำหรับการประยุกต์ใช้ในทางคลินิกเนื่องจากความสามารถอันหลากหลายของเซลล์ ได้แก่ ความสามารถในการเพิ่มจำนวนในห้องปฏิบัติการ ศักยภาพในการเปลี่ยนแปลงไปเป็นเซลล์ที่จำเพาะได้หลายชนิด คุณสมบัติในการปรับระบบภูมิคุ้มกัน รวมทั้งง่ายต่อการเก็บเซลล์จากแหล่งที่ไม่ติดปัญหาทางด้านจริยธรรม งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาพัฒนาวิธีการเพิ่มจำนวน WJ-MSCs และการพัฒนา WJ-MSCs ให้เป็นเซลล์ตับ การศึกษาการเพิ่มจำนวนเซลล์โดยทำการเลี้ยงเซลล์ในอาหารเลี้ยงเซลล์ 4 ชนิดดังนี้ ชนิดที่ 1 Dulbecco's modified Eagle's medium with 1.0 g/L glucose (DMEM-LG) + 10% FBS (ออกซิเจน 20% และคาร์บอนไดออกไซด์ 5%) ชนิดที่ 2 DMEM-LG + 10% FBS (ออกซิเจน 5% และคาร์บอนไดออกไซด์ 5%) ชนิดที่ 3 Embryonic stem cells conditioned medium (ESCM) และชนิดที่ 4 ESCM + 10 ng/mL EGF โดยชนิดที่ 3 และ 4 เลี้ยงในสภาวะที่มีออกซิเจน 5% และคาร์บอนไดออกไซด์ 5% จากการทดลองพบว่าอาหารเลี้ยงเซลล์ชนิดที่ 1 เซลล์มีการแบ่งตัวน้อยมากและแก่เร็ว ในขณะที่การเลี้ยงเซลล์ชนิดที่ 2 3 และ 4 สามารถเพิ่มจำนวนเซลล์ได้ดีมากอย่างมีนัยสำคัญ โดยสามารถเพิ่มได้ 113.77 $\pm$ 7.89 204.66 $\pm$ 10.39 และ 424.88 $\pm$ 14.62 เท่า ในวันที่ 12 ตามลำดับ จากผลการทดลองนี้แสดงให้เห็นว่า สภาวะปริมาณออกซิเจนต่ำส่งเสริมการเพิ่มจำนวนของเซลล์ได้ดีกว่าสภาวะที่มีออกซิเจนปกติ และการเลี้ยงเซลล์ชนิดที่ 4 ให้ผลการเพิ่มจำนวนของเซลล์ดีที่สุด นอกจากนี้พบว่าเซลล์ที่ถูกเลี้ยงด้วยอาหารชนิดที่ 3 และ 4 ใช้เวลาในการแบ่งตัวโดยเฉลี่ยเร็วกว่าที่ถูกเลี้ยงด้วยชนิดที่ 2 ผลการทดลองเหล่านี้บ่งชี้ว่าอาหารเลี้ยงเซลล์ ESCM ทั้งที่มีหรือไม่มี EGF เป็นส่วนประกอบมีศักยภาพในการเพิ่มจำนวน WJ-MSCs ได้ดีกว่าอาหารเลี้ยงเซลล์ทั่วไป (DMEM-LG + 10% FBS) สำหรับการศึกษาคุณลักษณะของเซลล์พบว่า เซลล์ที่ถูกเลี้ยงด้วยอาหารชนิดที่ 2 3 และ 4 สามารถรักษาคุณสมบัติของความเป็นเซลล์ต้นกำเนิดเมสเซนไคม์มอลได้เป็นอย่างดีทั้งในด้านของรูปร่างเซลล์ การแสดงออกของโปรตีนบนผิวเซลล์ (CD_{29}^{+} CD_{44}^{+} CD_{90}^{+} CD_{34}^{-} CD_{45}^{-}) ความสามารถในการเปลี่ยนแปลงไปเป็นเซลล์ที่จำเพาะ (เซลล์กระดูกแข็ง เซลล์กระดูกอ่อน และเซลล์ไขมัน) และการแสดงออกของยีนที่บ่งชี้ความเป็นเซลล์ต้นกำเนิด อย่างไรก็

ตาม เป็นที่น่าสังเกตว่าเซลล์ที่ถูกเลี้ยงด้วยอาหารเลี้ยงเซลล์ชนิดที่ 3 และ 4 สามารถรักษาระดับการแสดงออกของยีนที่บ่งชี้ความเป็นเซลล์ต้นกำเนิดคือ *Oct-4* และ *Nanog* ได้ดีกว่าชนิดที่ 2 นอกจากนี้ WJ-MSCs ที่ถูกเลี้ยงด้วยอาหารชนิดที่ 2 3 และ 4 มีความสามารถในการเปลี่ยนแปลงไปเป็นเซลล์ตับหรือ hepatocyte-like cells ได้อีก ซึ่งพบหลักฐานจากการเปลี่ยนแปลงรูปร่างเซลล์ รวมทั้งการแสดงออกของยีนและโปรตีนที่บ่งชี้ความเป็นเซลล์ตับ ความสามารถในการทำหน้าที่ของเซลล์ตับ เช่น การเก็บสะสมไกลโคเจน การนำโลว์เดนซิตี้ไลโปโปรตีนเข้าเซลล์ การหลั่งอัลบูมินและการสร้างยูเรีย เป็นที่น่าสนใจว่าเซลล์ตับที่ได้จากการเหนี่ยวนำเซลล์ที่ถูกเลี้ยงด้วยอาหารชนิดที่ 3 และ 4 มีความสามารถในการทำหน้าที่ของเซลล์ตับได้ดีกว่าเซลล์ที่ถูกเลี้ยงด้วยอาหารชนิดที่ 2 จากผลการทดลองทั้งหมดนี้บ่งชี้ได้ว่า อาหารเลี้ยงเซลล์ ESCM ทั้งที่มีหรือไม่มี EGF เป็นส่วนประกอบไม่เพียงแต่มีคุณประโยชน์ในการใช้เลี้ยงเพื่อเพิ่มจำนวน WJ-MSCs ได้เป็นอย่างดี แต่ยังช่วยส่งเสริมให้เซลล์มีความสามารถเปลี่ยนแปลงไปเป็นเซลล์ตับได้อีกด้วย นอกจากนี้งานนี้ได้ทำการศึกษาผลของการใช้สารสกัดเอทานอลจากใบของ *Gynura procumbens* หรือแป๊ะตำปึงต่อความสามารถในการเปลี่ยนแปลงไปเป็นเซลล์ตับของ WJ-MSCs ผลการทดลองพบว่า สารสกัดนี้ช่วยส่งเสริมให้เซลล์มีความสามารถเปลี่ยนแปลงไปเป็นเซลล์ตับเมื่อกระตุ้นเซลล์ด้วยสารสกัดนี้ร่วมกับอาหารที่ใช้เหนี่ยวนำในช่วงแรกของการเหนี่ยวนำ โดยสรุปแล้วการศึกษานี้ได้แสดงให้เห็นแง่มุมใหม่อย่างน้อย 3 ข้อดังนี้ 1) ESCM เป็นแหล่งรวมของสารอาหารที่จำเป็นสำหรับการเลี้ยงและเพิ่มจำนวนของ WJ-MSCs โดยเฉพาะในช่วงระยะเวลาสั้นๆ ซึ่งจำเป็นต่อการนำไปใช้ทางคลินิก 2) WJ-MSCs เหมาะที่จะเป็นทางเลือกใหม่ในการนำมาเลี้ยงให้เป็นเซลล์ตับหรือ hepatocyte-like cells เพื่อนำไปประยุกต์ใช้รักษาโรคตับในอนาคต 3) สารสกัดจากใบของ *G. procumbens* ช่วยส่งเสริมให้ WJ-MSCs มีความสามารถเปลี่ยนแปลงไปเป็นเซลล์ตับได้ดียิ่งขึ้น

สาขาวิชาจุลชีววิทยา
ปีการศึกษา 2555

ลายมือชื่อนักศึกษา Patcharm
ลายมือชื่ออาจารย์ที่ปรึกษา
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม

PATCHAREE PRASAJAK : DEVELOPMENT OF NEW METHODS FOR WHARTON'S JELLY MESENCHYMAL STEM CELLS EXPANSION AND HEPATOCYTE DIFFERENTIATION. THESIS ADVISOR : ASST. PROF. WILAIRAT LEEANANSAKSIRI, Ph.D. 168 PP.

WHARTON'S JELLY-DERIVED MESENCHYMAL STEM CELLS /HEPATOCYTE-LIKE CELLS/*GYNURA PROCUMBENS*/LIVER DISEASES/EMBRYONIC STEM CELLS CONDITIONED MEDIUM

At present, WJ-MSCs are considered as promising tool for clinical application based on their several advantages. These include extensive *in vitro* expansion, multi-lineages differentiation potential, immunomodulatory properties including easy accessibility with little or no ethical concern. This study aimed to investigate appropriate culture conditions for *in vitro* expansion of WJ-MSCs including development of hepatic lineage differentiation in WJ-MSCs. Here, we cultured the cells in various conditions as followed, C1 (Dulbecco's modified Eagle's medium with 1.0 g/L glucose (DMEM-LG) + 10% FBS under 20% O_2 and 5% CO_2), C2 (DMEM-LG + 10% FBS under 5% O_2 and 5% CO_2), C3 (Embryonic stem cells conditioned medium (ESCM)), and C4 (ESCM + 10 ng/mL EGF). Cells in C3 and C4 were cultured under 5% O_2 and 5% CO_2. Our results revealed that cells in C1 had low proliferation and fast senescense. On the contrary, cells cultured in C2, C3, and C4 yielded significant proliferations as 113.77 ± 7.89, 204.66 ± 10.39, and 424.88 ± 14.62 folds at day 12, respectively. These findings indicate that C4 is the best culture condition and hypoxic condition is more suitable for *in vitro* WJ-MSC expansion than normoxic condition. Furthermore, we observed that the expanded cells from C3 and C4 had faster mean population doubling time (PDT) than that from C2. These results demonstrate that ESCM with or without additional EGF provided greater potential of WJ-MSCs expansion than conventional medium

(DMEM-LG + 10% FBS). The expanded cells from C2-4 could preserve common characteristics of MSCs including cells morphology, cell surface marker expressions (CD_{29}^{+}, CD_{44}^{+}, CD_{90}^{+}, CD_{34}^{-}, CD_{45}^{-}), differentiation potential (osteoblasts, chondroblasts, adipocytes), and stemness marker expressions. However, it was remarkable that the expanded cells from C3 and C4 could maintain the expression of stemness gene markers, *Oct-4* and *Nanog*, more than that from C2. Moreover, the expanded cells from C2-4 had ability to differentiate into hepatic lineage or hepatocyte-like cells (MSCDHC) which had evidenced by morphological changing including hepatic-specific gene and protein expressions. The MSCDHC cells contain hepatic functions, such as, glycogen storage, LDL uptake, albumin secretion, and urea production. Interestingly, capacity of hepatocyte differentiation of cells from C3 and C4 is higher than that from C2. Taken together, ESCM with or without additional EGF is not only appropriation for *in vitro* WJ-MSCs expansion but also promoting WJ-MSCs differentiation into hepatic lineage. Additionally, this study revealed that ethanolic leaves extract of *Gynura procumbens* or paetumpung could accelerate MSCDHC production. This study, thus, opens a new insight at least three points. Firstly, ESCM serves as a rich source of several factors required for supportive WJ-MSCs expansion, especially in a short period of time which is crucial for clinical use. Secondly, WJ-MSCs can serve as an alternative source of hepatocyte-like cells generation which can be applied for treatment of liver diseases in the future. Thirdly, *G. procumbens* leaves extract provides a benefit in supportive hepatogenic differentiation capacity of WJ-MSCs.

School of Microbiology

Academic Year 2012

Student's Signature Patchara

Advisor's Signature

Co-advisor's Signature Chavaboon Uthaipibull

ACKNOWLEDGEMENTS

This thesis would not be possible without the contribution, guidance and support of many individuals.

Firstly, I would like to express my sincerest gratitude and appreciation to my supervisor, Asst. Prof. Dr. Wilairat Leeanansaksiri, who has supported me throughout my thesis with her kindness and excellent guidance.

I would like to express my gratitude to my co-advisor, Dr. Chavaboon Dechsukhum, for his guidance and valuable suggestions.

I would like to thank Asst. Prof. Dr. Nooduan Muangsan, Chair of School of Microbiology/Biology, Suranaree University of Technology, for her generous and kind support.

I would like to thank the other members of my committee, Assoc. Prof. Dr. Tassanee Saovana and Prof. Dr. Sittisak Honsawek for their valuable guidances and suggestions.

I would like to thank all lecturers and staffs in the School of Microbiology/Biology, and The Center for Scientific and Technology Equipment at Suranaree University of Technology.

I would like to thank all physicians, nurses, and medical staffs from Por Pat Hospital, St. Mary Hospital, and Fort Suranaree Hospital for their kindnesses and warm assistances.

Many thanks also go to past and present members of the Stem Cell Therapy and Transplantation Research Group at Institute of Science, Suranaree University of Technology, especially Ms. Kamonnaree Chotinantakul, Ms. Piyaporn Rattananinsruang, and Ms. Duangnapa Dejjuy, for their assistance and warm friendship. This also to whom it may concern for their contribution to my studies during this work. All things I have received will be a long term memory with gratefulness.

Finally, I would like to thank my family including my parents, my aunts, my uncles, my grandmother and grandfather with most of my heart for their loving and great supporting throughout my study.

Patcharee Prasajak

CONTENTS

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

AFP	=	alpha-fetoprotein
ALB	=	albumin
ALT	=	alanine aminotransferase
AST	=	aspartate aminotransferase
AT-MSCs	=	adipose tissue-derived mesenchymal stem cells
bFGF	=	basic fibroblast growth factor
BM	=	bone marrow
BM-MSCs	=	basic fibroblast growth factor
BMP	=	bone morphogenic protein
BSA	=	bovine serum albumin
ºC	=	degree Celsius
C5/C5a	=	complement component 5/5a
CCL	=	chemokine (C-C motif) ligand
CCl_4	=	carbon tetrachloride
cDNA	=	complementary DNA
CFU	=	colony-forming unit
CK-18	=	cytokeratin 18
CO_2	=	carbon dioxide
CXCL	=	chemokine (C-X-C motif) ligand
D	=	day

LIST OF ABBREVIATIONS (Continued)

DAPI	=	4,6-diamidino-2-phenylindole
Dil-Ac-LDL	=	1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-acetylated-low density lipoprotein
DMEM	=	Dulbecco's modified Eagle's medium
DP-MSCs	=	dental pulp-derived mesenchymal stem cells
EDTA	=	ethylenediaminetetraacetic acid
EGF	=	epidermal growth factor
ELISA	=	enzyme-linked immunosorbent assay
ES	=	embryonic stem cells
ESCM	=	embryonic stem cells conditioned medium
FBS	=	fetal bovine serum
FGF	=	fibroblast growth factor
g	=	gram
G-CSF	=	granulocyte colony-stimulating growth factor
GM-CSF	=	granulocyte-macrophage colony-stimulating growth factor
GROα	=	growth-related oncogene alpha
h	=	hour
HDGF	=	hepatoma-derived growth factor
HepG2	=	hepatocellular carcinoma cell line
HGF	=	hepatocyte growth factor

LIST OF ABBREVIATIONS (Continued)

HLA-G5	=	soluble human leukocyte antigen G
IDO	=	indoleamine 2,3-dioxygenase
IGF-1	=	insulin-like growth factor 1
IL	=	interleukin
iNOS	=	inductible nitric oxide synthase
ITS	=	insulin, transferrin and selenium
KO-DMEM	=	knockout-DMEM
LDL	=	low-density lipoprotein
LIF	=	leukemia inhibitory factor
M-CSF	=	macrophage colony-stimulating factor
MCP-1	=	monocyte chemoattractant protein 1
MHC	=	major histocompatibility complex
MIF	=	migration inhibitory factor
MMP	=	matrix metalloproteinase
mRNA	=	messenger RNA
MSCs	=	mesenchymal stem cells
NH_4Cl	=	ammonium chloride
NOD-SCID	=	non-obese diabetic severe combined immunodeficiency
O_2	=	oxygen
OSM	=	oncostatin M
PBS	=	phosphate buffer saline
PD-MSCs	=	placenta-derived mesenchymal stem cells

LIST OF ABBREVIATIONS (Continued)

PDT	=	mean population doubling time
PGE_2	=	secreted prostaglandin E2
PIGF	=	placental growth factor
RNA	=	ribonucleic acid
RT-PCR	=	reverse transcription polymerase chain reaction
SCF	=	stem cell factor
SDF-1	=	stromal derived factor-1
Serpin E1	=	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
sICAM-1	=	soluble intercellular adhesion molecules 1
TGF-β	=	transforming growth factor-beta
TIMP-2	=	tissue inhibitor of metalloproteinase-2
Treg	=	regulatory T cells
VCAM-1	=	vascular cell adhesion molecule-1
VEGF	=	vascular endothelial growth factor
WJ-MSCs	=	Wharton's jelly-derived mesenchymal stem cells
Wnt	=	Wingles

CHAPTER I

INTRODUCTION

1.1 Introduction

The liver is one of vital organs of the body that performs many essential functions such as producing bile for lipid digestion, generating plasma proteins and metabolic enzymes, detoxifying toxic substances, storing glycogen, and regulating blood clotting system. Therefore, numerous effects on the liver can cause the loss of liver functions that lead to liver failure and death. Liver disease is a common term describing any diseases that cause liver inflammation, tissue damage, and affect liver functions. Causes may include by various factors such as viral infection, injury, exposure to drugs or toxic compounds, an autoimmune process, and a genetic defect. These causes can lead to hepatitis, cirrhosis, obstruction of bile ducts by stones blockages, fatty liver, and liver cancer (American Association for Clinical Chemistry, 2011). Liver disease is a serious problem and widely impacts the economy and public health worldwide. To date, orthotopic liver transplantation is proved to be an effective therapeutic choice for patients with end stage of liver disease (Wedro, 2011). Although the patients have benefited from liver transplantation, the shortage of donor organs is still a limitation of this treatment. Moreover, the recipient has at risk of side effects by taking immunosuppressive drugs that are required for transplantation. Because of these limitations, other alternative therapeutic approaches are needed. Recently, Mesenchymal stem cells (MSCs) are interested as stem cells-based therapy

in several diseases including liver disease. Since, MSCs possess several characteristics which make them variable applying in various clinical trials. These include self-renewal capacity, multi-lineages differentiation potential, homing and migration ability, immunomodulatory properties, and paracrine effect (Newman, Yoo, LeRoux, and Danilkovitch-Miagkova, 2009).

Mostly, bone marrow derived mesenchymal stem cells (BM-MSCs) are widely used for research and clinical application because they are extensively expanded *in vitro* and broadly differentiation capacity. However, numbers of isolated cells from bone marrow are rather low and their differentiation capacity depends on the age of donor (Hass, Kasper, Bohm, and Jacobs, 2011). Recently, umbilical cord Wharton's jelly has been revealed as an attractive source of MSCs. This tissue is derived from more primitive origin than other sources and enrichment with MSCs. Several studies found that Wharton's jelly derived mesenchymal stem cells (WJ-MSCs) have common characteristics of MSCs similar to those obtained from other sources as mentioned above (Baksh, Yao, and Tuan, 2007; Wang et al., 2004). Additionally, WJ-MSCs have multi-lineages differentiation potential into various cell types such as hepatocyte-like cells (Zhang, Lie, and Wei, 2009), cardiomyocyte (Pereira, Khushnooma, Madkaikar, and Ghosh, 2008), nerve cells (Peng, Wang, Zhang, Zhao, Zhao, Chen, Guo, Liu, Sui, Xu, and Lu, 2011), and insulin-producing cells (Chao, Chao, Fu, and Liu, 2008). Based on differentiation capacity of MSCs, generation of a new hepatocytes or hepatocyte-like cells for replacing the old damage cells is a one alternative choice to overcome the scarcity of donor livers. To date, several studies have successfully generated functional hepatocyte-like cells *in vitro.* In addition, MSCs-derived hepatocyte-like cells also could improve the liver function of

animal models both acute and chronic liver injury (Puglisi et al., 2011). These findings indicate that MSCs are promising cells for therapeutic purpose in liver disease.

In clinical use, however, large scale expansion of cells is necessary before transplantation. Thus, *in vitro* expansion is still needed to obtain an enough cells for therapeutic purpose. Normally, MSCs are difficult to expand and maintain stem cells properties in a culture under normoxic condition (20% oxygen). Interestingly, recent study has reported that MSCs could proliferate more rapidly at hypoxic condition or lower oxygen tension (Dos Santos et al., 2010). Thus, the influence of oxygen tension is importance for an *in vitro* expansion. Regarding culture medium, Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) has commonly used as a conventional medium for culturing MSCs. However, the risk of using FBS must be concerned for prion or xenogeneic proteins transmission in clinical use. Thus, new supplementary growth needs to be substituted the use of animal serum. Interestingly, xeno-free expansion medium for Embryonic stem cells (ES) showed four to five fold higher proliferation rate of MSCs than those cultured in conventional medium (Battula et al., 2007). However, the effect of embryonic stem cells conditioned medium (ESCM) on proliferation rate of MSCs has not been investigated yet. Actually, culture medium for ES cells is quite expensive and need to be changed and discarded every day for preserving their pluripotency capacity. Thus, a lot of ESCM will be discarded every day as a waste. Here, this is the first study that investigated whether ESCM can be reused as *in vitro* supportive culture medium for WJ-MSCs expansion. This study aims to investigate the benefit of ESCM for WJ-MSCs expansion *in vitro* in comparison with the most commonly used

basal culture medium for MSCs as DMEM supplemented with 10% FBS under hypoxic condition. In addition to WJ-MSCs expansion, the expanded cells were further determined the liver lineage differentiation potential *in vitro* by using a novel cytokine-based cocktail medium with or without medicinal plant extract from *Gynura procumbens* leaves. The achievement of these goals may serve as an exploration of a new expansion medium for WJ-MSCs that can save the cost of cell culture by reusing the discarded medium from ES culture system. Additionally, WJ-MSCs may serve as a novel source of hepatocyte-like cells that can be used as cell-based therapy in liver disease. This study also opens a new insight of using *G. procumbens* leaves extract in combination with a novel differentiation medium for promoting liver lineage differentiation of WJ-MSCs.

1.2 Research objectives

1.2.1 To investigate the efficacy of ESCM with or without additional epidermal growth factor (EGF) on supportive proliferation of WJ-MSCs *in vitro* in comparison with the most commonly used basal culture medium for MSCs as DMEM supplemented with 10% FBS and the expanded cells will be further characterized both in cellular and molecular levels.

1.2.2 To determine the *in vitro* liver-lineage differentiation potential of the expanded cells by using a new cytokine-based cocktail medium and further characterize both phenotype and functions of the differentiated cells.

1.2.3 To investigate the effect of medicinal plant extract from *Gynura procumbens* leaves on WJ-MSCs differentiation into liver-lineage *in vitro* and both phenotype and functions of the cells will be further characterized.

CHAPTER II

LITERATURE REVIEW

2.1 Liver disease

2.1.1 Epidemiology

Currently, the incidence of liver disease is rising and trends to be a serious public health problem in worldwide. The epidemiology study of non-alcoholic fatty liver disease (NAFLD) between in 1980-2010 showed that 10-35% of prevalence rates were found in the United States population. In the rest of the world, prevalence rates ranged from 6-35% (Vernon, Baranova, and Younossi, 2011). Other study revealed that patients with NAFLD and type II diabetes were at risk for liver related mortality (Rafiq et al., 2009). Cirrhosis was the twelfth leading cause of death in the United States (U.S.). Additionally, the etiologies of liver disease were found to relate with chronic viral hepatitis, alcoholic liver disease and NAFLD. In the U.S, the most of leading cause of mortality was accounted for 40% by Hepatitis C-related cirrhosis and led to liver transplantation (BMJ Evidence Centre, 2011). Incidence rates of liver cancer were increasing in worldwide including the U.S. and central Europe (Bosetti et al., 2008; Kohler et al., 2011). In the U.S., liver cancer was the ninth leading cause of cancer death (Altekruse, McGlynn, and Reichman, 2009). Chronic hepatitis B and C virus infection were associated with the majority cases of liver cancer (Perz, Armstrong, Farrington, Hutin, and Bell, 2006). In worldwide, hepatocellular carcinoma (HCC) that is a one type of liver cancer showed various

incidence rates depending on geographic location. For instance, high-incidence regions were sub-Saharan Africa, the People's Republic of China, Hong Kong, and Taiwan. Intermediate-incidence areas included several countries in eastern and western Europe, Thailand, Indonesia, Jamaica, Haiti, New Zealand (Maoris), and Alaska (Eskimos). Low-incidence areas were north and south America, most of Europe, Australia and parts of the Middle East (Schwartz and Carithers, 2011).

In Thailand, Bureau of Epidemiology reported that morbidity rate of hepatitis cases was found 16.44 per 100,000 of population in 2011. In addition, morbidity rates of viral hepatitis A, B, and C cases were 0.71, 9.93, and 2.16 per 100,000 of population, respectively (Center of Epidemiological Information, Bureau of Epidemiology, Ministry of Public Health, Thailand, 2011). Prevalence rate of cirrhosis cases was found 75.3 per 100,000 of Nakhon Nayok population in 2007. Moreover, the cause related with alcohol was more prevalent than non-alcohol about 2.6 folds (Rattanamongkolgul, Wongjitrat, and Puapankitcharoen, 2010). In northeast part of Thailand, liver flukes (*Opisthorchis viverrini*) infection is one of the major causes of hepatitis and subsequent liver cancer (cholangiocarcinoma) (Sripa et al., 2007). The incidence rate of liver fluke infection was 21.6% person-years in a rural community of Thailand (Rangsin et al., 2009). The prevalence of nonalcoholic steatohepatitis was found in 76.1% of Thai patients. Most patients exhibited unexplained chronic hepatitis that caused by other factors excluding viral hepatitis B and C infection such as obesity, diabetes mellitus, and dyslipidemia (Kladchareon, Treeprasertsuk, Mahachai, Wilairatana, and Kullavanijaya, 2004). The retrospective study in patients diagnosed with thrombosis of the portal system during 2000-2009 at Siriraj hospital, Thailand found that these patients associated with hepatocellular

carcinoma (52.5%), liver cirrhosis (9.3%), and abdominal infections or inflammations (9.3%). Among the patients with cirrhosis, the common causes were hepatitis B viral infection (38.8%), alcohol (15.3%), hepatitis C viral infection (12.2%), and the combination of hepatitis B and C viral infections with alcohol intake (11.2%) (Lertpipopmetha and Auewarakul, 2011). These data indicate that many kinds of liver disease are still a serious problem in Thailand.

2.1.2 Type of liver disease

Liver disease is the term of many diseases or disorders that affect the function of liver or cause abnormal function of the liver. Various causes may lead to damage of liver function including infection, injury, exposure to drugs or toxins, an autoimmune process, and a genetic defect. The types of liver disease are classified as followed (American Association for Clinical Chemistry, 2011).

2.1.2.1 Hepatitis

Hepatitis is an inflammation of the liver that is classified as acute and chronic forms. Acute hepatitis persists within six months whereas it takes time longer in chronic form. The major cause of hepatitis is infectious viruses, however, other factors may also lead to hepatitis such as toxin, alcohol, autoimmune, and other infectious pathogens. The three viruses mainly associated with hepatitis are hepatitis A, B, and C. Hepatitis A is spread through contaminated food and water that is common cause of acute hepatitis. Symptoms may include jaundice, nausea, diarrhea, and fatigue. Hepatitis B can be spread by exposure to contaminated blood, sexual transmission, and through infected mother to baby. Symptoms may be flu-like or acute hepatitis, however, some infected people about 1-3% will become carriers and chronically infected that may lead to chronic hepatitis. Hepatitis C

is spread by the same way of hepatitis B. Hepatitis C can result in acute hepatitis but less common than hepatitis B. The most of infected people commonly become to chronic hepatitis and may develop cirrhosis after prolonged infection.

2.1.2.2 Cirrhosis

Cirrhosis refers to advanced fibrous scar tissue in the liver after prolonged exposure to chronic liver diseases such as viral hepatitis infection, alcoholism, and fatty liver disease. Cirrhosis leads to irreversible loss of liver function and some case may develop to liver cancer. Cirrhosis can cause chronic liver failure and life-threatening complications including ascites, hepatic encephalopathy, and portal hypertension.

2.1.2.3 Toxic or drug-induced hepatitis

Many drugs, toxins, and chemical substances have a potential to cause hepatitis in some people. These factors can cause injury of the liver by obstruction a normal metabolism of the liver. Long term consumption of alcohol is a common cause of hepatitis and may lead to progressive damage the liver. About 10-20% of heavy drinkers may develop to cirrhosis. Symptoms may be acute or chronic hepatitis depending on the cause and exposure time.

2.1.2.4 Non-alcoholic fatty liver disease (NAFLD)

NAFLD refers to excess fat deposition in the liver that finally leads to fibrous scar tissue and common causes chronic inflammation of the liver. This condition seems to be related with obesity, diabetes, metabolic syndrome, and hypertension. Typically, there are no obvious signs and symptoms of hepatitis, however, some cases can progress to chronic hepatitis and liver failure.

2.1.2.5 Liver cancer

There are two forms of liver cancer that start in the liver and start in other organs but then spread to the liver. The most common type of liver cancer is hepatocellular carcinoma that develops in the liver cells. Cholangiocarcinoma is a rare type of liver cancer that develops in the bile ducts. Chronic viral hepatitis B and C infections including cirrhosis are high risk of progression in liver cancer. Liver cancer is usually asymptomatic at early stage and there are no specific symptoms. Therefore, patients that have risk of liver cancer should be screened for liver cancer as early.

2.1.3 Treatment of liver disease

2.1.3.1 Conventional treatment

Prevention is the best choice for treatment in liver disease. For example, protection from the risk factors that lead to hepatitis such as viral hepatitis A and B vaccination, avoiding from excessive uptake of alcohol or some drugs and avoiding obesity condition. Moreover, limitation of further liver damage is needed for treatment as well as supporting remaining liver function. Normally, treatment of liver disease is focus on improvement in the consequence conditions of the liver. In patients with ascites fluid complication, removing of excess fluid is required to treat this condition. In a case of portal hypertension, it needs to minimize the risk of bleeding by operation. To date, orthotopic liver transplantation is the final effective choice for patients with end stage of liver disease (Wedro, 2011). Survival rate of liver transplanted patients has progressively improved over the decades. In the U.S., the percentage of one year graft survival among liver transplanted patients increased from 70% in 1991 to 85% in 2009 (Organ Procurement and Transplantation Network

and Scientific Registry of Transplant Recipients, 2011). Although this treatment is promising, the lack of donor organs is still a major limitation. To date, the current numbers of the U.S. patients waiting for liver transplantation are estimated more than 17,000 people whereas the transplantation was performed about 6,300 cases (The Organ Procurement and Transplantation Network, 2012). In Thailand, a number of liver transplantation has increased by 3 times over the past 5 years. It has been reported that 230 cases of Thai patients have listed for liver transplantation in 2011, but only 26.1% of them were got this therapy (The Thai Red Cross, 2012). Other techniques, hepatocytes transplantation has shown as an effective treatment in some conditions such as liver-based metabolic disorders. However, this technique is limited by the lack of donor organs that can supply a sufficient cells number for transplantation as well as the limited replication potency of hepatocytes *in vitro* (Soltys et al., 2010). Recently, stem cells-based therapy is considering as a novel and promising therapeutic strategy to overcome this limitation. Stem cells display as an unlimited source of immature cells that have ability to self-renewal and differentiate into various cell types. Among adult stem cells, mesenchymal stem cells (MSCs) exhibit as a promising cells for applying in several diseases including liver disease. The detail of MSCs will be reviewed later in the next topic.

2.1.3.2 Alternative treatment with herbal medicines

The beneficial effects of herbal medicines have been applied for treatment of liver diseases for a long time. A number of herbs show effective activities to treat and prevent liver diseases in different ways such as anti-fibrosis and inhibit hepatitis viral replication. For example, *Silybum marianum* has been shown to inhibiting fibrosis formation *in vitro* and this effect also observed *in vivo* by reducing

hepatic collagen deposition which is a one factor of hepatic fibrogenesis. In clinical, *Silybum marianum* has been shown to attenuate progression of fibrosis in patients with chronic viral hepatitis C. Antiviral activity has been found in *Phyllanthus amarus* and *Glycyrrhiza glabra* that were evaluated by several studies. *Glycyrrhiza glabra* has been shown to treat in patients with chronic viral hepatitis B and C by reducing enzyme markers of liver injury. *Phyllanthus amarus* had an effective impact on clearance of viral hepatitis B in clinical. The mechanism of this effect is still unknown but possibly exert by interference replication and transcription of hepatitis viral (Stickel and Schuppan, 2007). Another herb, *Gynura procumbens* (Lour.) Merr. is widely distributed and used as folk medicine in southeast Asia including Thailand (Figure 2.1) (Globinmed, 2012). The plant has been known as paetumpung in Thailand that is usually eaten their leaves as Thai salad with Nam phrik. The plant is a member of Compositae family which has been proved to treat in many disorders such as gastric ulcer, inflammation, rheumatism, viral diseases of skin, kidney diseases, rashes, fever, migraine, constipation, and cancer (Perry and Metzger, 1980). Its active chemical constituents are flavonoids and sterol glycosides which are found in their leaves (Akowuah, Sadikun, and Mariam, 2002; Sadikun, Aminah, Ismail, and Ibrahim, 1996). The biological effects of the plant are still unclear but it seems to involve in several activities including anti-inflammatory (Iskander, Song, Coupar, and Jiratchariyakul, 2002), anti-hypertensive (Hoe, Lee, Mok, Kamaruddin, and Lam, 2011), anti-ulcerogenic (Mahmood, Mariod, Al-Bayaty, and Abdel-Wahab, 2010), antioxidant, and antitumor (Maw, Mon, and Oo, 2011). Although the treatable effect of the plant is still not exist in liver disease, but it was evidence that the ethanolic extract of *Gynura procumbens* leaves not only restored

blood glucose level of streptozotocin-induced type 2 diabetic rats but also improved their liver function in previous study of Dr. Wilairat Leeanansaksiri's Laboratory. Thus, these promising results may open a new insight of *Gynura procumbens* for treatment in liver disease.

Figure 2.1 The leaves of *Gynura procumbens* (Lour.) Merr.
Source: http://www.globinmed.com/index.php?option=com_content&view=article&id=62750:gynura-procumbens-lour-merr&catid=371:g.html.

2.2 Mesenchymal stem cells (MSCs)

2.2.1 Definition and history

Mesenchymal stem cells (MSCs) are multipotent adult stem cells that have characteristics as self-renewal and differentiation potency into several specialized cell types at least in mesodermal-lineages cells. MSCs were first isolated from rodent bone marrow by Friedenstein and their colleagues in 1976 (Friedenstein, Gorskaja, and Kulagina, 1976). These cells could adhere to plastic culture dishes and

have feature as spindle-shape cells like fibroblast. Moreover, the adherent cells had clonogenic feature or clonal density form which was defined as colony-forming unit fibroblasts (CFU-F). Also, this group was named the cells as multipotent stromal precursor cells. Over the past years, subsequent studies found that these cells could differentiate into mesodermal-lineages cells such as oseteoblast, adipocytes as well as chondrocytes *in vitro* (Ashton et al., 1980; Bab et al., 1986; Castro-Malaspina et al., 1980). Many research groups have been termed the cells in different names such as marrow stromal cells, multipotent stromal cells, bone marrow stromal stem cells, and multi-potent adult progenitor cells. However, it is notable that most studies are widely mentioned these cells as mesenchymal stem cells. This term was first proposed by Arnold Caplan in 1991 (Caplan, 1991). He named the cells based on their differentiation potential toward mesodermal tissues or all connective tissues such as adipose, bone, and cartilage.

2.2.2 Sources, characteristics, and differentiation potential

Since pilot studies by Friedenstein and their colleagues in 1976, MSCs are extensively interesting in their biology, phenotypes, and characteristics. In addition to rodent bone marrow, further studies have been reported that MSCs could be isolated from other mammalian species including monkey, goat, sheep, dog, pig, and human (Rozemuller et al., 2010). Although MSCs were originally isolated from bone marrow, other adult connective tissues such as adipose tissue (Zuk et al., 2001), muscle (Asakura, Komaki, and Rudnicki, 2001), dental pulp (Perry et al., 2008), and peripheral blood (Chong, Selvaratnam, Abbas, and Kamarul, 2012) have been found as additional sources of MSCs. Furthermore, MSCs can be harvested from fetal origin such as placenta (In't Anker et al., 2004), amniotic fluid (Tsai, Lee, Chang, and

Hwang, 2004), umbilical cord Wharton's jelly (Troyer and Weiss, 2008), and umbilical cord blood (Lee et al., 2004). Several studies have been reported variable immunophenotype of MSCs due to the fact that each laboratory has been studied MSCs from different sources and culture methods. According to these findings, MSCs have absence of unique specific antigens but slight different expression of cell surface antigens depending on their origins which were summarized in Table 2.1 (Hass, Kasper, Bohm, and Jacobs, 2011). To avoid the confusion, the International Society for Cellular Therapy (ISCT) has proposed minimal criteria for defining MSCs in 2006 (Dominici et al., 2006). The characteristics of MSCs can be defined at least as followed. Firstly, MSCs must adhere to plastic culture flasks after cultured in standard culture conditions. Secondly, they must be positive staining for some surface antigens as CD44, CD73, CD90, and CD105 and negative staining for human haematopoietic stem cells surface antigens as CD34, CD45 including lack expression of human leukocytes surface antigens as CD11b, CD14, CD19, and CD79α as well as human major histocompatibility complex (MHC) class II antigen or HLA-DR. Thirdly, they must be able to differentiate into mesodermal-lineage cells including osteoblasts, adipocytes, and chondroblasts *in vitro*.

Table 2.1 Cell surface antigen expressions of MSCs isolated from various tissues origins. Modified from Hass's study (Hass, Kasper, Bohm, and Jacobs, 2011).

Tissues	Positive markers	Negative markers
BM-MSCs	CD13, CD44, CD73 (SH3), CD90, CD105 (SH2), CD166, STRO-1	CD14, CD34, CD45
AT-MSCs	CD9, CD13, CD29, CD44, CD54, CD73 (SH3), CD90, CD105 (SH2), CD106, CD146, CD166, STRO-1, HLA I	CD11b, CD14, CD19, CD31, CD34, CD45, CD79α, CD133, CD144, HLA-DR
PB-MSCs	CD44, CD54, CD90, CD105 (SH2), CD166	CD14, CD31, CD34, CD45
WJ-MSCs	CD10, CD13, CD29, CD44, CD90, CD73 (SH3), CD105 (SH2), HLA I	CD14, CD31, CD33, CD34, CD45, CD56, HLA-DR
DP-MSCs	CD13, CD29, CD44, CD59, CD90, CD73 (SH3), CD105 (SH2), CD146	CD11b, CD14, CD24, CD19, CD34, CD45, HLA-DR

BM-MSCs, bone marrow-derived MSCs; AT-MSCs, adipose tissue-derived MSCs; PB-MSCs, peripheral blood-derived MSCs; WJ-MSCs, Wharton's jelly-derived MSCs; DP-MSCs, dental pulp-derived MSCs.

Based on mesodermal differentiation potential of MSCs *in vitro*, the original mesengenic process pathway was proposed in responding to haematopoiesis lineage diagrams as shown in Figure 2.2 (Caplan, 2009; Singer and Caplan, 2011). This pathway attempts to describe function of MSCs *in vivo* that is responsible for maintenance of the turnover rate of adult mesenchymal tissues in the body such as

bone, cartilage, muscle, fat, and other connective tissues. In addition to be as supply source of mesoderm lineage cells including cardiomyocyte (Pereira, Khushnooma, Madkaikar, and Ghosh, 2008), results from many studies found that MSCs have broad differentiation capacity into ectoderm and endoderm lineages *in vitro* such as neuron like cells (Liao, Gong, Li, Tan, and Yuan, 2010) and endothelial cells (Chen, Lie, Li, and Wei, 2009; Liang et al., 2005) in ectoderm lineage as well as insulin-producing cells (Chao, Chao, Fu, and Liu, 2008; Wu, Wang, Liu, and Wei, 2009) and hepatocyte-like cells (Banas et al., 2007; Zhang, Lie, and Wei, 2009) in endoderm lineage.

Overall, MSCs isolated from different tissues display similar phenotypic characteristics. However, fetal MSCs and adult MSCs have been shown some difference in proliferation and differentiation potential *in vitro*. Human first-trimester fetal MSCs showed higher proliferation rate in comparison with adult MSCs. This finding can be explained that fetal MSCs come from more primitive origin than adult MSCs which relate to fetal MSCs have longer telomeres than the other (Campagnoli et al., 2001; Guillot, Gotherstrom, Chan, Kurata, and Fisk, 2007). Additionally, MSCs isolated from umbilical cord blood showed lower adipogenic differentiation capacity than those harvested from bone marrow and adipose tissue (Kern, Eichler, Stoeve, Kluter, and Bieback, 2006; Rebelatto et al., 2008). Although there have some different features, MSCs from diverse sources remain share common characteristics according to the basic criteria for MSCs characterization.

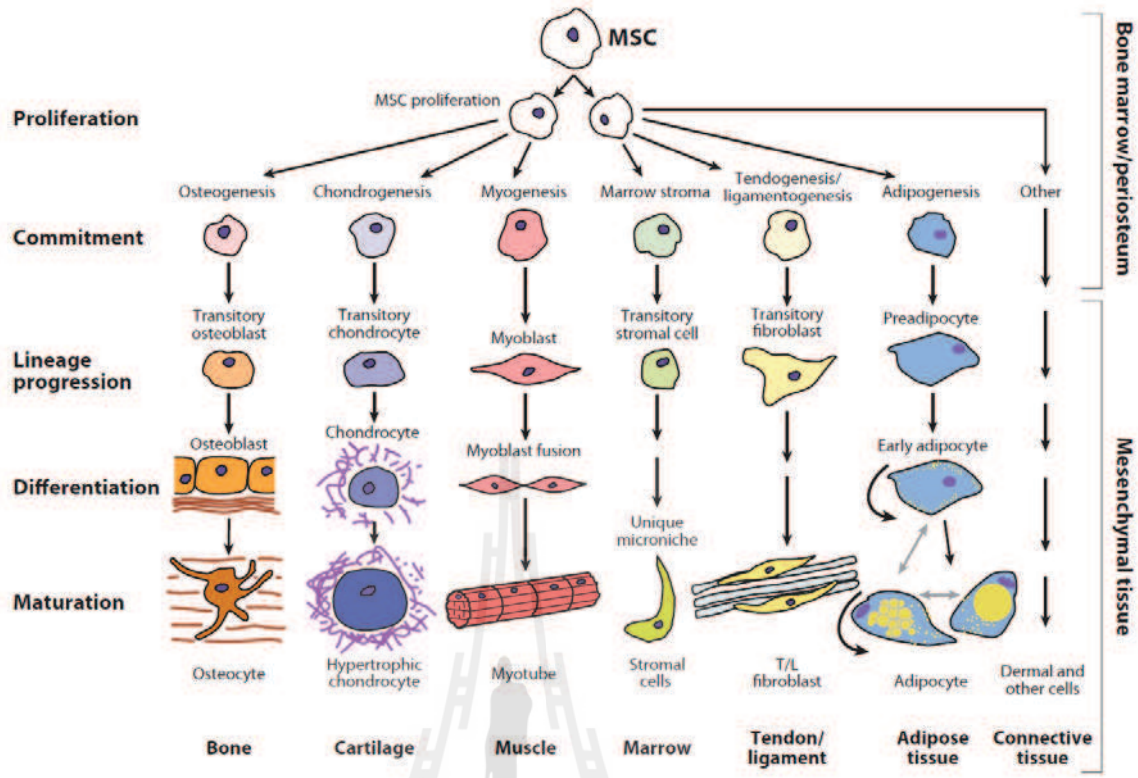


Figure 2.2 The mesengenic process pathway of MSCs in bone marrow (Caplan, 2009; Singer and Caplan, 2011).

2.2.3 Stem cells niche

To date, the native location or stem cell niche which MSCs reside is still unclear. In bone marrow, it is well known that MSCs play a role in supportive the haematopoietic stem cells niche which is crucial for maintaining haematopoietic stem cells in a quiescent state (Kiel and Morrison, 2008). In addition to bone marrow, various tissues of post-natal organs have been found as additional sources of MSCs. Recent researches showed increasing of evidences that MSCs may be derived from or identical to pericytes. Pericytes are described as the cells located surrounding small blood vessels (perivascular) including capillaries and microvessels that exist in entire body. The widespread of vascularized connective tissues throughout the body seems

to be a reason that why can be isolated MSCs from a various source of tissues and organs. The functions of pericytes play a role in blood vessel stabilization, tissue regeneration, immune system homeostasis, and reconstruction of injured tissue or blood vessel walls. Like MSCs, it has been shown that pericytes were similar to MSCs both in phenotypes and functions. For example, pericytes had ability to secrete diverse growth factors or cytokines similar to MSCs that seemed to support the regenerative capacity of them. Additionally, pericytes could express MSCs markers and exhibited multi-lineages differentiation potential into osteoblast, adipocytes, and chondrocytes as well as they had long term viability *in vitro* (Chen et al., 2009).

In mice model, it has been found that the isolated cells from perivascular of capillary, aorta, and vena cava had common properties of MSCs. Moreover, the distribution of the MSCs-like cells was found in a variety of adult organs including kidney, brain, spleen, liver, bone marrow, lung, and muscle (da Silva Meirelles, Chagastelles, and Nardi, 2006). Similarity, perivascular cells purified from multiple organs of human including skeletal muscle, pancreas, adipose tissue, and placenta exhibited a phenotype similar to MSCs such as mesodermal-lineages differentiation potential and MSCs markers expression (Crisan et al., 2008). Taken together, these findings suggest that the wide distribution of MSCs *in vivo* relates with microvasculature system that presents throughout the entire body. This close relationship between pericytes and MSCs makes possibility hypothesis that pericytes may be a native ancestor of MSCs or equivalent of MSCs *in vivo*. Based on this knowledge, perivascular is believed to be specific location niche of MSCs *in vivo* that reserves precursors of progenitor cells for tissue regeneration especially mesenchymal tissues in response to tissue injury circumstance.

2.2.4 Self-renewal and expansion

Typically, a variety of basal media containing 10% fetal bovine serum (FBS) can expand MSCs in a humid environment 5% CO_2 at 37ºC. To avoid the clinical risk of prion or xenogeneic proteins transmission from animal serum, other substances are considered to replace it. For instance, human platelet lysates supplemented medium has been observed to promote proliferation of MSCs while still maintained their biological characteristics (Doucet et al., 2005; Schallmoser et al., 2007; Xu et al., 2011). Other factors such as recombinant human platelet-derived growth factor-BB (PDGF-BB), basic fibroblast growth factor (bFGF), and transforming growth factor (TGF)-β1 could enhance proliferation of MSCs under serum-free condition (Chase, Lakshmipathy, Solchaga, Rao, and Vemuri, 2010). In addition, some studies have supported that epidermal growth factor (EGF) stimulated MSCs proliferation while still retained their stemness properties (Tamama, Kawasaki, and Wells, 2010). Although many cytokines or growth factors have been identified as inducer of MSCs proliferation, the exact underlying mechanism of signaling pathways that regulate their self-renewal is not fully understood. Among several signaling pathways, the Wnt and BMP signalings are the most candidates for regulation of MSCs fate. Currently, Wnt and BMP signalings are well known to regulate MSCs differentiation into bone and cartilage (Lin and Hankenson, 2011). It has evidences that these two pathways also play a role in regulation of MSCs proliferation, although available data are not enough to elucidate the network of molecular mechanisms.

2.2.4.1 Wnt signaling

Wnt signaling is a conserved pathway that regulates several processes of various organisms including development, cell proliferation, cell

polarity, cell fate decision, and cell adhesion. This pathway is activated by the specific bindings between Wnt proteins and their receptors, Frizzled (Fz) family of proteins, on the plasma membrane. Wnt signaling can be categorized into canonical and non-canonical Wnt pathways depending on their specific ligands. It has been reviewed that Wnt1, 2, 3, 3a, 8, and 8b involved in canonical Wnt signaling whereas Wnt4, 5a, 5b, 6, 7a, and 11 involved in non-canonical Wnt signaling. Previous studies have been found that non-canonical Wnt signaling did not involve in β-catenin activation but associated with several pathways. Planar Cell Polarity (PCP) and Wnt/Calcium pathways have shown to associate with non-canonical Wnt signaling which plays an important role in regulation of cell migration. However, the molecular mechanism of non-canonical Wnt signaling is poorly understood and needs to be further investigated. Thus, the molecular mechanism of canonical Wnt signaling is reviewed here.

Canonical Wnt signaling is induced through Frizzled (Fz) receptors with LRP5/6 co-receptor and further suppresses the degradation of β-catenin in cytoplasm leading to initiate transcription of target genes. Actually, β-catenin is constitutively degraded by the Axin complex which is consist of Axin, casein kinase 1α (CK1α), glycogen synthase kinase 3 (GSK3), and the tumor suppressor adenomatous polyposis coli (APC). The degradation of β-catenin is rendered by the phosphorylation through their amino terminal side by GSK3β and CK1α. Consequently, phosphorylated β-catenin is target for destruction by proteasome-mediated degradation. In the absence of β-catenin, the target genes are still inactivated by Groucho which plays a role as inhibitor of transcription factors T cell factor/lymphoid enhancer factor (TCF/LEF) of the target genes. Once Wnt

signaling is activated, it activates Dishevelled (Dsh) scaffolding protein to phosphorylate the receptors and further degrades the Axin complex as a result in accumulation of β-catenin in cytoplasm. Consequently, β-catenin translocates into the nucleus and further displaces transcription inhibitor Groucho which allows to mediate transcription of the target genes (Figure 2.3) (Eisenmann, 2005).

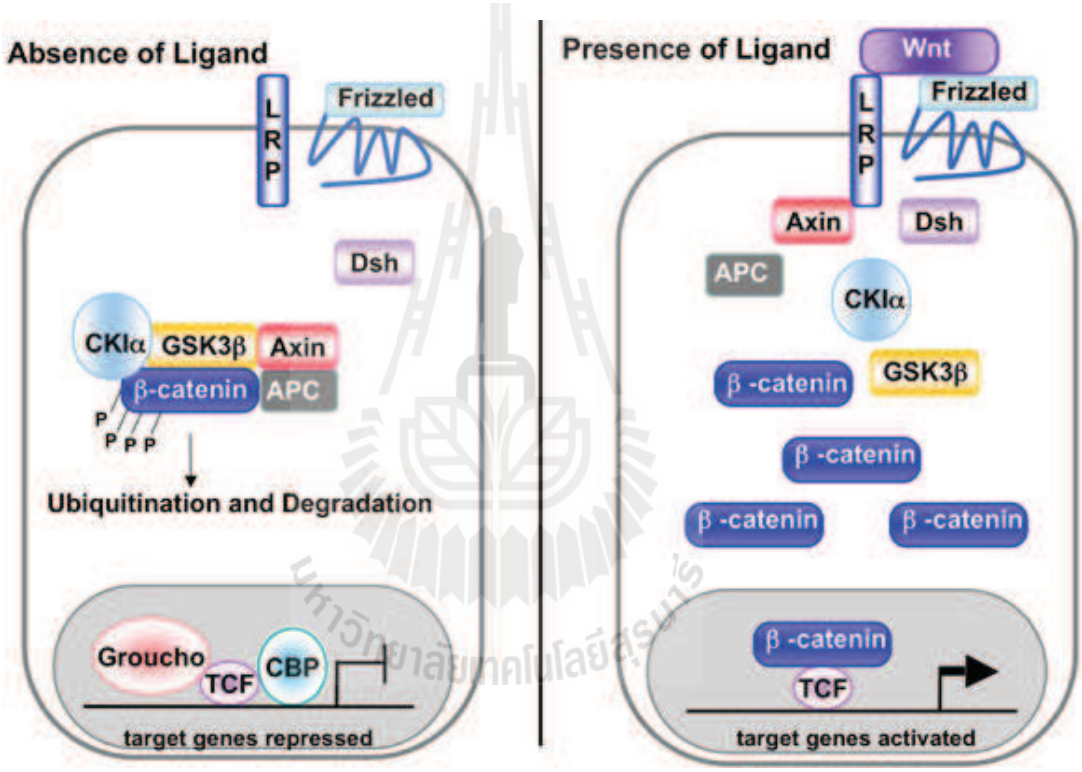


Figure 2.3 Regulation of canonical Wnt signaling pathway (Eisenmann, 2005).

It has been evidenced that undifferentiated MSCs maintained their stemness by endogenous canonical Wnt signaling. This finding was confirmed by the expression of several Wnt ligands and Fz receptors in MSCs. Previous study has been assumed that Wnt3a could activate the canonical Wnt signaling by

up-regulation of cell cycle regulator genes, cyclin D1, and c-Myc, as a result in promoting cells growth (Baek et al., 2003). Conversely, Wnt5a could activate the non-canonical Wnt signaling by down-regulation of cyclin D1 leading to suppress MSCs proliferation (Baksh, Boland, and Tuan, 2007; Baksh and Tuan, 2007). Further study found that the effect of canonical Wnt signaling on MSCs proliferation related to intensity of the signal. Low signal showed stimulating proliferation while high signal exerted to the opposite role (De Boer, Wang, and Van Blitterswijk, 2004). However, some studies have been reported the inhibition effect of canonical Wnt signaling on MSCs proliferation (Alfaro et al., 2008; Gregory, Singh, Perry, and Prockop, 2003; Qiu et al., 2007). Thus, additional studies are needed to complete the answer of these discrepancy findings.

2.2.4.2 Bone morphogenic protein (BMP) signaling

BMP signaling is transduced through ligands-specific receptors binding. BMP is a member of the superfamily of TGF-β. This signaling is involved in several processes in various organisms such as cell growth, differentiation, and apoptosis. BMP signaling is activated by the heteromeric complex formation of BMP ligands, BMP2, 4, and 7, and their receptors as type I and type II serine/threonine kinase subunits. Consequently, the serine/threonine kinase of the type II subunit phosphorylates intracellular domain in GC box of the type I receptor. This binding leads to further phosphorylation of R-Smad1, 5, and 8 which are members of the receptor-regulated Smad proteins. Further downstream, the complex of R-Smads with Smad4 is formed and translocates into the nucleus leading to control transcription of the target genes in association with transcription factors. Negative regulation of this signaling includes competitive binding to their receptors by BMP antagonists as

gremlin, noggin, chordin, and cerberus as well as inhibition of intracellular phosphorylation processes by Smad6 and Smad7 (Figure 2.4) (Robin and Durand, 2010).

Interestingly, previous studies have found that endogenous BMP signaling was involved in survival of MSCs under serum-free condition (Solmesky, Abekasis, Bulvik, and Weil, 2009). Recent study revealed the effect of BMP4 on biological activities of MSCs as dose dependent manner. High dose of BMP4 showed increasing apoptosis and reducing proliferation whereas low dose converted these activities. Although MSCs were extensive proliferation when treated with low dose of BMP4, they still be preserved the expression of stemness genes such as *Nanog* and *Oct-4* (Vicente Lopez et al., 2011). Also, BMP3 was shown to promote MSCs proliferation through TGF-β/Activin signaling pathway (Stewart, Guan, and Yang, 2010). In addition, recent study found that high dose of BMP7 could enhance both proliferation and osteogenic differentiation of MSCs (Pountos et al., 2010). Although these data seem to be promising, a few evidences are reported about the role of BMP signaling in self-renewal and proliferation of MSCs. Therefore, further studies are required to support the regulation effect of BMP signaling on MSCs proliferation.

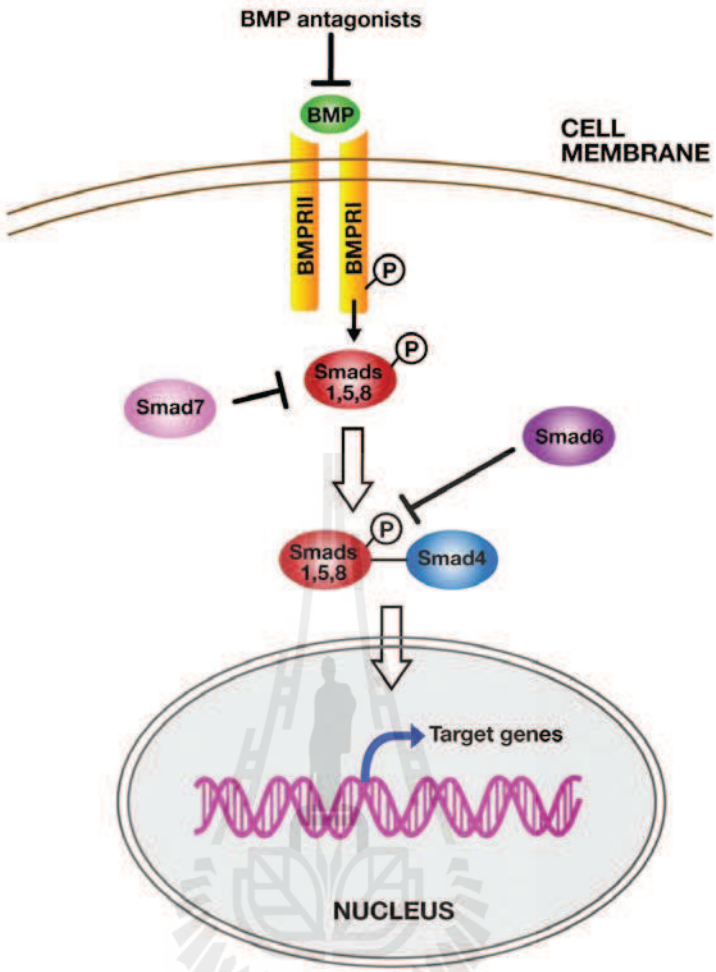


Figure 2.4 Regulation of BMP-Smad signaling pathway (Robin and Durand, 2010).

2.2.5 Paracrine effects

MSCs can secrete a number of bioactive molecules that affect to biological changing of other cells or known as paracrine effect. These paracrine effects are categorized into six main activities as immunomodulation, anti-apoptosis, angiogenesis, supporting the growth and differentiation local stem and progenitor cells, anti-scarring, and chemoattraction which are summarized in Table 2.2 based on review study of Meirelles Lda et al. (2009). Several studies have shown that MSCs

secreted a variety of angiogenic factors including basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), placental growth factor (PIGF), monocyte chemoattractant protein 1 (MCP-1), and interleukin 6 (IL-6). These paracrine factors are shown to promote local angiogenesis or the formation of new blood vessels that is important for tissue repair process. Additionally, MSCs secreted large amounts of chemokines which play a role in recruitment of leukocytes to a site of injury and further initiating the immune response. MSCs can limit the area of tissue injury by their anti-apoptosis activity. VEGF, hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF-1), Stanniocalcin-1, transforming growth factor beta (TGF-β), bFGF, and granulocyte-macrophage colony-stimulating growth factor (GM-CSF) were found to reduce apoptosis of the normal tissues around the injured tissues. Anti-scarring or anti-fibrotic is a one activity of paracrine factors secreted by MSCs. It has been demonstrated that HGF, bFGF, and adrenomedullin involved in prevention of fibrosis in animal model. Moreover, MSCs could support the growth of haematopoietic stem cells *in vitro* via secretion of paracrine factors including stem cell factor (SCF), leukemia inhibitory factor (LIF), IL-6, and macrophage colony-stimulating factor (M-CSF). Furthermore, pericytes which are known as MSCs *in vivo* were recruited and supported neural progenitors in animal model. Finally, immunomodulatory effects of MSCs on the immune system were exerted by a number of paracrine factors. For example, secreted prostaglandin E2 (PGE-2), TGF-β, HGF, indoleamine 2,3-dioxygenase (IDO), and LIF not only involved in suppression of T cell proliferation but also enhance secretion of anti-inflammatory cytokines. The secretion of M-CSF, PGE_2, and IL-6 contributed to inhibition of dendritic cell maturation that led to defect in initiating T cell response. Additionally,

MSCs-derived paracrine factors as IDO, TGF-β, and PGE_2 also showed the inhibition effect on NK cell proliferation (Meirelles Lda, Fontes, Covas, and Caplan, 2009). Taken together, all these activities are believed to involve the therapeutic potency of MSCs that make them interesting for cell-based therapy.

Table 2.2 Paracrine factors secreted by MSCs (Meirelles Lda, Fontes, Covas, and Caplan, 2009).

Effects	Paracrine factors
Anti-apoptotic	VEGF, HGF, IGF-1, Stanniocalcin-1, TGF-β, bFGF, GM-CSF
Immunomodulatory	PGE-2, TGF-β, HGF, mpCCL2, IDO, iNOS, HLA-G5, LIF
Anti-scarring	bFGF, HGF, Adrenomedullin
Supportive	SCF, LIF, IL-6, M-CSF, SDF-1, Angiopoietin-1
Angiogenic	bFGF, VEGF, PIGF, MCP-1, IL-6, Extracellular matrix molecules
Chemoattractant	CCL2, CCL3, CCL4, CCL5, CCL7, CCL10, CCL20, CCL26, CX3CL1, CXCL1, CXCL2, CXCL5, CXCL8, CXCL11, CXCL12

VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor 1; TGF-β, transforming growth factor beta; bFGF, basic fibroblast growth factor; GM-CSF, granulocyte-macrophage colony-stimulating growth factor; PGE-2, secreted prostaglandin E2; mpCCL2, MMP-processed CCL2; IDO, indoleamine 2,3-dioxygenase; iNOS, inductible nitric oxide synthase; HLA-G5, soluble human leukocyte antigen G; LIF, leukemia inhibitory factor; SCF, stem cell factor; IL-6, interleukin 6; M-CSF, macrophage colony-stimulating factor; SDF-1, stromal cell-derived factor 1; PIGF, placental growth factor; MCP-1, monocyte chemoattractant protein 1; CCL2, chemokine (C-C motif) ligand 2; CCL3, chemokine (C-C motif) ligand 3; CCL4, chemokine (C-C motif) ligand 4; CCL5, chemokine (C-C motif) ligand 5; CCL7, chemokine (C-C motif) ligand 7; CCL10, chemokine (C-C motif) ligand 10; CCL20, chemokine (C-C motif) ligand 20; CCL26, chemokine (C-C motif) ligand 26; CX3CL1, chemokine (C-X3-C motif) ligand 1; CXCL5, chemokine (C-X-C motif) ligand 5; CXCL11, chemokine (C-X-C motif) ligand 11; CXCL1, chemokine (C-X-C motif) ligand 1; CXCL2, chemokine (C-X-C motif) ligand 2; CXCL8, chemokine (C-X-C motif) ligand 8; CXCL12, chemokine (C-X-C motif) ligand 12.

2.2.6 Immunomodulatory effects

MSCs have low immunogenic property because they have natural features as low expression of major histocompatability complex (MHC) class I antigens and lack of MHC class II including co-stimulatory molecules expressions. Additionally, MSCs also have ability to secrete paracrine factors that can regulate the immune systems. Several studies found that MSCs had immunomodulatory effects on both the innate and adaptive immune systems such as inhibition of T cell proliferation and cytokine secretion, inhibition of B cell proliferation and immunoglobulin synthesis including inhibition of monocytes differentiation to dendritic cells (Figure 2.5). The underlying mechanisms are still unknown, but it has evidences that paracrine factors secreted by MSCs and direct cell-to-cell contacts are involved in these effects.

T lymphocytes or T cells play a major role of cell-mediated immune responses in adaptive immunity. Once stimulation by pathogens, the proliferation of T cells occurs and further releases inflammatory cytokines to destroy the pathogens. It has been demonstrated that MSCs were able to suppress T cells proliferation *in vitro*. This effect was involved inhibition of cell cycle by accumulation of cells in the G_0/G_1 phase. A number of paracrine factors secreted by MSCs such as TGF-β, HGF, PGE_2, IDO, LIF, IL-6, and NO as well as cell-to-cell interactions were found to effect on T cells in different manner including suppression of T cells proliferation, suppression of pro-inflammatory cytokine secretion, inhibition of cytotoxic T cells activation, and promotion of anti-inflammatory cytokine secretion. Moreover, MSCs can generate and expand regulatory T cells (Treg) which are important for regulating immune responses by their anti-inflammatory effect. HLA-G5 secreted by MSCs exhibited as

key factor that was required for Treg generation. B lymphocytes or B cells play a role in humoral immune response and response to produce antibodies against antigens. Several studies revealed the regulatory effects of MSCs on B cells including inhibition of B cell proliferation and immunoglobulin synthesis. Like T cells, MSCs could arrest B cell proliferation at G_0/G_1 cell cycle phase. Additionally, all of these effects are believed to associate with the interactions of paracrine factors and direct cell-to-cell contacts (Newman, Yoo, LeRoux, and Danilkovitch-Miagkova, 2009).

Natural killer cells or NK cells are a one of effector cells in innate immunity. NK cells response to destroy the target cells, virus-infected cells and tumor cells, by releasing cytotoxic cytokines that makes the lysis of target cells. IDO, TGF-β, HLA-G5, and PGE_2 secreted from MSCs are found to affect on NK cells both inhibition of cell proliferation and cytokine secretion. It has evidences that MSCs could suppress cytotoxicity function of NK cells through downregulated expression of NK cells receptors. This effect was associated with both paracrine factors as well as direct cell-to-cell contacts. Dendritic cells act as antigen presenting cells that are derived from monocytes. The major role of dendritic cells is initiation of immune response by presenting antigens to naïve T cells which further stimulate T cells activation and proliferation. Several studies demonstrated the inhibition effects of MSCs on dendritic cells including dendritic cells maturation, co-stimulatory receptors expression, and inflammatory cytokine secretion. MSCs-derived paracrine factors as M-CSF, PGE_2, and IL-6 including direct cell-to-cell contacts were involved in these effects (Newman, Yoo, LeRoux, and Danilkovitch-Miagkova, 2009). Although most findings are come from *in vitro* studies, some results from *in vivo* studies in animal models also support the capacity of MSCs to regulate the immune systems.

Additionally, these immunomodulatory effects could be observed in patients who have applied MSCs-based therapy according to clinical trials regimen in several diseases. Taken together, all evidences confirm the immunomodulatory effects of MSCs that provide a superior benefit in cell-based therapy.

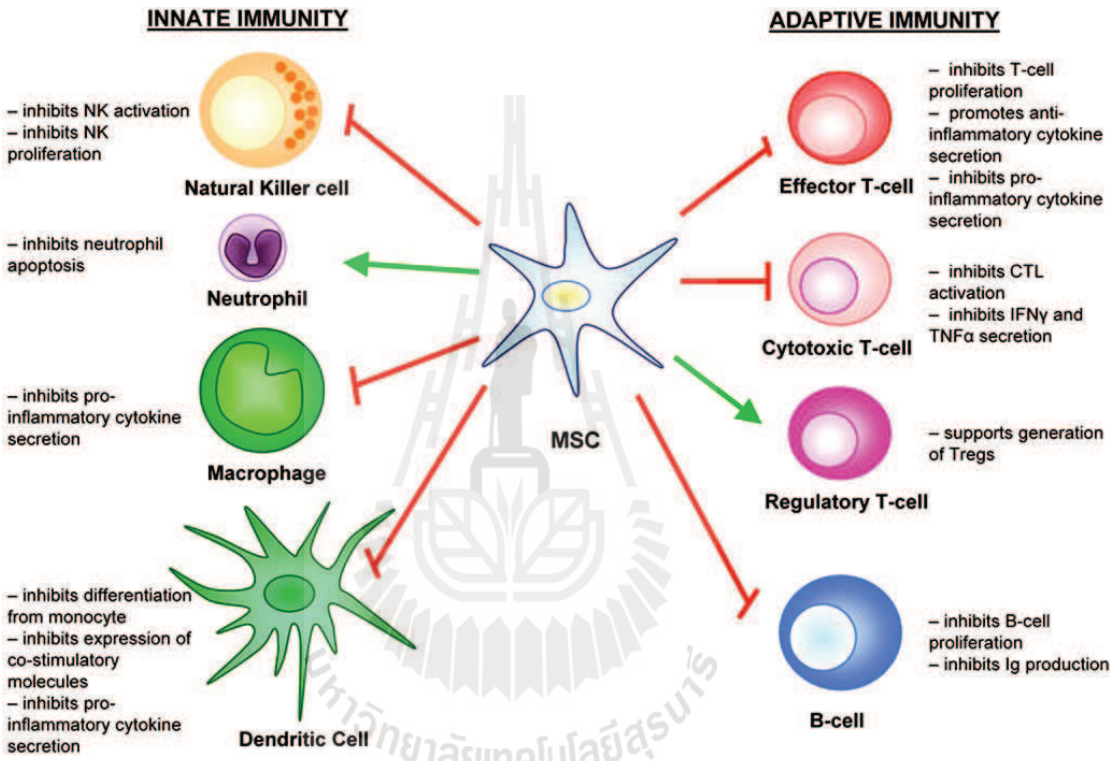


Figure 2.5 Immunomodulatory effects of MSCs on various immune cells both in innate and adaptive immune systems (Newman, Yoo, LeRoux, and Danilkovitch-Miagkova, 2009).

2.2.7 Homing and wound healing

MSCs have ability to migrate to the injured tissue or called homing process. This ability is shown to be crucial roles in wound healing and tissue

regeneration (Mansilla et al., 2005). It has been purposed that MSCs can improve damaged tissues by engraftment at the site of injury and differentiation toward the new cells instead of injured cells. In addition, paracrine factors secreted by MSCs are believed as a major mechanism responding to tissues repair. These factors exhibit as mediators of wound healing that regulate inflammation, angiogenesis, and epithelialization as well as recruit native inflammatory cells in response to tissues repair (Hocking and Gibran, 2010).

Both local and systemic infusions of MSCs demonstrated that MSCs could selectively home to the site of injury in various animal models (Chen et al., 2010; Mouiseddine et al., 2007; Rojas et al., 2005). Systemic infusion of MSCs has believed to mimic the trafficking of endogenous MSCs from bone marrow to injured site through vascular system. It is well known that MSCs appear at perivascular along blood vessels throughout the body, thus, it has been questioned whether the homing of MSCs from other distant sources is still needed. Here, the trafficking of MSCs from bone marrow is reviewed as a model of the homing process to the site of injury. The mechanism of MSCs homing is still unclear but it has been purposed similar to the homing process of leukocytes toward the site of inflammation (Luster, Alon, and von Andrian, 2005). First, MSCs are mobilized from bone marrow via blood circulation to home to injured site in response to inflammatory cytokines or chemokines. Subsequently, circulating MSCs adhere to the endothelium of blood vessel and transmigrate across extracellular matrix into injured site.

Homing process is involved in several factors including inflammatory cytokines or chemokines, specific receptors, growth factors and extracellular matrix components. MSCs have been reported to express different profiles of chemokine

receptors such as CCR1, CCR7, CCR9, CXCR4, CXCR5, and CXCR6 (Honczarenko et al., 2006). These chemokines play essential roles in mobilization and homing regulation of stem cells and progenitor cells. Many studies have been found that the interaction of CXC chemokine stromal derived factor-1 (SDF-1) and their CXCR4 receptor are involved in MSCs migration. *In vitro* experiment, the migration of MSCs corresponding to SDF-1 was observed in dose-dependent manner (Wynn et al., 2004). *In vivo* studies showed the crucial effect of SDF-1 ligand and CXCR4 receptor on MSCs migration to injured tissue which further enhancing tissue repair in various conditions of animal models such as bone injury (Kitaori et al., 2009) and myocardium infraction (Cheng et al., 2008). Altogether, these evidences have proved that the interaction of SDF-1 ligand and CXCR4 receptor is crucial for migration of MSCs. In addition to chemokines, some growth factors could trigger the migration of MSCs to injured site including bFGF, IGF-1, HGF, EGF (Ozaki et al., 2007), and matrix metalloproteinase 2 (MMP-2) (De Becker et al., 2007).

Adhesion molecules such as integrin are found to facilitate strong adhesion of leukocytes to vascular endothelial cells. Like leukocytes, MSCs have shown to express a member of integrins including integrin β1 and integrin α4 which are important role in firm adherence of cells to endothelial ligand, the vascular cell adhesion molecule 1 (VCAM-1) (Fox, Chamberlain, Ashton, and Middleton, 2007). The migration across vascular endothelial cells and extracellular matrix are a final step of MSCs invasion into injured site. Although the underlying mechanism of this process is still unknown, it has been evidence that matrix metalloproteinases (MMPs) enzyme involved in MSCs invasion. Previous studies showed ineffective MSCs invasion across extracellular matrix after exposure to synthetic MMP inhibitors

against to MMP-2, membrane type 1 MMP (MT1-MMP), and tissue inhibitor of metalloproteinase 2 (TIMP-2) (Ries et al., 2007). Actually, MMPs are a group of enzyme that participates in breakdown of extracellular matrix components in various conditions such as embryonic development, wound healing, angiogenesis, and tissue remodeling (Visse and Nagase, 2003). In this circumstance, MMPs may support MSCs invasion through extracellular matrix toward the site of injury in the same manner.

2.2.8 Clinical applications

MSCs are well known to possess several properties including extensive expansion *in vitro*, broad differentiation potential, immunomodulatory properties and other paracrine effects such as tissue repair and anti-inflammation. These superior effects make MSCs as promising cells for regenerative medicine. In recent years, several clinical trials with MSCs have registered on the United States National Institutes of Health's clinical trial website (http://clinicaltrials.gov). Based on this database, MSCs have been widely applied for therapeutic purpose as many as 193 clinical trials which are categorized by disease as shown in Figure 2.6 (The United States National Institutes of Health, 2012).

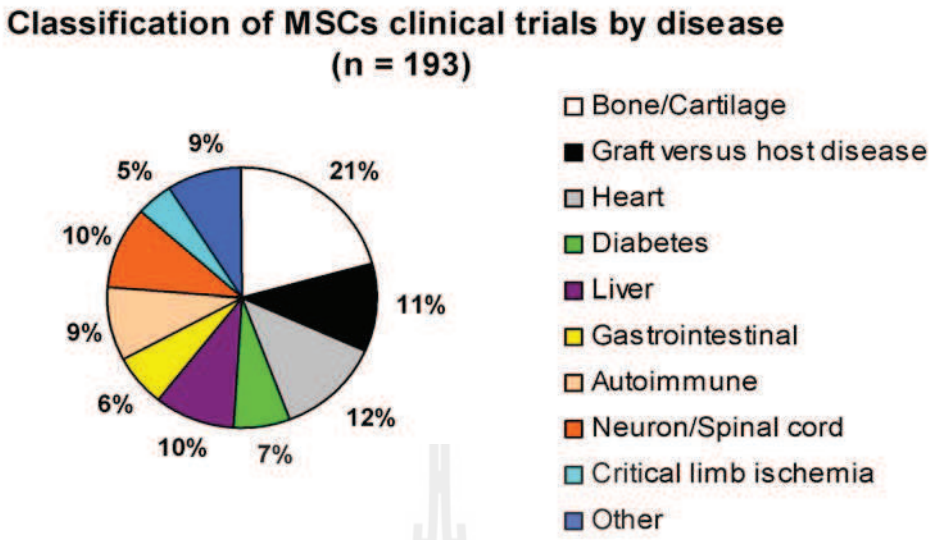


Figure 2.6 Classification of MSCs clinical trials which are categorized by disease (n = number of trials).

Source: http://clinicaltrials.gov/ct2/results?term=mesenchymal+stem+cells&recr=&no_unk=Y.html.

The most of these clinical trials are being under investigation and are stayed in phase I and II which have aim to investigate safety and efficacy of the treatment, respectively. To date, the therapeutic effects of MSCs are investigated in both autogeneic and allogeneic transplantations. Based on this data, autogeneic MSCs (MSCs are isolated from patients themselves) were commonly used in various diseases including bone defect, myocardial infarction, spinal injury, critical limb ischemia, and multiple sclerosis. In addition, Allogeneic MSCs (MSCs are isolated from healthy donor) were preferred to apply in some conditions such as graft versus host disease and crohn's disease. The most widely used MSCs are isolated from bone marrow and adipose tissue. Furthermore, around 5% of all studies have entered to phase III clinical trials which aim to investigate the efficacy of a new treatment

compared to standard treatment in a large number of patients. These included acute myocardial infarction, graft versus host disease, cartilage injury, diabetes mellitus, and crohn's disease. Although around 25% of all clinical trials were completed, the results from some studies are available to access. Here, this review focuses on the published results only.

It has been demonstrated that autologous BM-MSCs transplantation could improve regional contractility of a chronic myocardial scar in 8 patients with myocardial infarction (Williams et al., 2011). Recently, the safety and efficacy of intramyocardial injection with autologous BM-MSCs were observed in patients with stable coronary artery disease and refractory angina as long as one year after treatment. These effects included improvement in exercise capacity, Seattle Angina Questionnaire evaluations, decreasing angina attacks per week and reducing nitroglycerine consumption which is used for heart disease treatment (Haack-Sorensen et al., 2013). Phase I/II clinical trial with allogeneic MSCs transplantation showed effective treatment in patients with acute and chronic graft-versus-host disease. In acute graft-versus-host disease, complete response, partial response and no response were found in 1, 6, and 3 out of 10 patients, respectively. In chronic graft-versus-host disease, 1 out of 8 patients achieved complete response, 3 had a partial response, and 4 had no response (Perez-Simon et al., 2011). Recent study found the safety and improvement in type 2 diabetes patients with islet cell dysfunction after transplantation with human placenta-derived MSCs (PD-MSCs). 40% (4 out of 10) of all patients showed reducing of insulin requirement more than 50% after transplantation for 6 months (Jiang et al., 2011). Moreover, it has been demonstrated that intramuscularly BM-MSCs injection could improve the

patients with complications of type 2 diabetes including critical limb ischemia and foot ulcer (Lu et al., 2011). Regarding ulcerative colitis treatment, allogeneic BM-MSCs transplantation successfully improved clinical and morphological characteristics of the ulcers compared to standard therapy. Moreover, this therapy also reduced the cost of treatment by decreasing immunosuppressive drugs use (Lazebnik, Gusein-Zade, Kniazev, Efremov, and Ruchkina, 2010). Taken together, MSCs-based therapy trends to be safe and shows promising effect in several diseases. However, these therapeutic effects seem to be transient and need to be validated in a long term. Furthermore, routine follow-up is needed to monitor the side effects in a large group of patients before using MSCs as alternative treatment.

2.2.9 Therapeutic potential in liver disease

2.2.9.1 MSCs and *in vitro* studies in liver disease

Generation of new hepatocytes or hepatocyte-like cells for replacing the old damaged cells is a one alternative choice to overcome the scarcity of donor livers. MSCs are capable of unlimited source of self-renewal cells that have ability to differentiate into many cell types including hepatocytes. To date, numerous studies have successfully generated hepatocyte-like cells *in vitro* by different ways as followed.

2.2.9.1.1 Induction by soluble growth factors or cytokines-defined medium

A number of studies use the sequential exposure of exogenous growth factors or cytokines as a cocktail to induce MSCs differentiate into hepatocytes. These factors are applied following the understanding of liver development during mouse and human embryogenesis (Zorn, 2008). The most factors

that were commonly added into the first stage of induction are FGF-1, FGF-2, and FGF-4 which are required to initiate the early hepatogenesis. Subsequently, the combination of HGF, oncostatin M (OSM), and dexamethasone were widely used for hepatic maturation step. HGF is required for supportive fetal hepatocytes during mid-stage hepatogenesis. OSM is produced by the haematopoietic cells which play an important role in secreting the factor for maturation of fetal hepatocytes. Dexamethasone is a synthetic glucocorticoid hormone which is needed to induce liver enzymes involved in gluconeogenesis such as phosphoenolpyruvate carboxykinase (PEPCK) and tyrosine aminotransferase (TAT) (Lavon and Benvenisty, 2005). Several studies have successfully generated hepatocyte-like cells from MSCs by various cocktail media with different induction period ranging from 14-21 days.

Dong et al. (2010) demonstrated that FGF-4 and HGF were necessary in the first step of hepatic differentiation, because of early hepatic specific genes, α-fetoprotein (*AFP*), and hepatocyte nuclear factor-3β (*HNF3β*), were down-regulated after removing these cytokines from induction medium. Moreover, they also investigated that OSM was important for hepatic maturation by observing down regulation of late hepatic specific genes, albumin (*ALB*) and tyrosine aminotransferase (*TAT*), after withdrawing from the system. Pournasr et al. (2011) added FGF-4 and HGF in the first stage medium and used the cocktail of HGF, insulin-transferrin-sodium selenite (ITS), and dexamethasone in the last stage medium of differentiation. They successfully generated hepatocyte-like cells from human BM-MSCs after complete induction. The differentiated cells had functional ability as normal hepatocytes including glycogen storage, albumin secretion, urea detoxification, low-density lipoprotein (LDL) uptake, and hepatic specific markers

expression both gene and protein levels. Pulavendran et al. (2010) applied a novel technique to gradually deliver HGF to murine BM-MSCs by incorporating HGF into chitosan nanoparticles. This technique successfully induced cells to differentiate into functional hepatocytes via sustainable HGF releasing to the target cells. Saulnier et al. (2010) revealed the molecular mechanism underlying the hepatic differentiation of human AT-MSCs. The cells were exposed to HGF and FGF-4 in the early stage and the mixture of OSM with nicotinamide in the maturation stage. They found the down regulation of mesenchymal lineage genes relative to the over expression of epithelial-related genes during differentiation. These results indicate that the transition of molecular pathway occurs during differentiation from mesenchymal to epithelial lineage that is a feature of hepatocytes. Chivu et al. (2009) showed incomplete hepatogenic differentiation of human BM-MSCs when HGF, nicotinamide, and dexamethasone were separately added in differentiation medium. Conversely, hepatocyte-like cells features were observed after reunion all these factors in the culture.

Dental pulp mesenchymal cells also had differentiation potency toward hepatic lineage after inducing by the cocktail medium as other studies used. They observed that the differentiated cells acquired the characteristics of hepatocytes both in morphology and function (Ishkitiev et al., 2010). Amniotic fluid-derived MSCs (AF-MSCs) could be induced into hepatocyte-like cells by using FGF-4 and HGF in early stage and combination of trichostatin A, dexamethasone, ITS, and HGF in last stage (Zheng et al., 2008). Umbilical cord matrix stem cells also had differentiation potential into hepatic lineage after treatment with the mixture of HGF, bFGF, ITS, and nicotinamide following by the mixture of

OSM, ITS, and dexamethasone. The differentiated cells exhibited functional hepatocytes such as expression of hepatic specific markers, production of urea, storage of glycogen, and induction of cytochrome P450 (CYP) 3A4 or CYP3A4 activity (Campard, Lysy, Najimi, and Sokal, 2008). Similarly, mesenchymal cells derived from amniotic membrane could differentiate into hepatic lineage after treatment with induction medium as 10% FBS, HGF, bFGF, OSM, and dexamethasone. The differentiated cells acquired hepatocytes characteristics including glycogen storage and hepatic lineage expression both at gene and protein levels (Tamagawa, Oi, Ishiwata, Ishikawa, and Nakamura, 2007). Another study successfully promoted hepatic differentiation of mesenchymal stromal cells derived from umbilical cord Wharton's jelly by one step protocol. After induction with 1% FBS, HGF, and FGF-4, the differentiated cells expressed hepatic specific markers both at gene and protein levels, stored glycogen as well as had ability to uptake LDL which is a one characteristic of functional hepatocytes (Zhang, Lie, and Wei, 2009).

2.2.9.1.2 Induction by co-culture with other cell types

Many studies have been shown that MSCs could be induced into hepatocyte-like cells by co-culture together with other cell types such as hepatic stellate cell (HSC) and mature hepatocytes. Secreting factors derived from these cells are assumed to induce hepatic differentiation (Antoine et al., 2007). Deng et al. (2008) observed the hepatocyte-like cells after co-culture rat BM-MSCs with activated HSC. The differentiated cells also had hepatocytes phenotypes both in morphology and function such as glycogen storage capacity. In addition to HSC, mature hepatocytes are commonly used for hepatic induction in MSCs. Qihao et al. (2007) showed the supportive effect of adult liver cells on the hepatic differentiation

of rat MSCs. They observed spheroid formation of the differentiated cells which could express liver specific markers such as albumin, α-fetoprotein, and cytokeratin 18 at gene and protein levels. Moreover, the heterotypic interaction of porcine hepatocytes/MSCs at ratio 2:1 was found as optimal ratio for hepatic lineage induction. The differentiated cells were approved by testing liver function including albumin secretion, urea production, and CYP3A1 activity (Gu, Shi, Zhang, and Ding, 2009).

2.2.9.1.3 Induction by biomaterial scaffolds

Recent studies are focusing on using biomaterial scaffolds in combination with stem cells. Biomaterial scaffolds provide 3 dimensional structures resembling extracellular matrix environment *in vivo* (Willerth and Sakiyama-Elbert, 2008). Several studies observe promising outcomes of using biomaterial scaffolds associated with induction medium to promote MSCS differentiation into hepatocyte-like cells. Alginate scaffold is derived from natural polysaccharide-based biomaterials that provide extracellular matrix structure allowing for cells adhesion. Lin et al. (2010) showed the supportive effect of alginate scaffold on hepatic differentiation of rat BM-MSCs. The differentiated cells displayed hepatocytes phenotypes including albumin secretion, urea production, glycogen storage, and liver specific markers expression. However, natural biomaterials have some disadvantages especially the variability of materials between lot to lot. In addition to alginate scaffold, nanofibrous scaffold is a one of synthetic polymer-based biomaterials that are widely used for stem cells culture. These scaffolds are made from defined chemical materials allowing easy control the quality and reproducibility of product. Kazemnejad et al. (2009) investigated the hepatic differentiation potential

of human BM-MSCs seeded on 3 dimensional nanofibrous scaffold with differentiation medium compared to 2 dimensional culture system. They found that nanofibrous scaffold enhanced the cells to differentiate into functional hepatocyte-like cells more than 2 dimensional culture system. The differentiated cells derived from the scaffold could express liver specific markers such as albumin, α-fetoprotein, cytokeratin 18, cytokeratin 19, and CYP3A4. Moreover, the levels of albumin, urea, transferrin, serum glutamic pyruvic transaminase, and serum oxaloacetate aminotransferase in culture medium of the differentiated cells derived from nanofibrous scaffold were greater than those cultured in 2 dimensional culture system. Another group also studied the hepatic differentiation efficacy of MSCs after seeding on synthetic extracellular matrix, ultraweb nanofibers. They observed significant increasing of albumin, urea, and α-fetoprotein levels including CYP3A4 activity in conditioned medium derived from the differentiated cells seeded on ultraweb nanofibers coated plate compared to those derived from none coated plate (Piryaei, Valojerdi, Shahsavani, and Baharvand, 2011). The effect of collagen-coated poly (lactic-co-glycolic acid) (C-PLGA) scaffold on hepatic differentiation of rat BM-MSCs has been investigated. The researchers found that the differentiated cells derived from C-PLGA scaffolds more expressed liver specific markers both at gene and protein levels earlier than in the control group, a monolayer culture system. In addition, the differentiated cells derived from C-PLGA scaffolds could acquire hepatic function such as glycogen storage (Li et al., 2010).

2.2.9.2 MSCs and *in vivo* studies in liver disease

2.2.9.2.1 Studies in preclinical models (animal)

Several studies have been hypothesized that paracrine signals from MSCs seem to be a crucial factor for improvement of liver injury in animal models. It has been shown that secreting factors from undifferentiated MSCs were likely to play important roles in regeneration and recovery of the damaged cells by stimulating the endogenous host cells repair. In a model of hepatotoxic chemical-induced acute liver injury, paracrine signals from MSCs exhibited as a major role in improvement the disease. One study demonstrated the effective treatment in rat model with fulminant hepatic failure by using MSCs conditioned medium. This medium consisted of MSCs-derived molecules including growth factors, chemokines, and cytokines involved in immunomodulation and liver regeneration which could prevent hepatocytes apoptosis and increasing survival rate of rat model (Parekkadan, van Poll, Suganuma, Carter, Berthiaume, Tilles, and Yarmush, 2007). Furthermore, van Poll et al. (2008) showed the evidence that systemic infusion of MSCs conditioned medium could promote the survival rate of rat model with acute liver injury. Based on *in vitro* study, they assumed that this effect was accompanied by reducing hepatocellular death and increasing hepatocytes proliferation which were mediated by MSCs-derived molecules.

In addition to using the conditioned medium, direct infusion with adipose tissue-derived stem cells also had ability to improve liver function in mice with acute liver injury as soon as one day after transplantation. The researchers suggested that paracrine effects derived from undifferentiated cells may exert to ameliorate liver injury (Kim, Park, Lee, Kim, and Kim, 2011). Mice bone

marrow stem cells also had ability to home in recipient liver of acute liver injury mice as well as could improve liver function and survival rate (Jin et al., 2012). Similarly, Zhang et al. (2012) observed the therapeutic treatment in mice model with carbon tetrachloride (CCl_4)-induced acute liver failure by using human MSCs from umbilical cord matrix. This study revealed that the effective treatment was likely to mediate by paracrine effects derived from MSCs which could stimulate endogenous liver regeneration rather than transdifferentiation into hepatocytes. This conclusion was supported by *in vitro* experiments which the regeneration and albumin secretion of mouse model with damaged hepatocytes were improved by MSCs.

In addition to paracrine effects, transdifferentiation of the engrafted MSCs toward hepatocyte-like cells *in vivo* is found to facilitate the improvement of disease. One recent study observed the effective treatment for D-galactosamine-induced fulminant hepatic failure in pig model by transplantation immediately with human BM-MSCs after liver failure induction. The transplanted cells contributed to liver regeneration and transdifferentiation into hepatocytes in recipient liver which accounted as 30% of the total hepatocytes. Moreover, surviving animals were found in transplanted group for more than 6 months while control group died within 96 h (Li et al., 2012). Similarly, Cao et al. (2012) also demonstrated the therapeutic potential of transplanted human placenta-derived MSCs (PD-MSCs) in pig with D-galactosamine-induced acute liver failure. The transplanted cells could not only differentiate into hepatocyte-like cells *in vivo* but also improving the liver function as well as reducing liver inflammation and increasing liver regeneration in recipient host. The survival rate of transplanted group was prolonged significantly as more than 5 months compared with control group.

In a model of hepatotoxic chemical-induced chronic liver injury, several studies have been shown the anti-fibrosis effect of transplanted MSCs on the recipients. The putative mechanism underlying the anti-fibrotic effect of MSCs may involve in the expression of matrix metalloproteinases (MMPs). MMP-2 and MMP-9 were shown to degrade the extracellular matrix and induce apoptosis of hepatic stellate cells (Higashiyama et al., 2007; Parekkadan, van Poll, Megeed, Kobayashi, Tilles, Berthiaume, and Yarmush, 2007). Transdifferentiation of the engrafted MSCs toward hepatocyte-like cells *in vivo* is shown as a one strategy to improve the liver function. Many studies showed evidences that MSCs could engraft into liver and committed toward hepatic lineage cells *in vivo* ranging from 4-8 weeks post-transplantation. Zhang et al. (2011) observed the therapeutic effects of human amniotic membrane-derived MSCs (AM-MSCs) after transplantation into mice model with CCl_4-induced liver cirrhosis. These effects included reducing hepatic stellate cells activation which can enhance fibrosis, decreasing hepatocytes apoptosis, and promoting liver regeneration. Moreover, the engrafted cells could differentiate into human albumin or α-fetoprotein-expressing hepatocyte-like cells in the damaged liver. This finding is similar to the results of Jung et al. (2009) study. They found that human umbilical cord blood-derived MSCs (UB-MSCs) could improve liver fibrosis in rat model with CCl_4-induced liver cirrhosis by decreasing expression of transforming growth factor-β1 (TGF-β1), alpha-smooth muscle actin (α-SMA), and collagen type I which are mediators of liver fibrosis. In addition, the engrafted cells could differentiate into human albumin or α-fetoprotein-expressing hepatocyte-like cells which may improve the liver function by decreasing serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels.

MSCs from human umbilical cord also had therapeutic effect on liver injury mice by decreasing serum AST and ALT levels, inflammation, apoptosis, and denaturation of hepatocytes (Yan et al., 2009). Similarly, Abdel-Aziz et al. (2007) observed that rat BM-MSCs could differentiate toward hepatocyte-like cells in rats with CCl_4-induced liver injury and further improvement the liver function through increasing albumin level and decreasing ALT level. Moreover, they also found the relation of anti-fibrotic effect with the low expression of collagen type I which is a marker of liver fibrosis. Interestingly, human adipose tissue-derived multilineage progenitor cells infusion could reduce total cholesterol in rabbit model of familial hypercholesterolemia within 4 weeks after transplantation. This effect was maintained for 12 weeks correlated with hepatic differentiation of the engrafted cells in recipient liver (Okura et al., 2011). Conversely, Tsai et al. (2009) did not observe the transdifferentiation toward hepatocyte-like cells of human MSCs from umbilical cord Wharton's jelly while the percentage of liver fibrosis was significantly reduced in rat model with CCl_4-induced liver fibrosis. This study suggested that bioactive cytokines released from undifferentiated MSCs may play important roles in reducing hepatic inflammation and restoring liver function in recipients. Notably, these studies have shown a low percentage of engrafted MSCs at maximum level of 20% in recipient liver of animal model. These results led to the question whether hepatocyte-like cells differentiation of MSCs *in vivo* have enough to compensate the liver function and improvement in liver injury.

Recently, some studies are focusing on using MSCs-derived hepatocyte-like cells for transplantation instead of undifferentiated cells. Several studies generated hepatocyte-like cells *in vitro* by chemically defined

cocktail medium before administration *in vivo*. Banas et al. (2009) successfully differentiated human AT-MSCs into hepatocyte-like cells under appropriate condition *in vitro*. The differentiated cells could restore the liver function of acute liver failure mice by decreasing serum ALT, AST, and ammonia levels within 24 h post-transplantation. Similarity, The hepatocyte-like cells clusters derived from human AT-MSCs had hepatic characteristics after exposure to hepatogenic inducer not only *in vitro* but also *in vivo*. The improvement of serum albumin and total bilirubin levels were observed after transplantation the cell clusters into non-obese diabetic severe combined immunodeficiency (NOD-SCID) mice with chronic liver injury (Okura et al., 2010). Recent study demonstrated the reducing liver fibrosis effect of intravenous injection with rat bone marrow MSC-derived hepatocyte-like cells in rats with CCl_4-induced liver fibrosis. This study suggested that the transplanted cells may induce host immune response to rescue the disease by increasing expression of interleukin 10 (IL-10) which can further decrease the accumulation of liver fibrosis (Zhao et al., 2012). Hepatic lineage cells derived from pretreated mice MSCs with injured liver tissue provided promising therapeutic potential in CCl_4-induced liver fibrosis mice. This result was confirmed by reducing liver fibrotic area as well as improving serum levels of bilirubin and alkaline phosphatase (ALP) in transplanted group (Mohsin et al., 2011).

2.2.9.2.2 Studies in clinical models (human)

MSCs-based therapy has shown as promising tool for chronic liver diseases especially in cirrhosis condition. Most of these clinical trials stay in phase I or II that verified the safety and efficacy of the treatment. All studies have shown the efficacy of MSCs-based therapy without serious side effects or

complications. Autologous BM-MSCs were successfully transplanted into patients with decompensated liver cirrhosis. The cells could improve the overall of disease particularly decreasing the model for end-stage liver disease (MELD) score. Moreover, the quality of life of all patients was improved that was observed by increasing the mean physical component scale and the mean mental component scale at 1 year post-transplantation (Mohamadnejad et al., 2007). Another study showed the therapeutic effect of autologous BM-MSCs transplantation in patients with end-stage liver disease. The liver function of all patients was improved within 24 weeks after transplantation that was monitored by decreasing MELD score as well as serum creatinine level (Kharaziha et al., 2009). Recently, El-Ansary et al. (2012) compared the therapeutic effect of autologous transplantation between undifferentiated BM-MSCs and differentiated cells (hepatocyte-like cells) in patients with liver cirrhosis. The improvement of liver function was observed in patients after transplantation with both undifferentiated and differentiated cells such as increasing prothrombin and serum albumin levels including decreasing bilirubin and MELD score. Additionally, they did not observe significant difference in clinical improvement between two groups of patients. In consistency with El-Ansary's study, Amer et al. (2011) demonstrated the safety and short-term therapeutic effect of autologous transplantation with BM-derived hepatocyte-like cells in patients with end-stage liver failure. Clinical improvement was verified by Child score, MELD score, fatigue scale, performance status, and serum albumin level. Recent study investigated the safety and efficacy of umbilical cord-derived MSC (UC-MSC) in patients with decompensated liver cirrhosis. During 1 year follow-up period, the significant reduction of ascites volume and the improvement of liver function were

observed in transplanted patients compared with control group (Zhang et al., 2012). Similarly, recent study observed the short-term efficacy of autologous BM-MSCs transplantation in liver failure patients caused by viral hepatitis B. The liver function and MELD score were significantly improved in the transplantation group compared with control group for 2-3 weeks post-transplantation and these effects could extend for 36 weeks (Peng, Xie, Lin, Liu, Zhu, Xie, Zheng, and Gao, 2011). All data indicate that MSCs can be used as regenerative medicine in liver disease, although it seems to exert effective treatment in a short term. Furthermore, increasing studies are required to design experiments that can complete define the therapeutic effects of MSCs in clinical use.

CHAPTER III

MATERIALS AND METHODS

3.1 Isolation and expansion of WJ-MCSs

3.1.1 Isolation and culture of WJ-MSCs

Fresh human umbilical cords were collected after birth by cesarean section in the Department of Obstetrics and Gynecology at Por-Pat Hospital, St. Mary Hospital, and Fort Suranari Hospital, Nakhon Ratchasima, Thailand. Written inform of consent was obtained from the mother under the approval of Institute of Research and Development of Suranaree University of Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand. The umbilical cords were collected in phosphate buffer saline (PBS) supplemented with 200 U/mL penicillin (Sigma-Aldrich), 200 µg/mL streptomycin (Sigma-Aldrich), and 4 µg/mL amphotericin B (E.R. Squibb & Sons) prior to restore at 4ºC for further WJ-MSCs isolation. After removal of blood vessels, tissues were dissected into small pieces and then washed with an equal volume of PBS (200 U/mL penicillin (Sigma-Aldrich), 200 µg/mL streptomycin (Sigma-Aldrich), and 4 µg/mL amphotericin B (E.R. Squibb & Sons). The suspension was centrifuged at 1200 rpm, 4ºC for 5 minutes and supernatant was discarded. The precipitate (mesenchymal tissue) was digested with collagenase type I (2 mg/mL) (Gibco-Invitrogen) at 37ºC for 16-18 hours with agitation. An equal volume of Dulbecco's modified Eagle's medium with 1.0 g/L glucose (DMEM-LG) (Gibco-Invitrogen) containing 50% fetal bovine serum (FBS)

(HyClone) was added and centrifuged at 1200 rpm, 4ºC for 10 minutes. Cell pellet was then resuspended, counted and equally seeded into T25 cm^2 culture flask (Greiner Bio-One) and cultured them in various media and conditions as followed 1) C1 : DMEM-LG supplemented with 10% FBS (HyClone), 100 U/mL penicillin (Sigma-Aldrich), 100 µg/mL streptomycin (Sigma-Aldrich), and 2 µg/mL amphotericin B (E.R. Squibb & Sons), in 20% O_2 and 5% CO_2, 2) C2 : DMEM-LG supplemented with 10% FBS (HyClone), 100 U/mL penicillin (Sigma-Aldrich), 100 µg/mL streptomycin (Sigma-Aldrich), and 2 µg/mL amphotericin B (E.R. Squibb & Sons), in 5% O_2 and 5% CO_2, 3) C3 : Embryonic stem cells conditioned medium (ESCM), in 5% O_2 and 5% CO_2 and 4) C4 : ESCM supplemented with 10 ng/mL epidermal growth factor (EGF, Peprotech) in 5% O_2 and 5% CO_2. After 3-4 days of seeding, non-adherent cells were discarded while adherent cells were replaced with their respective fresh medium and incubated at 37ºC with 20% O_2 and 5% CO_2 for C1 and 5% CO_2 and 5% O_2 for C2-4. Primary culture was maintained for 8 days, thereafter, the cells were subpassaged every 3-4 days and expanded at a ratio of 1 to 3 or 4 until passage 8 or 4 weeks.

3.1.2 Proliferation and population doubling times analysis

To determine the effect of these different four conditions on WJ-MSCs proliferation *in vitro*, cells were plated at a low density of 5×10^3 cells/cm^2 in order to minimize surface area limitation and further cultured for 12 days. Cell proliferation was determined using haemocytometer and vital Trypan blue staining. Moreover, fold increase in total cell number was calculated by dividing the number of cells of each day by the number of cells at day 0. To determine WJ-MSCs doubling time, cells were plated at a density of 5×10^3 cells/cm^2 in a 6-well plate (TPP) and fed with their

respective growth medium as described above. The cells were counted daily at the same time for 6 days by using haemocytometer and vital Trypan blue staining. The population doubling time (PDT) was calculated by using online formula for PDT (www.doubling-time.com) which based on the formula: PDT = tlg2/(lgNH-lgNI) where NI is the inoculum cell number, NH is the cell harvest number, and t is the time of the culture (in hours).

3.1.3 Embryonic stem cells conditioned medium (ESCM) preparation

ESCM were generated from culturing H9 human ES cells line on human foreskin fibroblast feeders with serum-free medium that was prepared by using Knockout-DMEM (Gibco-Invitrogen) containing 20% Knockout serum replacement (Gibco-Invitrogen), 1% non-essential amino acids (Sigma), 1 mmol/L L-glutamine (Gibco-Invitrogen), 0.1 mmol/L β-mercaptoethanol (Gibco-Invitrogen), and 5 ng/mL basic fibroblast growth factor (bFGF, Peprotech). After 24 hours later, the culture medium was removed to be used as conditioned medium for culture of WJ-MSCs. This conditioned medium was filtrated by 0.2 μm syringe filter and stored at -20ºC until used. Unconditioned medium was identically prepared as ESCM but it did not expose to the cells.

3.1.4 Flow cytometry

Cultured cells at passage 4 and 7 were washed twice with 2% FBS/PBS and suspended in 2% FBS/PBS with saturating concentration of the following conjugated mouse-anti-human antibodies: CD29-phycoerythrin (PE), CD34-PE, CD44-PE, and CD45-fluorescein isothiocyanate (FITC) (all from BD Biosciences), and the unconjugated antibody: CD90 (BD Biosciences) for 1 hour at 4ºC. Unconjugated primary antibody was treated with a goat-anti-mouse-FITC-conjugated

secondary antibody (BD Biosciences) for 1 hour at room temperature. Thereafter, cells were washed twice with 2% FBS/PBS and fixed with 4% paraformaldehyde in PBS. Control samples were incubated with PBS instead of the primary antibody. Samples were analyzed using FACS Calibur (Becton Dickinson) collecting 20,000 events and the data analyzed by Cell Quest Software (Becton Dickinson).

3.1.5 RNA extraction and RT-PCR

Cultured cells were subjected to total RNA extraction using Total RNA Mini Kit (Tissue) (Geneaid) and treated with RNase inhibitor (Gibco-Invitrogen) according to the manufacturer's protocol. The cDNA was generated by RevertAid^{TM} First Stand cDNA Synthesis Kit (Fermentas) according to the manufacturer's protocol. The cDNA was then added into PCR master mix containing 10X PCR buffer, 1 U Taq polymerase, 25 mmol/L $MgCl_2$, 10 mmol/L dNTP mixed, and 10 µmol/L of each primer set for the corresponding target gene. The primer sequences were shown in Table 3.1. Amplification conditions were as followed: initial denaturation at 95ºC for 5 minutes followed by 35 cycles of denaturation at 95ºC for 45 seconds, annealing at 55-64ºC for 30 seconds (see table 3.1 refer to temperatures used), extension for 1 minute at 72ºC, and a final extension at 72ºC for 10 minutes. The samples were separated on a 2% agarose gel, stained with ethidium bromide and photographed under UV light.

Table 3.1 Primer sequences and PCR conditions involved in stemness gene expression.

Gene	Primer sequence (5' to 3')	Annealing temp. (ºC)	Product size (bp)	Reference
Oct-4	GAGCAAAACCCGGAGGAGT TTCTCTTTCGGGCCTGCAC	60	310	(Yao et al., 2006)
Nanog	GCTTGCCTTGCTTTGAAGCA TTCTTGACTGGGACCTTGTC	60	255	(Yao et al., 2006)
Oct-3/4	CTCACCCTGGGGGTTCTATT CTCCAGGTTGCCTCTCACTC	60	230	(Darr, Mayshar, and Benvenisty, 2006)
C-myc	TGGTCTTCCCCTACCCTCTCAAC GATCCAGACTCTGACCTTTTGCC	55	265	(Han et al., 2007)
Klf-4	GTTTTGAGGAAGTGCTGAG CAGTCACAGTGGTAAGGTTT	55	332	(Chiambaretta et al., 2004)
Sox-2	GCCCCCAGCAGACTTCACA CTCCTCTTTTGCACCCCTCCCATTT	64	170	(Tsukamoto et al., 2005)
GAPDH	AGCCACATCGCTCAGACACC GTACTCAGCGGCCAGCATCG	60	302	(Yao et al., 2006)

3.1.6 Mesodermal-lineage differentiation assays

WJ-MSCs were differentiated into adipocytes, chondroblasts, and osteoblasts by using STEM PRO Adipogenesis Differentiation Kit, STEM PRO Chondrogenesis Differentiation Kit, and STEM PRO Osteogenesis Differentiation Kit (all from Gibco-Invitrogen) according to the manufacturer's protocol, respectively. The medium was changed every 3 days. For adipocyte differentiation, after induction for four weeks, cells were fixed in 10% formalin and stained with fresh oil red O solution (Sigma-Aldrich) to detect fat deposition in the cells. For chondrogenic differentiation, after induction for three weeks, cells were fixed in 4% paraformaldehyde and stained with 1% alcian blue solution (Sigma-Aldrich) to detect proteoglycan synthesis. For osteogenic differentiation, to detect mineralization (calcium deposits), cells were fixed in 4% paraformaldehyde and stained with 2% alizarin red S solution (Sigma-Aldrich) after induction for two weeks. The differentiated cells were visualized and photographed under inverted microscope (Olympus CKX41, Olympus Corporation). Negative controls consisted of expanded MSCs from the same passage number which cultured in expansion medium and did not treat with osteogenic, adipogenic, and chondrogenic differentiation media.

3.1.7 Cytokine array analysis

Embryonic stem cells conditioned medium (ESCM) was collected to determine their proteins component by using the human cytokine array panel A (proteome profiler™) (R&D Systems) according to the manufacturer's instructions. The array includes 36 human cytokines panel of C5/C5a, CD40, G-CSF, GM-CSF, GROα, I-309, sICAM-1, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, IL-13, IL-16, IL-17, IL-17E, IL-23, IL-27, IL-32α, IP-10, I-TAC,

MCP-1, MIF, MIP-1α, MIP-1β, Serpin E1, RANTES, SDF-1, TNF-α, and sTREM-1. Unconditioned medium was used as control medium to compare the relative expression levels of each cytokine. Data were captured by exposure to Kodak BioMax Light film. Arrays were scanned into a computer and the detected signals were quantified as the pixel density by using Image J software (Image J, 1.45, NIH).

3.1.8 Statistical analysis

All data were presented as mean ± standard deviation (SD) calculation. Statistical analysis was done by statistical software SPSS17.0 and results were analyzed by Student's t-test with significant difference at $P< 0.05$.

3.2 Induction of hepatic-lineage differentiation *in vitro*

3.2.1 Induction of hepatic-lineage differentiation

WJ-MSCs at 90-100% confluency were prepared for differentiation assays. Cells were cultured in stage 1 differentiation medium as DMEM-LG (serum free, 100 U/mL penicillin (Sigma), 100 µg/mL streptomycin (Sigma), and 2 µg/mL amphotericin B (Abbott)) supplemented with 20 ng/mL human hepatocyte growth factor (HGF, Peprotech), 10 ng/mL fibroblast growth factor-4 (FGF-4, Peprotech), and 5 mmol/L nicotinamide (Sigma) for 7 days. Subsequently, cells were further cultured in stage 2 differentiation medium as DMEM-LG (serum free, 100 U/mL penicillin (Sigma), 100 µg/mL streptomycin (Sigma), and 2 µg/mL amphotericin B (Abbott)) supplemented with 40 ng/mL oncostatin M (OSM, Sigma), 2 µmol/L dexamethasone (Sigma), and 20 µl/mL insulin, transferrin and selenium premix (ITS+premix, BD Biosciences) for up to 18 days. Differentiation medium was

changed twice a week. In this study, we termed the differentiated cells as mesenchymal stem cells-derived hepatocyte-like cells (MSCDHC).

For additional medicinal plant extract, 200 ng/mL ethanolic leaves extract of *Gynura procumbens* was supplemented into the differentiation cocktail medium at various time points as day 1-7, day 8-13, day 8-18, and day 1-18 of differentiation period. The formula of differentiation cocktail medium was similar as described above but minor modification in stage 2 differentiation medium by decreasing the concentration of cytokines down to one fold. Moreover, the induction medium without additional the hepatogenic cytokines but supplementation with this extract only was used to confirm their effect on hepatic differentiation of WJ-MSCs. *Gynura procumbens* leaves extract was kindly gifted from Asst. Prof. Dr. Wilairat Leeanansaksiri.

3.2.2 Preparation of plant extract

Gynura procumbens leaves were collected from home-garden, Khon Kaen province, Thailand. The leaves were washed well three times by tap water and then wash one time again by distilled water. The fresh leaves (1 kg) were ground with 95% ethanol (1 L) by an electrical grinder. It was then filtered through a filter paper, evaporated and freeze-dried. The yield of *G. procumbens* was 9 g/ 1 kg. The dried powder was stored at -20ºC until further use.

3.2.3 Immunocytochemical staining

On day 7, 12, and 18 of hepatocytes differentiation, the differentiated cells were fixed with ice cold absolute methanol or 4% paraformaldehyde for 10 minutes at room temperature and permeabilized thereafter with 0.3% Triton X-100 in PBS for 20 minutes. Nonspecific immunostaining was prevented by 30 minutes

incubation of cells in PBS solution containing 3% bovine serum albumin (BSA, Gibco-Invitrogen) at room temperature. Cells were incubated in blocking solution overnight at 4ºC with primary antibodies as followed: rabbit polyclonal anti-human albumin (ALB) and mouse monoclonal anti-human cytokeratin 18 (CK-18) (all 1:100, DakoCytomation). Cells were washed 3 times with PBS and incubated for 1 hour at room temperature with the secondary antibodies FITC-coupled polyclonal goat anti-mouse immunoglobulin G (1:100, BD Biosciences) or FITC-coupled polyclonal swine anti-rabbit immunoglobulin G (1:100, DakoCytomation). Nuclei was revealed by 3 minutes of staining with the nuclear dye 4',6-diamidino-2-phenylindole (DAPI, Gibco-Invitrogen). After washing 3 times, preparations were mounted with anti-fading solution (Vector Laboratories) and examined under fluorescence microscope (Olympus BX51, Olympus Corporation).

3.2.4 Periodic acid–schiff staining

After 4% paraformaldehyde fixation, the differentiated cells at day 7, 12, and 18 were incubated for 5 minutes in 1% periodic acid (Sigma), washed with distilled water and incubated with Schiff's reagent (Sigma) for 15 minutes. After washing with tap water for 5 minutes, the differentiated cells were then counterstained with Mayer's hematoxylin (Sigma) for 1 minute. The preparations were washed and visualized under inverted microscope (Olympus CKX41, Olympus Corporation).

3.2.5 Urea assay

On day 7, 12, and 18 of differentiation, the differentiated cells were incubated with DMEM-LG (serum free) containing 5 mmol/L NH_4Cl (Sigma) for 24 hours. The supernatants were collected and measured urea concentration by colorimetric assay as QuantiChrom^{TM} Urea Assay Kit (BioAssay Systems) according

to the manufacturer's instructions with an absorbance reader (Benchmark Plus, Bio-Rad Laboratories). Day 0 (refer to undifferentiated cells) was represented as negative control and human hepatocellular carcinoma cell line (HepG2) was represented as positive control.

3.2.6 Albumin assay

Conditioned media from the differentiated cells were collected at day 7, 12, and 18 of differentiation period and frozen at -20ºC until determination. Human albumin concentration in supernatants was screened by ELISA assay by using AssayMax Human Albumin ELISA Kit (Assaypro) according to the manufacturer's instructions with an absorbance reader (Benchmark Plus, Bio-Rad Laboratories). The conditioned media from day 0 or undifferentiated cells and HepG2 were represented as negative control and positive control, respectively.

3.2.7 Uptake of 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-acetylated-low Density Lipoprotein (Dil-Ac-LDL)

The capacity of low density lipoprotein uptake was determined in the differentiated cells at day 7, 12, and 18 using fluorescent probe conjugated Dil-Ac-LDL (Biomedical Technologies) according to the manufacturer's instructions. The preparations were washed and visualized under fluorescence microscope (Olympus BX51, Olympus Corporation).

3.2.8 RNA extraction and RT-PCR

On day 7, 12, and 18 of differentiation, total RNA of the differentiated cells was isolated by using Total RNA Mini Kit (Tissue) (Geneaid) and treated with RNase inhibitor (Gibco-Invitrogen) according to the manufacturer's protocol. The resulting products were amplified using primer sequences as shown in Table 3.2. The

cDNA was generated by using RevertAid^{TM} First Stand cDNA Synthesis Kit (Fermentas) according to the manufacturer's protocol. The corresponding cDNA was added into PCR master mix containing 10X PCR buffer, 1 U Taq polymerase, 25 mmol/L $MgCl_2$, 10 mmol/L dNTP mix, and 10 μmol/L of each primer for the corresponding forward and reverse gene target. Amplification conditions were as followed: initial denaturation at 95ºC for 5 minutes followed by 35 cycles of denaturation at 94ºC for 30 seconds, annealing at 56-62ºC for 30 seconds (see table 3.2 refer to temperatures used), extension for 45 seconds at 72ºC, and a final extension at 72ºC for 10 minutes. The samples were separated on a 2% agarose gel, stained with ethidium bromide and photographed under UV light. Undifferentiated cells and HepG2 were represented as a negative control and positive control, respectively.

Table 3.2 Primer sequences and PCR conditions involved in liver-specific gene expression.

Gene	Primer sequence (5' to 3')	Annealing temp. (ºC)	Product size (bp)	Reference
AFP	GCTTGGTGGTGGATGAAACA TCCTCTGTTATTTGTGGCTTTTG	62	157	(Talens-Visconti et al., 2006)
ALB	TGAGAAAACGCCAGTAAGTGAC TGCGAAATCATCCATAACAGC	62	265	(Talens-Visconti et al., 2006)
CYP3A4	CCTTACAT TACACACCCTTTGGAAGT AGCTCAATGCATGTACAGAATCCCCGGTTA	62	382	(Talens-Visconti et al., 2006)
HNF4α	GCCTACCTCAAAGCCATCAT GACCCTCCCAGCAGCATCTC	62	275	(Talens-Visconti et al., 2006)
AAT	ACATTTACCCAAACTGTCCATT GCTTCAGTCCCTTTCTCGTC	56	183	(Cai et al., 2007)
TTR	GGTGAATCCAAGTGTCCTCTGAT GTGACGACAGCCGTGGTGGAA	61	352	(Baharvand, Hashemi, Kazemi, and Farrokhi, 2006)
G6P	GCTGGAGTCCTGTCAGGCATTGC TAGAGCTGAGGCGGAATGGGAG	56	350	(Cai et al., 2007)

Table 3.2 (Continued).

Gene	Primer sequence (5' to 3')	Annealing temp. (ºC)	Product size (bp)	Reference
GAPDH	AGCCACATCGCTCAGACACC	60	302	(Yao et al., 2006)
	GTACTCAGCGGCCAGCATCG			

3.2.9 Statistical analysis

All data were presented as mean ± standard deviation (SD) calculation. Statistical analysis was done by statistical software SPSS17.0 and results were analyzed by Student's t-test with significant difference at $P< 0.05$.

CHAPTER IV

RESULTS

4.1 Cell morphology, proliferation, and population doubling time of WJ-MCSs

To determine whether ESCM is an appropriate medium for supporting proliferation of WJ-MSCs, the cells were cultured with ESCM (C3) and ESCM supplemented with 10 ng/mL EGF (C4) in comparison with the most commonly used culture medium for MSCs as DMEM-LG supplemented with 10% FBS both in normoxic condition 20% O_2, 5% CO_2 (C1) and hypoxic condition 5% O_2, 5% CO_2 (C2). Furthermore, the expanded cells were examined cell morphology, proliferation and population doubling time (PDT) at early and late passages.

This study successfully isolated and expanded WJ-MSCs *in vitro* by using the isolation protocol and various culture conditions as described in materials and methods. However, the cells cultured in C1 showed low proliferation capacity throughout *in vitro* culture period (Figure 4.1A). C1 could expand a maximum of cell numbers only $4.30 \pm 0.60 \times 10^4$ at day 23 and the growth rate continually declined after that. Moreover, decreasing cell growth correlated with altered cell morphology from small spindle shape like fibroblast into large, flat, and broad shape after P6 onward (Figure 4.1B). These findings indicate the limited life span and senescence of the expanded cells from C1. Based on these results, we excluded these cells for

further experiments because C1 could not maintain normal WJ-MSCs morphology and support WJ-MSCs expansion to reach enough cells for further characterization. These results demonstrate that normoxic condition is not appropriate for *in vitro* expansion of WJ-MSCs. On the contrary, WJ-MSCs could be significantly expanded by C2-4 under hypoxic condition. Thus, the expanded cells from C2-4 were further characterized for cell proliferation, fold increase, and population doubling time during *in vitro* culture. To determine cell proliferation, WJ-MSCs at early passages (P2-4) were cultured in their respective medium for 2 weeks and counted every 3 days to plot proliferation curve. From a low initial cell numbers, these cells could be expanded in maximal cell numbers of $5.68 \pm 2.18 \times 10^6$ from C2, $10.23 \pm 2.07 \times 10^6$ from C3, and $21.24 \pm 2.58 \times 10^6$ from C4 within 12 days (Figure 4.2A). C4 could more promote WJ-MSCs proliferation than C2 and C3 throughout the culture period. Notably, WJ-MSCs grown in ESCM-based medium C3 and C4 had more proliferation level than those cultured in conventional medium C2. Greater proliferation potential of cells grown in C3 and C4 was confirmed by higher fold increase rate. C4 gave the highest fold increase of 6.49 ± 0.16 (day 3), 24.11 ± 1.50 (day 6), 99.56 ± 3.00 (day 9), and 424.88 ± 14.62 (day 12) folds as compared with day 0. Subsequently, C3 gave the fold increase of 4.11 ± 0.26, 15.29 ± 1.19, 52.58 ± 2.18, and 204.66 ± 10.39 folds after cultivation for 3, 6, 9, and 12 days, respectively. The lowest fold increase was observed in the cultured cells from C2 which had capacity to expand on day 3 as 2.93 ± 0.40 fold, day 6 as 9.27 ± 0.52 fold, day 9 as 32.67 ± 1.73 fold, and day 12 as 113.77 ± 7.89 fold (Figure 4.2B). These results were consistent with mean population doubling time (PDT) calculation. The PDT data showed that WJ-MSCs cultured in C3 (37.13 ± 0.64 h) and C4 (33.57 ± 1.00 h) had a shorter mean doubling time as

compared to those cultured in C2 (41.10 ± 0.90 h) at early passage (P4). At late passage (P7), these cells had longer mean doubling time approximately 2 h from early passage as 43.30 ± 0.89 h, 39.00 ± 1.32 h, and 35.70 ± 1.60 h when culturing in C2, C3 and C4, respectively (Figure 4.3A-C). Taken together, these data support the extensive increasing cell growth after expanding in C3 and C4 for 2 weeks which gave maximum cell yield in a short period of time.

Although C2 poorly supported WJ-MSCs proliferation at early passage, the cells were continually expanded with time increasing to P15 whereas the expanded cells from C3 and C4 had slower cell growth after P8 onward (data not shown). These findings could be explained by the present of some cells aggregation into spheroid shape after long term maintaining in C3 and C4 from P8 onward (data not shown). Moreover, we found that these spheroid cells could be expanded as normal morphology as a monolayer after fed with C2. Based on these data, this feature may cause by long exposure to serum free-containing medium like C3 and C4. These results indicate that C3 and C4 are great for short term expansion but may not suitable for long term WJ-MSCs expansion. Focusing on cell morphology, primary culture of WJ-MSCs displayed as epithelioid cells in combination with short spindle shape in all conditions. Afterward, these cells transformed into long spindle shape like fibroblast cells (Figure 4.4). No significant difference of cell morphology was observed in the expanded cells from all conditions excluding at late passage which is described as above.

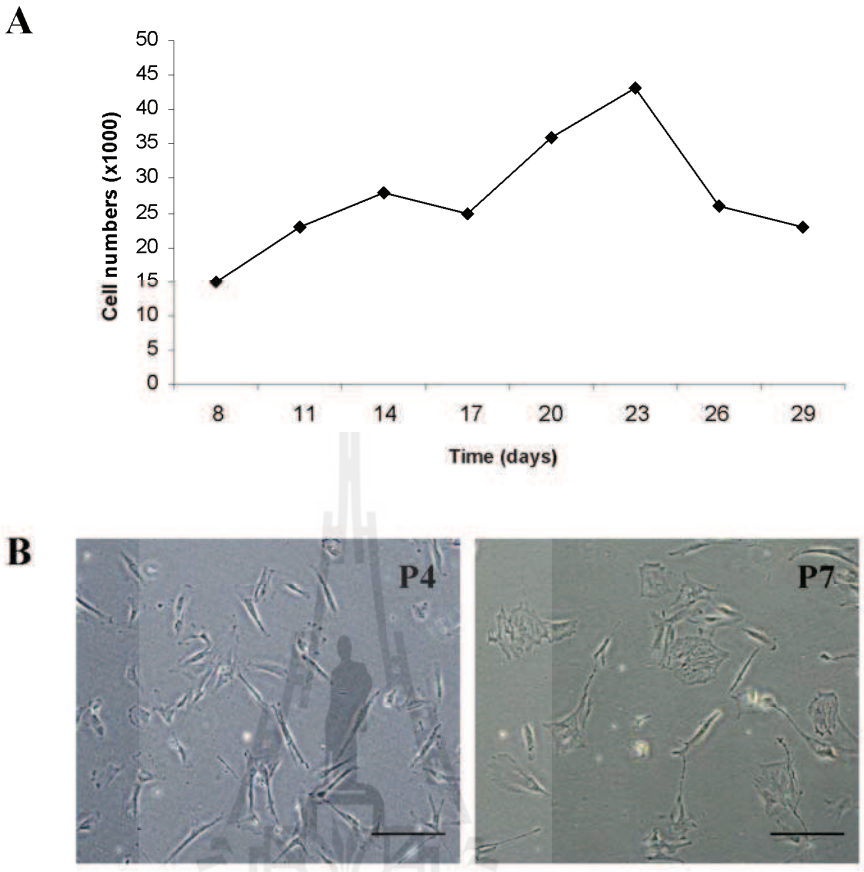


Figure 4.1 Growth characteristic and proliferation of WJ-MSCs cultured in C1 under normoxic condition (A). Cell morphology of WJ-MSCs cultured in C1 at P4 and P7 (B). All data are presented as mean ± SD (n = 3). Magnification x40. Scale bar = 300 μm.

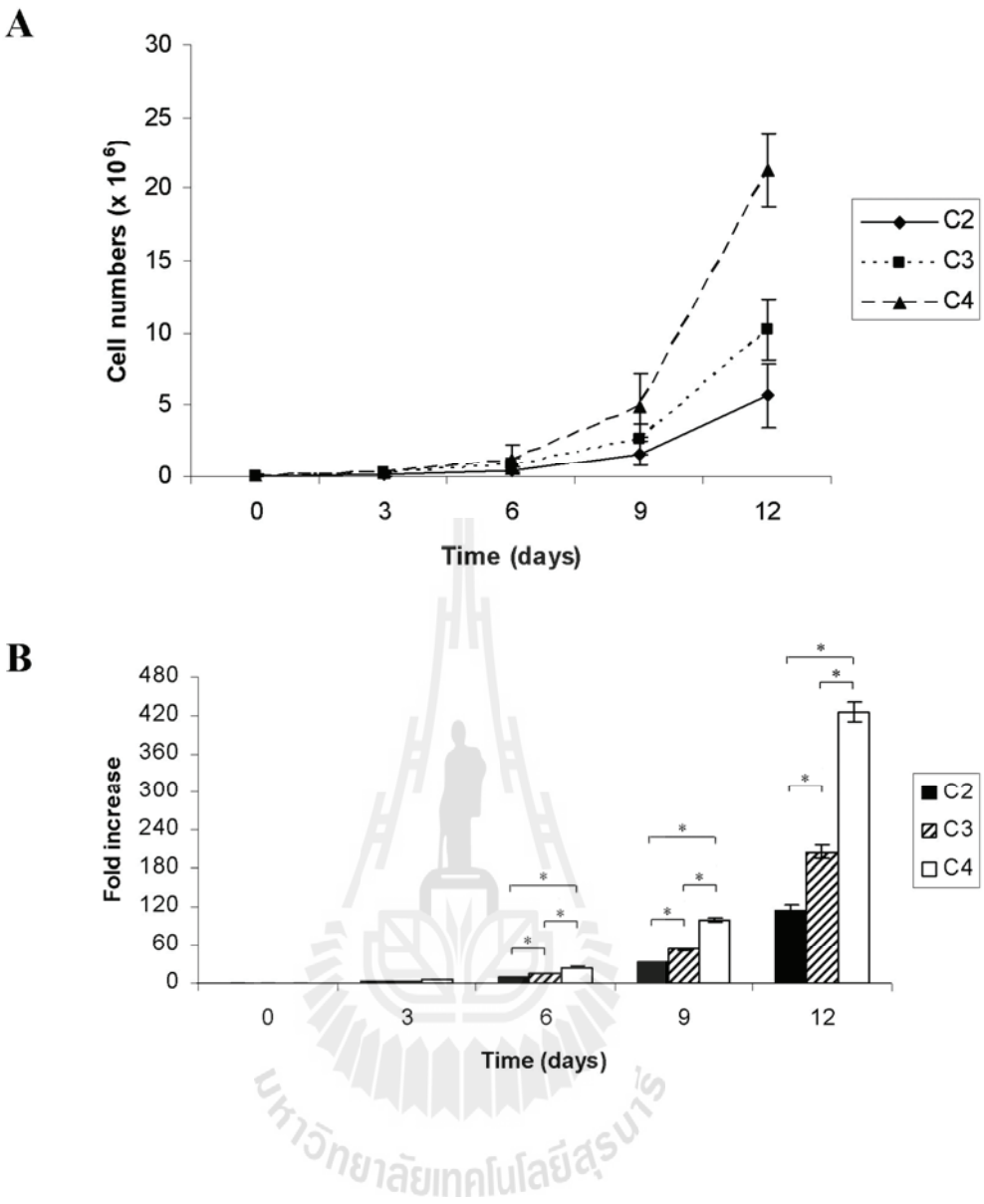


Figure 4.2 Proliferation and fold increase of WJ-MSCs cultured in C2-4 under hypoxic condition. Total cell numbers were determined throughout the culture time for 12 days (A). Fold increase values are presented as mean ± SD (n = 3) (B). *P< 0.05

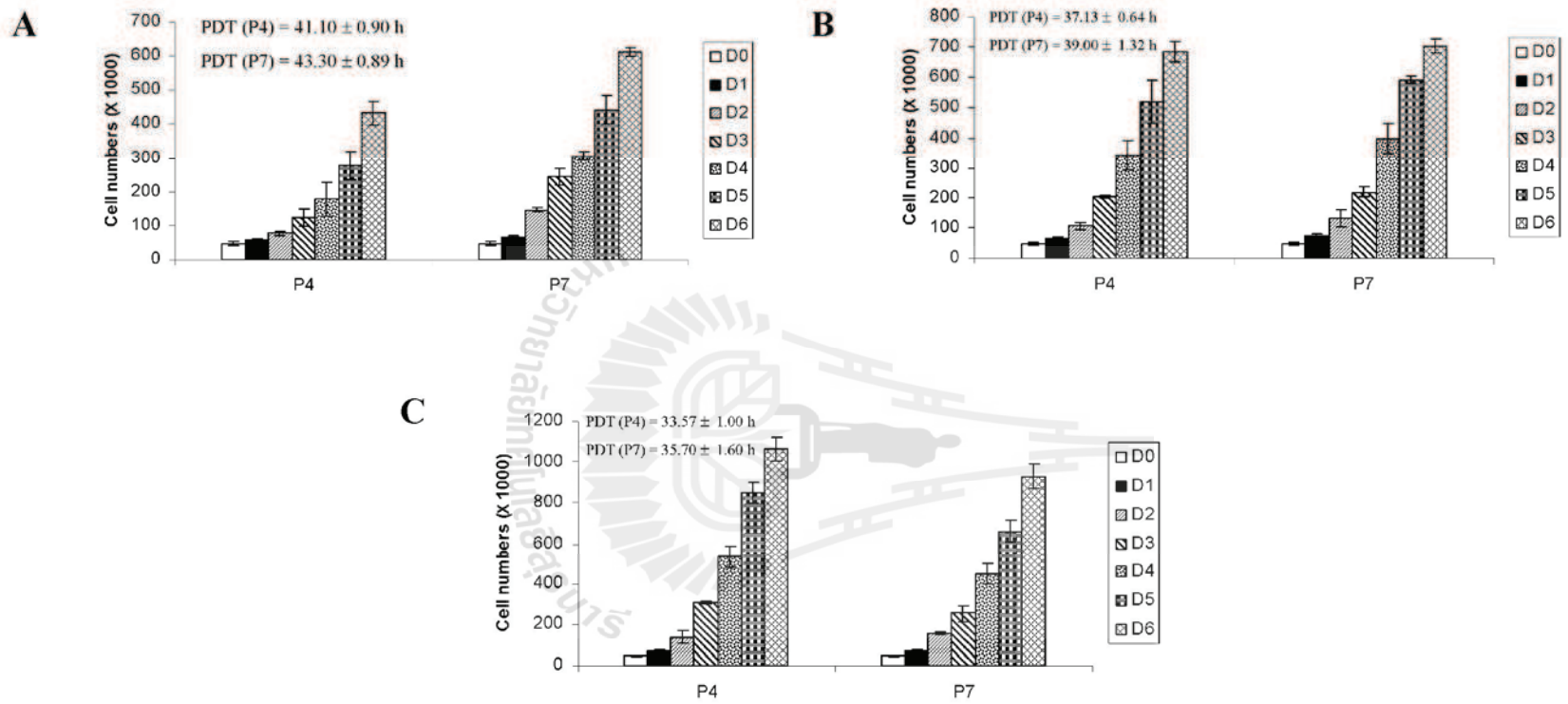


Figure 4.3 Population doubling time (PDT) of WJ-MSCs cultured in C2 (A), C3 (B), and C4 (C) at P4 and P7. All data are presented as mean ± SD (n = 3). D1-D6 = day in culture.

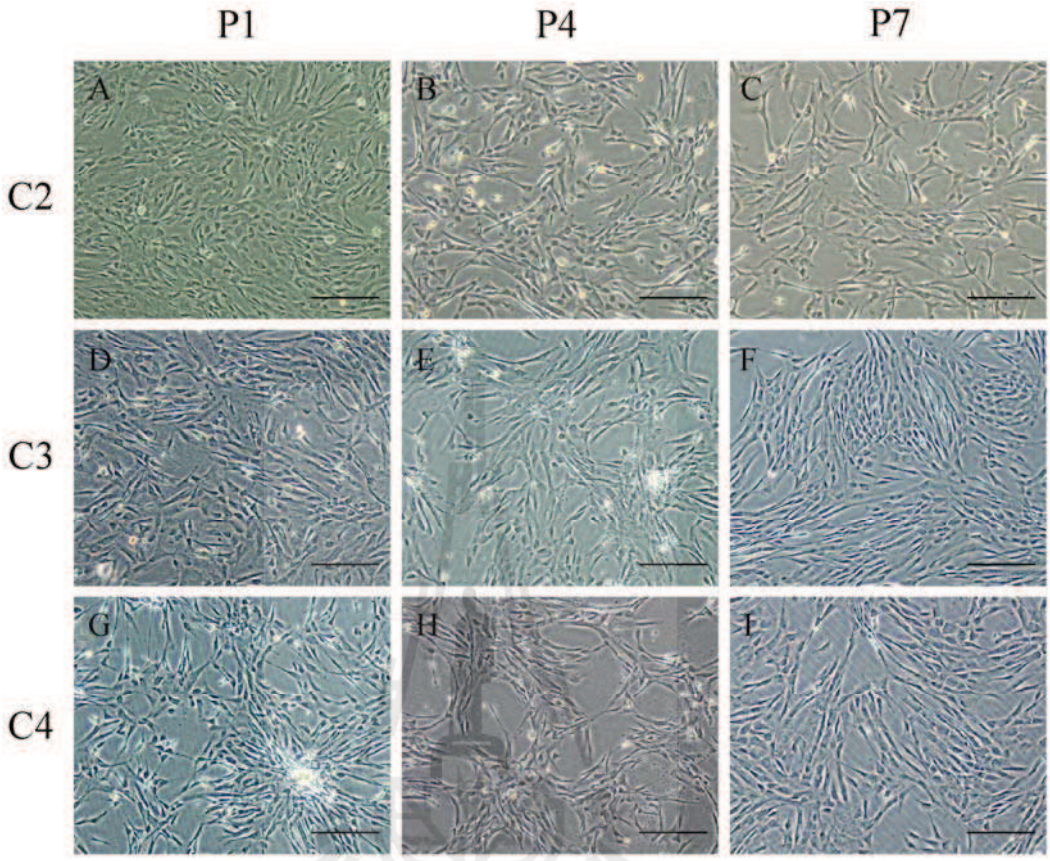


Figure 4.4 Cell morphology of WJ-MSCs cultured in C2 (A-C), C3 (D-F), and C4 (G-I) at various passages. Magnification x40. Scale bar = 300 μm.

4.2 Characterization of the expanded WJ-MSCs

To determine the effect of ESCM on MSCs characteristics, the expanded cells from C3 and C4 were further characterized in comparison with those cultured in conventional medium C2. All common characteristics of MSCs including immunophenotypes, stemness gene expressions, and mesodermal-lineage differentiation potential were tested in the expanded cells at early and late passages.

4.2.1 Immunophenotypes

WJ-MSCs cultured in C2-4 were characterized for specific cell surface antigens by flow cytometry analysis. The expanded cells from all conditions were positive for CD29, CD44, and CD90 which are commonly expressed on MSCs. Moreover, these cells were negative for haematopoietic stem cells markers as CD34 and CD45. Percent expression of these markers was quite similar in the expanded cells from different conditions (Table 4.1 and Figures 4.5-4.7). At early passage (P4), high positive percentages ranging from 89.87 ± 2.44% to 98.90 ± 0.20% were observed for CD29, CD44, and CD90. At late passage (P7), high positive percentages ranging from 94.98 ± 2.13% to 98.36 ± 0.29% were maintained for CD29 and CD44, whereas percent expression of CD90 was no significant decrease at C3 and C4 (Table 4.1 and Figures 4.5-4.7).

Table 4.1 Immunophenotypes characterization of WJ-MSCs cultured in C2-4 at P4 and P7 by flow cytometry analysis. The table shows mean values of percent positive cells ± SD to the total number of cells analyzed (n = 3).

	Condition	CD29	CD44	CD90	CD34	CD45
P4	C2	96.54 ± 1.63	94.33 ± 2.49	89.87 ± 2.44	0.61 ± 0.28	0.29 ± 0.08
	C3	95.64 ± 1.36	96.39 ± 2.16	94.83 ± 0.22	0.30 ± 0.04	0.27 ± 0.05
	C4	98.90 ± 0.20	98.44 ± 0.17	96.15 ± 1.33	0.97 ± 0.31	0.63 ± 0.26
P7	C2	95.47 ± 1.98	94.98 ± 2.13	89.86 ± 2.98	0.47 ± 0.14	0.43 ± 0.21
	C3	97.56 ± 1.06	96.58 ± 0.46	87.11 ± 4.83	0.88 ± 0.11	0.43 ± 0.06
	C4	98.36 ± 0.29	97.81 ± 0.05	88.30 ± 6.33	1.57 ± 0.14	0.77 ± 0.14

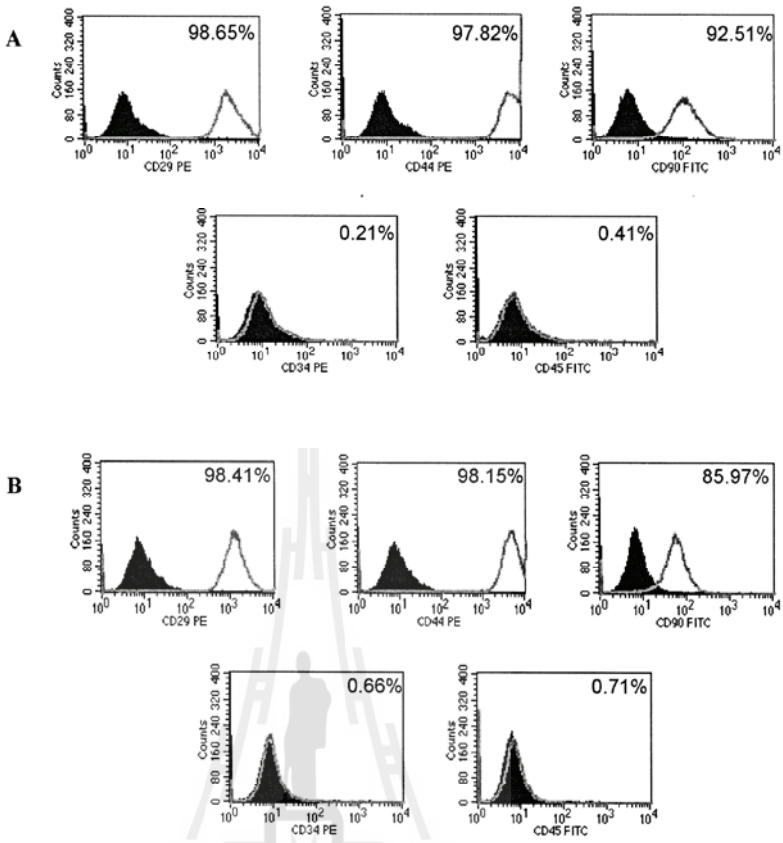


Figure 4.5 Representative immunophenotype characteristics of WJ-MCs cultured in C2 at P4 (A) and P7 (B) by flow cytometry analysis. Shaded histogram represents unstained controls, while open histogram represents positive staining with the indicated antibody.

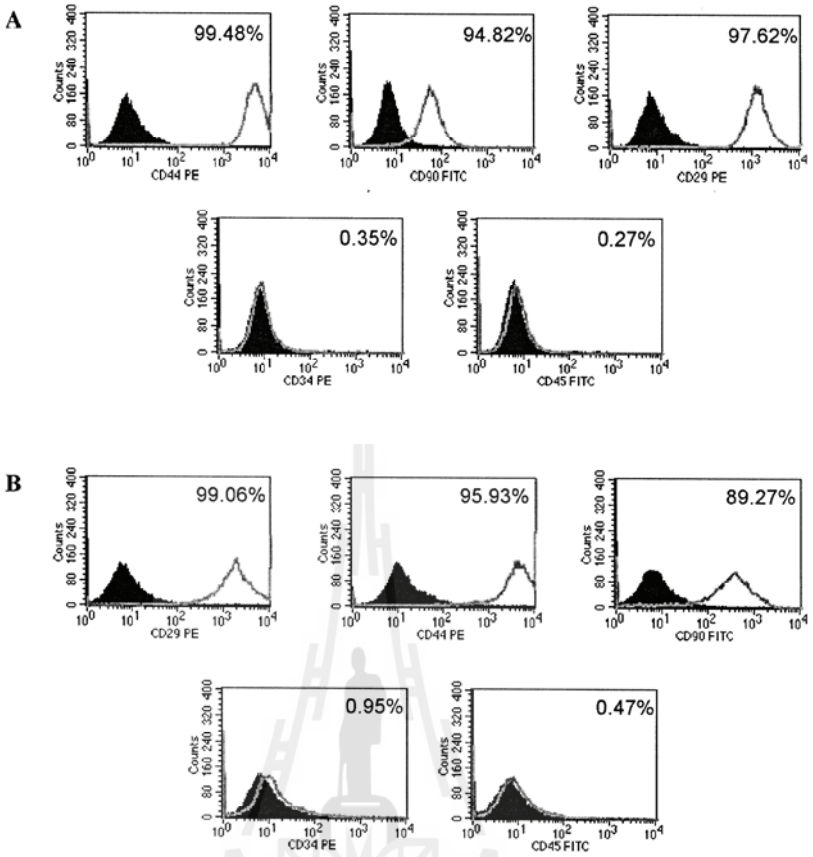


Figure 4.6 Representative immunophenotype characteristics of WJ-MCs cultured in C3 at P4 (A) and P7 (B) by flow cytometry analysis. Shaded histogram represents unstained controls, while open histogram represents positive staining with the indicated antibody.

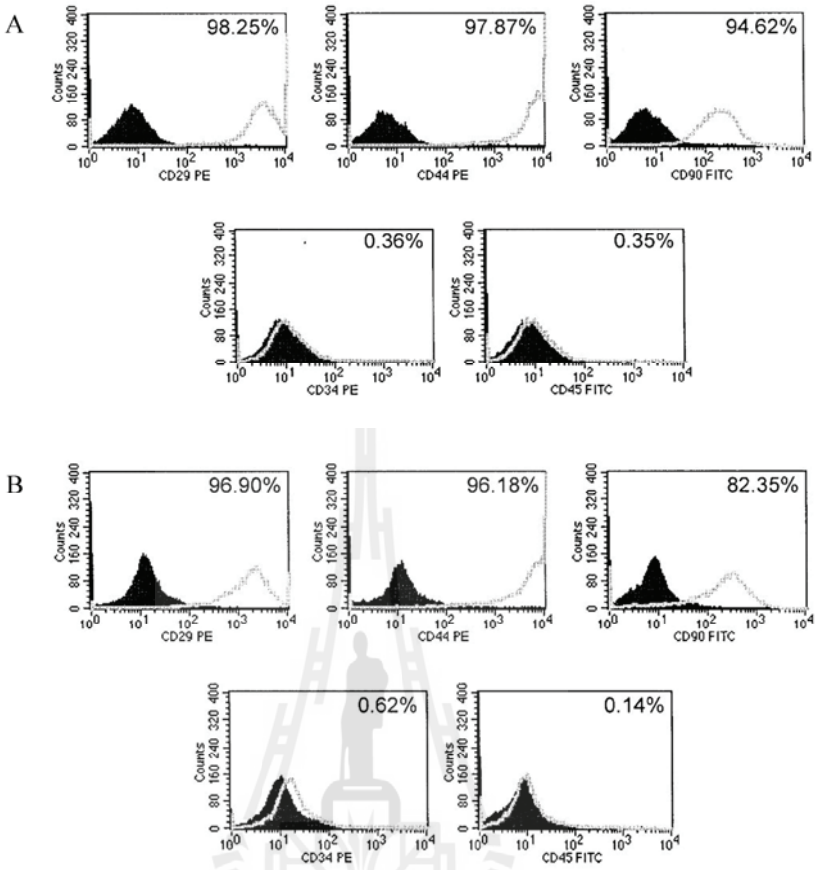


Figure 4.7 Representative immunophenotype characteristics of WJ-MCs cultured in C4 at P4 (A) and P7 (B) by flow cytometry analysis. Shaded histogram represents unstained controls, while open histogram represents positive staining with the indicated antibody.

4.2.2 Stemness gene expressions

The effect of an *in vitro* cultivation on stemness gene expressions of the expanded cells from different conditions was also investigated by RT-PCR analysis. These markers are key pluripotency-related genes essential for maintenance self-renewal and pluripotency properties of stem cells including embryonic stem cells. At early passage (P4), all stemness genes including *Oct-4*, *Oct-3/4*, *Nanog*, *Klf-4*, *C-Myc*, and *Sox-2* were expressed by the expanded cells from all conditions (Figure 4.8A). Additionally, the downregulation of *Oct-4* and *Nanog* were observed in the expanded cells from C2 at late passage (P7) as compared to those cultured in C3 and C4 (Figure 4.8B). These results indicate that ESCM-based medium C3 and C4 could more support pluripotency properties of WJ-MSCs than conventional medium C2 even entry to late passage. Moreover, cells cultured from C2 seem to decrease the expression of pluripotency markers, *Oct-4* and *Nanog*, with the increasing of cell passages number.

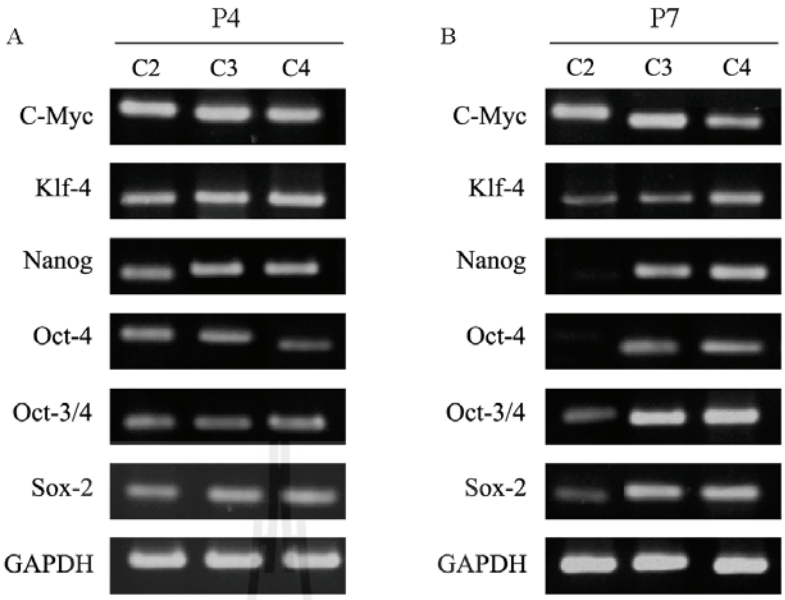


Figure 4.8 Stemness gene expressions of WJ-MSCs cultured in C2-4 at P4 (A) and P7 (B) by RT-PCR analysis. *GAPDH* mRNA expression was detected as PCR positive control and input template control.

4.2.3 Mesodermal-lineage differentiation potential

To investigate mesodermal-lineage differentiation potential of WJ-MSCs cultured in different conditions, the expanded cells at P4 and P7 were induced into adipogenic, chondrogenic, and osteogenic lineages by using commercial differentiation media. Both early passage (P4) and late passage (P7), the expanded cells from all conditions successfully differentiated toward adipogenic, chondrogenic, and osteogenic lineages which were identified by oil red O staining, 1% alcian blue staining, and 2% alizarin red S staining, respectively (Figures 4.9 and 4.10). For adipogenic differentiation, the highest lipid vacuoles formation was observed in the expanded cells from C2 as compared to those cultured in C3 and C4. These results may imply that the expanded cells from C3 and C4 had lower differentiation potential into adipogenic lineage. However, the expanded cells from all conditions had differentiation potential into chondrogenic and osteogenic lineages in the same level both at early and late passages. Altogether, these results indicate that WJ-MSCs cultured in ESCM-based medium C3 and C4 had differentiation potential into mesodermal lineages similar to common characteristics of MSCs.

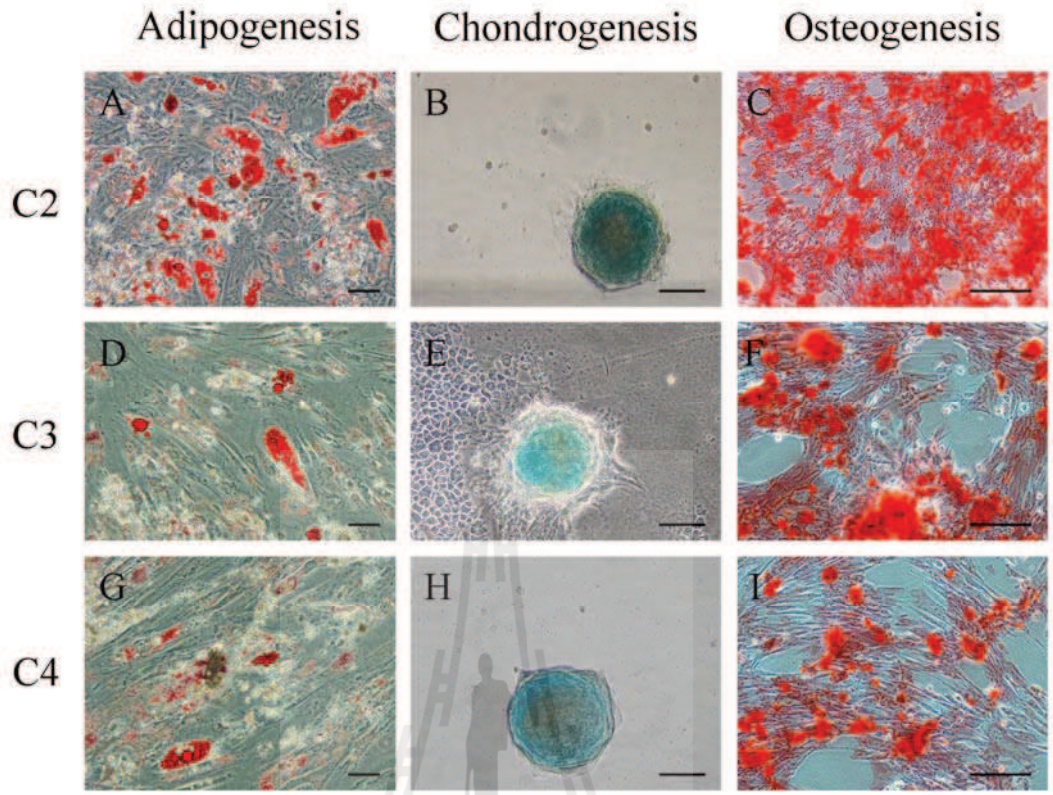


Figure 4.9 Adipogenic, chondrogenic, and osteogenic differentiations of WJ-MSCs cultured in C2 (A-C), C3 (D-F), and C4 (G-I) at P4, respectively. Adipogenic differentiation was evidence by oil red O staining of lipid vacuoles. Chondrogenic differentiation was examined by 1% alcian blue staining of proteoglycan synthesis. Osteogenic differentiation was confirmed by 2% alizarin red S staining of mineralized deposit. Magnification x200, x100, and x40 for adipogenic, chondrogenic, and osteogenic differentiations, respectively. Scale bar = 300 µm, 100 µm, and 50 µm for osteogenic, chondrogenic, and adipogenic differentiations, respectively.

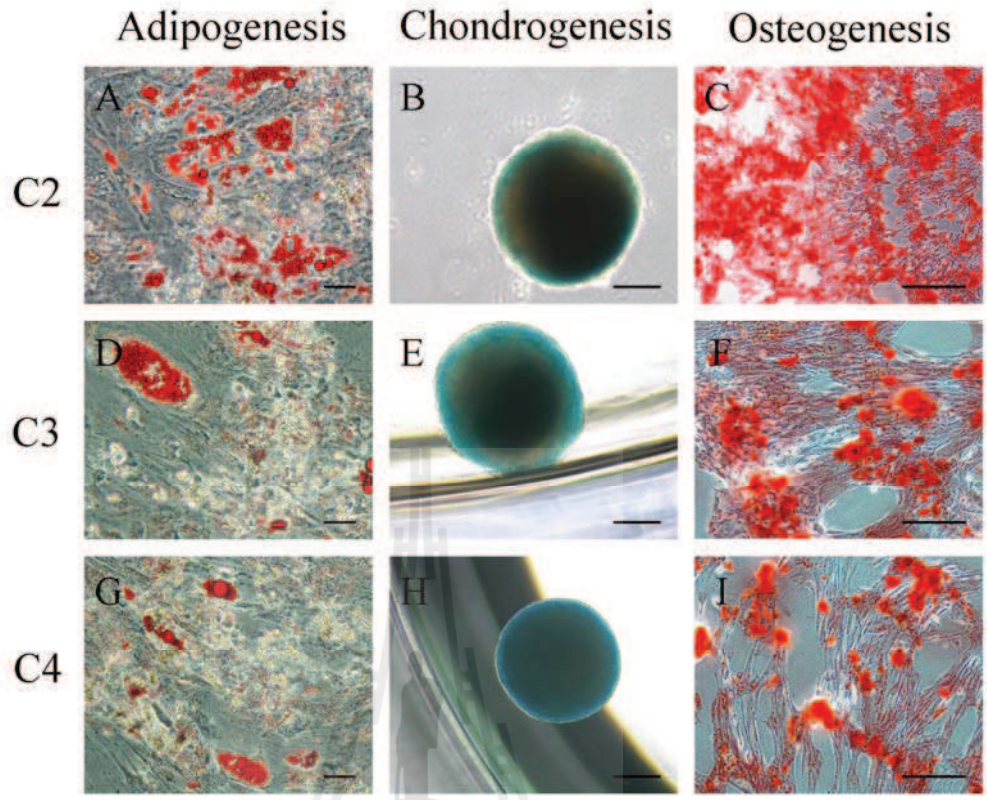


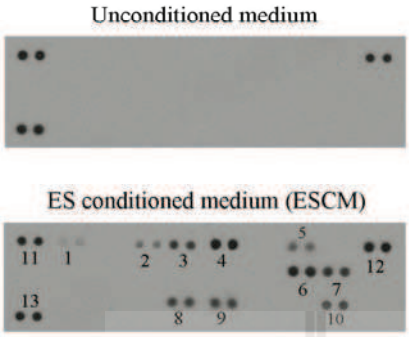
Figure 4.10 Adipogenic, chondrogenic, and osteogenic differentiations of WJ-MSCs cultured in C2 (A-C), C3 (D-F), and C4 (G-I) at P7, respectively. Adipogenic differentiation was evidence by oil red O staining of lipid vacuoles. Chondrogenic differentiation was examined by 1% alcian blue staining of proteoglycan synthesis. Osteogenic differentiation was confirmed by 2% alizarin red S staining of mineralized deposit. Magnification x200, x100, and x40 for adipogenic, chondrogenic, and osteogenic differentiations, respectively. Scale bar = 300 µm, 100 µm, and 50 µm for osteogenic, chondrogenic, and adipogenic differentiations, respectively.

4.3 Growth factors, chemokines, and cytokines component in ESCM

To investigate proteins component in ESCM that may involve in enhancing WJ-MSCs proliferation *in vitro*, cytokine array was performed in order to determine the existing growth factors in ESCM. Among 36 cytokines panel, ten of them were detected at significant higher levels in ESCM as compared to unconditioned medium which did not present any cytokines component (Figure 4.11A). The detected signals were translated as the intensity of pixel density which further calculated the relative fold change of cytokine levels between ESCM and unconditioned medium. The ten proteins presented in ESCM were complement component 5/5a (C5/C5a), granulocyte colony-stimulating growth factor (G-CSF), granulocyte-macrophage colony-stimulating growth factor (GM-CSF), growth-related oncogene α (GROα), soluble intercellular adhesion molecules 1 (sICAM-1), interleukin 6 (IL-6), interleukin 8 (IL-8), monocyte chemoattractant protein 1 (MCP-1), macrophage migration inhibitory factor (MIF), and serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 (Serpin E1). Among these proteins, GM-CSF, GROα, IL-6, IL-8, MCP-1, MIF, and Serpin E1 were increased by more than 15 folds in comparison with unconditioned medium (Figure 4.11B). MIF, MCP-1, and GROα are members of the chemokine family which play crucial roles in several functions such as regulation of inflammation, development, wound healing including angiogenesis. G-CSF and GM-CSF are growth factors which most widely used in clinical for mobilizing stem cells particularly haematopoietic progenitor cells from bone marrow into the peripheral blood circulation. Other proteins detected in ESCM included IL-6 and IL-8. These cytokines have multifunction involved in biological activities of immune cells, immune regulation, and inflammation. In

addition to chemokines, cytokines, and growth factors, a soluble form of cell adhesion molecule as sICAM-1 and a serine proteinase inhibitor as Serpin E1 were also detected in ESCM. These results indicate that undifferentiated H9 human ES cells secrete a variety of cytokines, chemokines, and growth factors into their microenvironment as culture medium. These factors may have potential to enhance WJ-MSCs proliferation via a paracrine mechanism. However, human foreskin fibroblast feeders may secrete some of these proteins in addition to undifferentiated human ES cells because feeder cells still maintained in human ES culture system. Therefore, all proteins detected in ESCM may be produced by undifferentiated human ES cells in combination and human foreskin fibroblast feeders.

A



Spot No.	Protein
1	C5/C5a
2	G-CSF
3	GM-CSF
4	GROα
5	sICAM-1
6	IL-6
7	IL-8
8	MCP-1
9	MIF
10	Serpin E1
11-13	Positive control

B

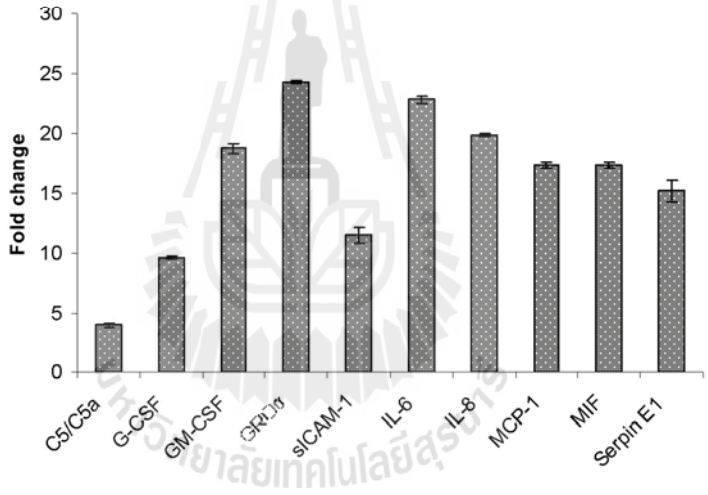


Figure 4.11 Growth factors, chemokines, and cytokines component in ESCM compared to unconditioned medium. The positive signals of each protein were shown on developed film (A). The relative fold change of cytokine levels in ESCM was normalized with those observed in unconditioned medium (B). All data are presented as mean ± SD (n = 3).

4.4 Hepatogenic differentiation potential of WJ-MSCs

In addition to expansion and characterization of WJ-MSCs cultured in different conditions, the expanded cells from C2-4 were further investigated whether they have broader capacity to differentiate into other cell lineages from their origin. To determine the hepatogenic differentiation potential of WJ-MSCs, the expanded cells from C2-4 were induced into hepatic lineage *in vitro* by using our new hepatogenic induction medium as described in materials and methods. The differentiated cells or MSCs-derived hepatocyte-like cells (MSCDHC) were determined both phenotypes and functions of hepatocytes including cell morphology, hepatic-specific marker expressions both at gene and protein levels, glycogen storage, low density lipoprotein (LDL) uptake, albumin secretion, and urea production.

4.4.1 Cell morphological changing

Cell morphology was determined on day 0, 7, 10, and 18 during differentiation. Undifferentiated cells (day 0) from all conditions had fibroblastic cell feature like common phenotype of MSCs. This feature still maintained throughout first stage of induction for 7 days. Upon entry into stage 2, WJ-MSCs cultured from C2 changed morphology from fibroblastic shape into epithelial shape earlier than those cultured from C3 and C4 on day 10 of differentiation period. However, about 80-90% of these cells showed morphological changing similar to hepatocytes that have phenotype like polygonal epithelioid shape cells from day 12 until day 18 (Figure 4.12). These findings indicate that WJ-MSCs cultured from C2-4 acquired hepatocyte-like cells phenotype after induction with our differentiation medium.

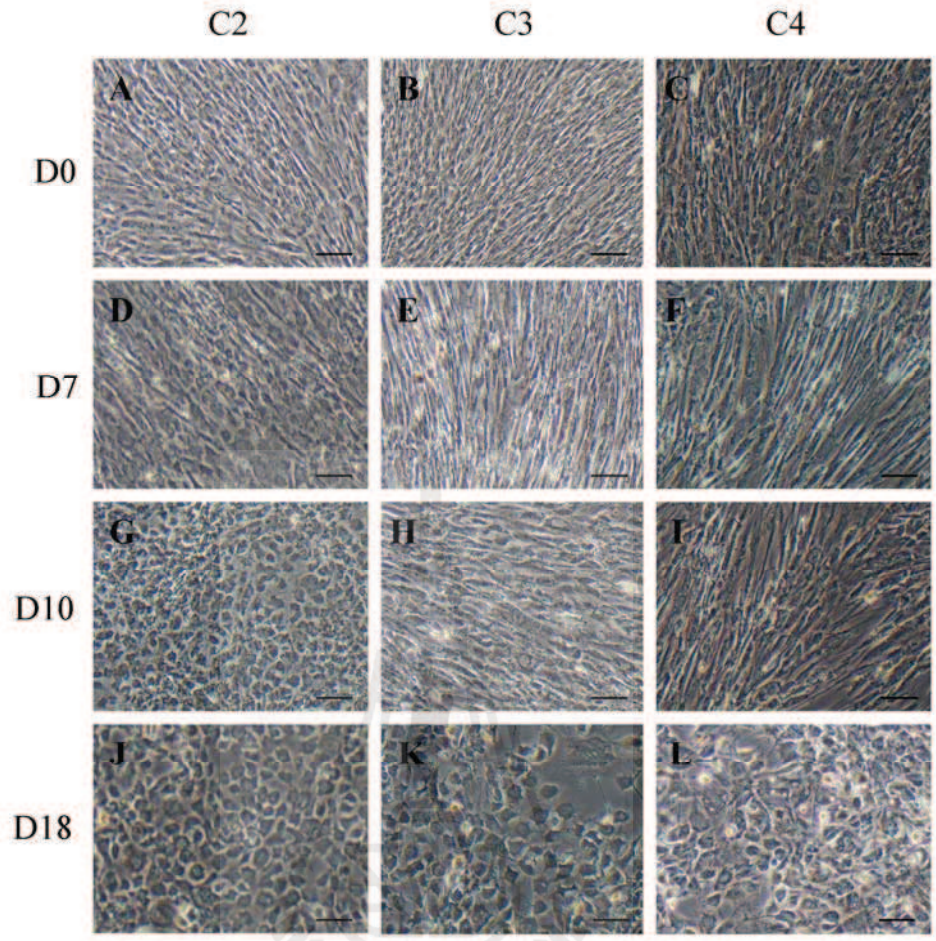


Figure 4.12 Hepatocyte-like cells phenotype of MSCDHC from C2-4. The cells from C2 (A, D, G, J), C3 (B, E, H, K), and C4 (C, F, I, L) gradually differentiated into hepatic lineage from day 0-18. The morphological changing of these cells was observed during differentiation in hepatogenic induction medium on day 0, 7, 10, and 18 of differentiation period. Magnification x100. Scale bar = 100 μm.

4.4.2 Hepatic-lineage gene and protein expressions

To determine hepatic lineage marker expressions of MSCs-derived hepatocyte-like cells (MSCDHC) during differentiation period, the expression profiles at the mRNA level were investigated by RT-PCR analysis. The expressions of some mature hepatic markers including α-1-antitrypsin (*AAT*), albumin (*ALB*), cytochrome P450 3A4 (*CYP3A4*), and glucose-6-phosphatase (*G6P*) were observed in undifferentiated cells from all conditions. However, these mature hepatic markers, *AAT*, *ALB*, and *CYP3A4*, were upregulated in a time-dependent manner by MSCDHC from all conditions after induction. Additionally, these cells showed compatible expression profiles between key hepatic transcription factor, hepatocyte nuclear factor 4 alpha (*HNF4α*), and mature hepatic marker (*ALB*) during differentiation. Moreover, the expression of *HNF4α* was detected in MSCDHC from C3 and C4 earlier than those detected in MSCDHC from C2. *HNF4α* expression is essential for inducing transcription of mature hepatic-specific genes during liver development. These findings imply that WJ-MSCs cultured from ESCM-based medium C3 and C4 had response to hepatogenic differentiation faster than those cultured from conventional medium C2. Altogether, the mRNA expressions of all hepatic-specific genes during hepatogenic differentiation were detected in MSCDHC from all conditions in the aspect of using different conditions (C2-4) to culture the cells prior hepatogenic induction (Figure 4.13).

To confirm *in vitro* hepatogenic differentiation of WJ-MSCs, the protein expressions of mature hepatic markers as albumin (ALB) and cytokeratin 18 (CK-18) were analyzed in MSCDHC by immunocytochemical staining. Undifferentiated cells (day 0) from all conditions did not express both ALB and CK-18 proteins. In contrast,

MSCDHC revealed the signal intensity of positive staining from weak signal at the first stage of induction until day 7. However, the positive signals became stronger after entry into stage 2 (day 12) and significant signals were observed at day 18. These significant up-regulations of ALB (Figure 4.14) and CK-18 (Figure 4.15) were detected in MSCDHC from all conditions in a time-dependent manner during differentiation. These results demonstrate that MSCDHC from ESCM-based medium C3 and C4 could express hepatic lineage markers both at gene and protein levels similar to MSCDHC from conventional medium C2. In addition, the expression profiles of hepatic lineage markers were consistent with the morphological changing of these cells during differentiation. These findings confirm that MSCDHC from all conditions acquired hepatocyte-like cells phenotypes both cell morphology and hepatic lineage marker expressions after hepatogenic lineage induction.

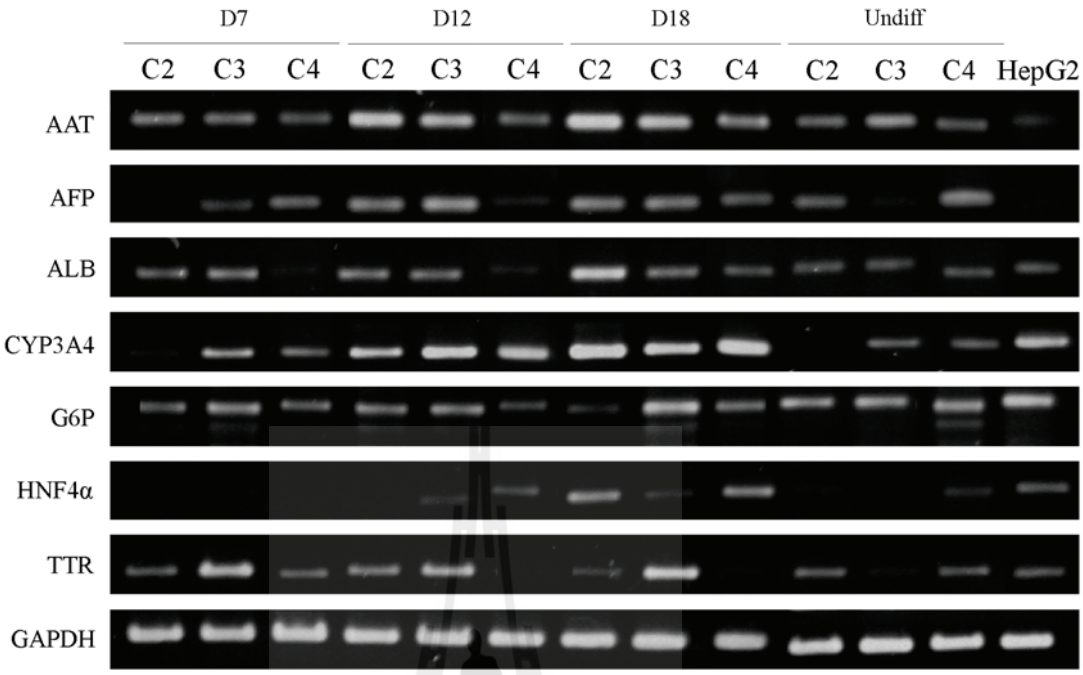


Figure 4.13 Hepatic marker expression profiles of MSCDHC from C2-4. Total RNA was isolated from the cells at different time points during differentiation and the mRNA expressions of hepatic specific genes were analyzed by RT-PCR. Undifferentiated cells were used as negative control. HepG2 was used as positive control. *GAPDH* mRNA expression was detected as input template control.

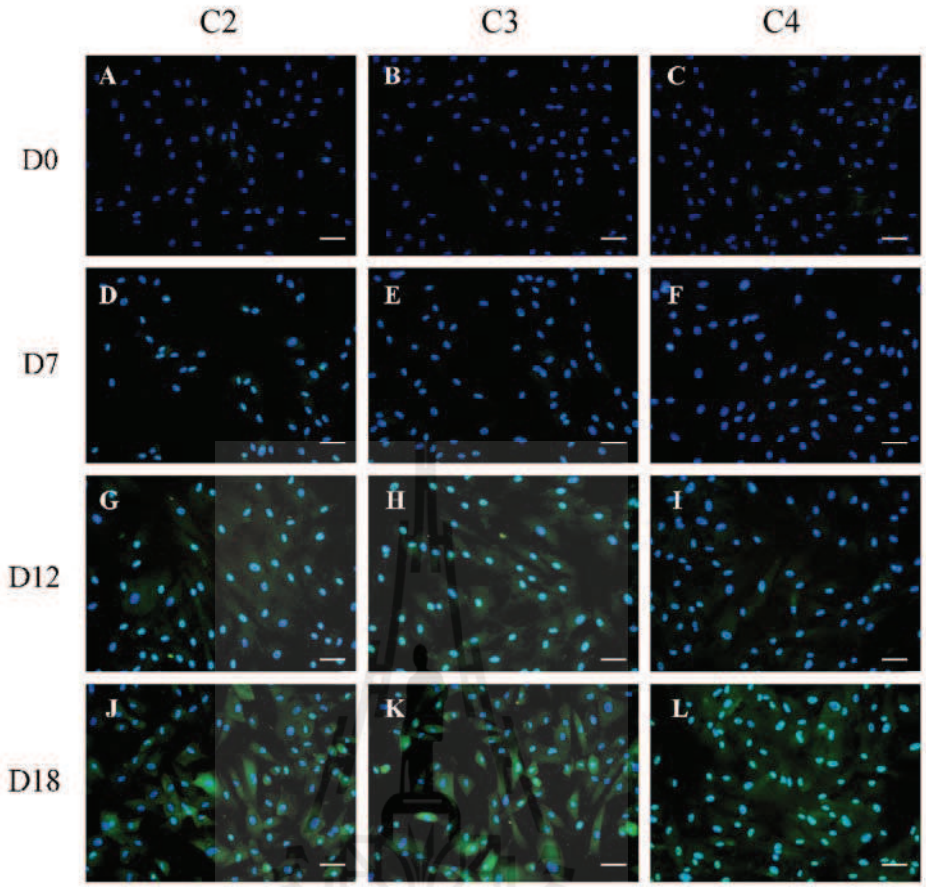


Figure 4.14 Immunocytochemistry analysis of albumin expressions (ALB) in MSCDHC from C2-4. These cells were stained with rabbit polyclonal anti-human albumin (ALB, green color) at different time points following differentiation. Cells nuclei were counterstained with DAPI (blue color). Magnification x200. Scale bar = 50 µm.

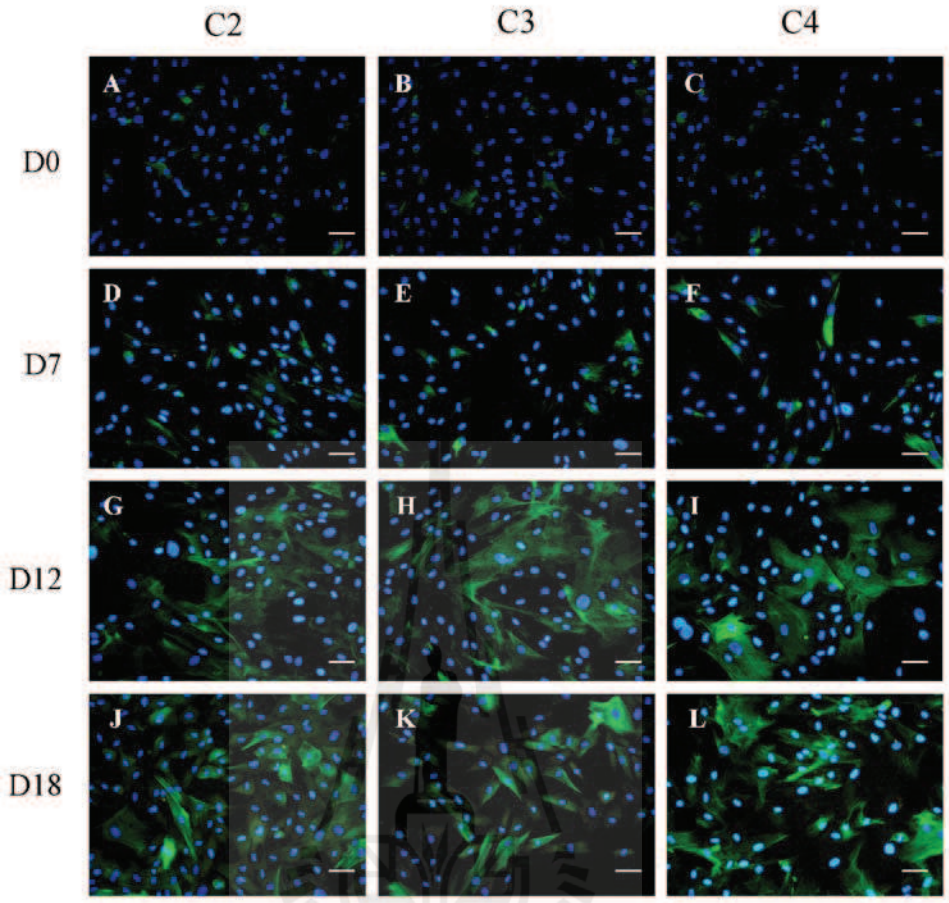


Figure 4.15 Immunocytochemistry analysis of cytokeratin 18 expressions (CK-18) in MSCDHC from C2-4. These cells were stained with mouse monoclonal anti-human cytokeratin 18 (CK-18, green color) at different time points following differentiation. Cells nuclei were counterstained with DAPI (blue color). Magnification x200. Scale bar = 50 μm.

4.4.3 Biological functions of MSCs-derived hepatocyte-like cells

To determine whether MSCDHC from all conditions had characteristics similar to functional hepatocytes, hepatic functions were evaluated in these cells during differentiation. PAS staining was performed to determine glycogen storage capacity of hepatocytes. In comparison to undifferentiated cells (day 0), MSCDHC from all conditions displayed positive for PAS staining which indicated the presence of glycogen inside the cells earlier on day 12 of differentiation. The positive signals were stronger on day 18 (Figure 4.16). Notably, MSCDHC from ESCM-based medium C3 and C4 showed positive PAS staining on day 18 stronger than those from conventional medium C2. Cellular uptake of low-density lipoprotein (LDL) is a one characteristic of functional hepatocytes. MSCDHC from all conditions could uptake LDL into the cells during differentiation period in a time-dependent manner whereas undifferentiated cells (day 0) did not perform this ability (Figure 4.17). Moreover, albumin secretion and urea production are major indicators of functional hepatocytes. Hepatocytes are responsible for plasma proteins synthesis which includes production and secretion of albumin to support homeostasis of blood circulation. Undifferentiated cells (day 0) from all conditions did not secrete detectable level of albumin even the mRNA expression was detected by RT-PCR analysis. Following differentiation, MSCDHC from all conditions continuously secreted albumin after entry into stage 2 of induction. On day 12, MSCDHC from C3 and C4 showed increasing of albumin secretion in comparison to those from C2 approximately 2 and 4 folds, respectively. Although the highest albumin secretion was observed in MSCDHC from C3 and C4, these levels were slightly decreased on day 18. Notably, MSCDHC from C2 could maintain albumin secretion capacity as the same range throughout induction period

(Figure 4.18). In comparison to HepG2, MSCDHC from ESCM-based medium C3 and C4 had higher capacity to secrete albumin while MSCDHC from conventional medium C2 could secrete albumin as the same range of HepG2 did. These data demonstrate that MSCDHC from all conditions can produce albumin like regular functional hepatocytes.

Urea is a by-product of toxic compound detoxification which is a one crucial activity of functional hepatocytes. The highest level of urea production was observed in MSCDHC from C2 at all points of differentiation period in comparison to those from C3 and C4. Notably, MSCDHC from all conditions could produce urea earlier on day 7 of induction. Furthermore, the urea levels produced by these cells did not increase in a time-dependent manner as it should be, but rather maintenance in steady levels throughout the induction period. In comparison to HepG2, MSCDHC from C2 had higher capacity to produce urea while MSCDHC from C3 and C4 could produce urea as the same range of HepG2 did (Figure 4.19). These data demonstrate that MSCDHC from all conditions could produce urea in respond to detoxification process similar to functional hepatocytes. Taken together, the data indicated that MSCDHC from C2 had superior detoxifying function over MSCDHC from C3 and C4. Conversely, MSCDHC from C3 and C4 had superior capacity to secrete albumin over MSCDHC from C2. According to all hepatic characteristics tests, these results confirm that WJ-MSCs from C2-4 had differentiation potential into hepatic lineage as functional hepatocyte-like cells.

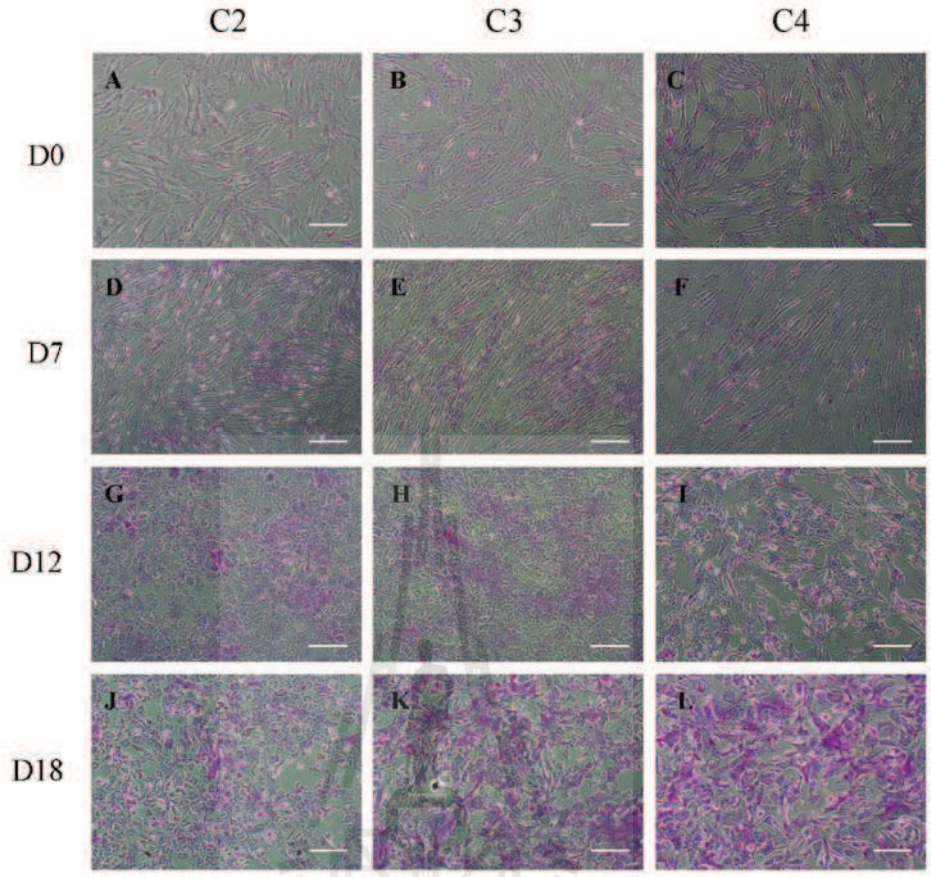


Figure 4.16 Periodic acid–schiff (PAS) staining of MSCDHC from C2-4. Positive staining refers to purple color of glycogen granules in the cells at different time points following differentiation. Magnification x100. Scale bar = 100 μm.

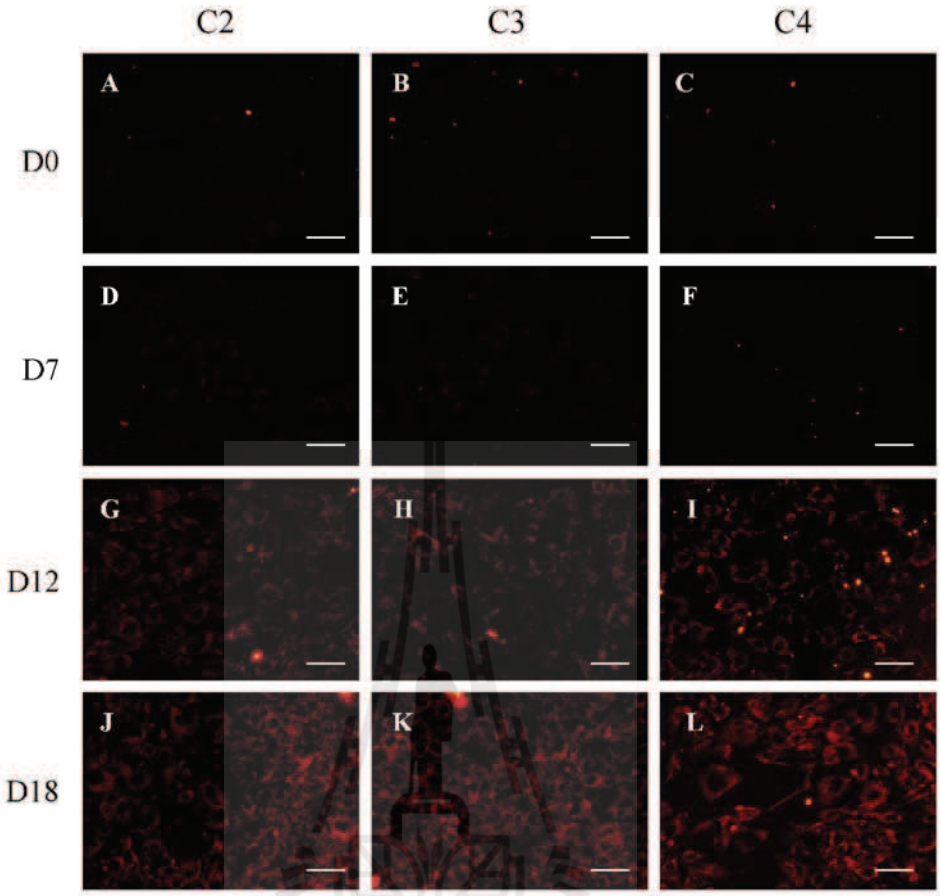


Figure 4.17 Low-density lipoprotein (LDL) uptake ability of MSCDHC from C2-4. LDL granules were detected by red color of LDL conjugated with fluorochromes in the cells at different time points following differentiation. Magnification x100. Scale bar = 100 μm.

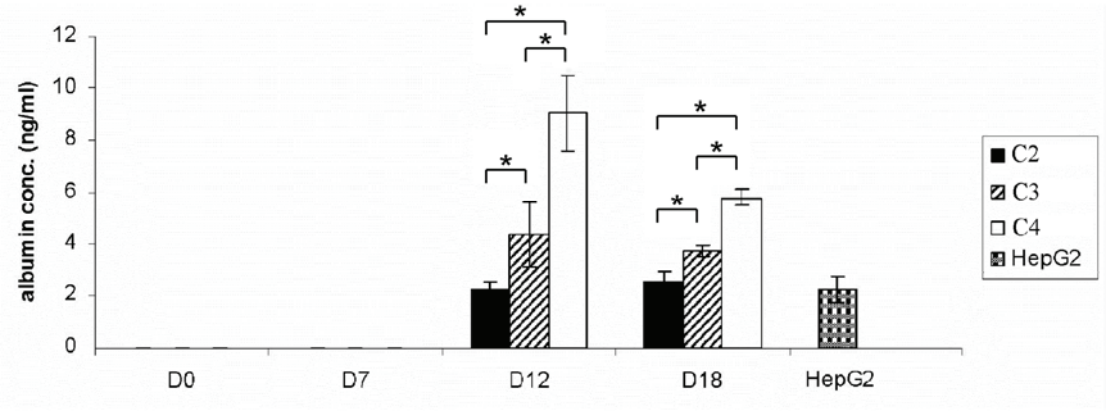


Figure 4.18 Albumin secretion by MSCDHC from C2-4. Albumin concentrations were determined by ELISA at different time points during differentiation. HepG2 was used as positive control. All data are presented as mean ± SD (n = 3). *P< 0.05

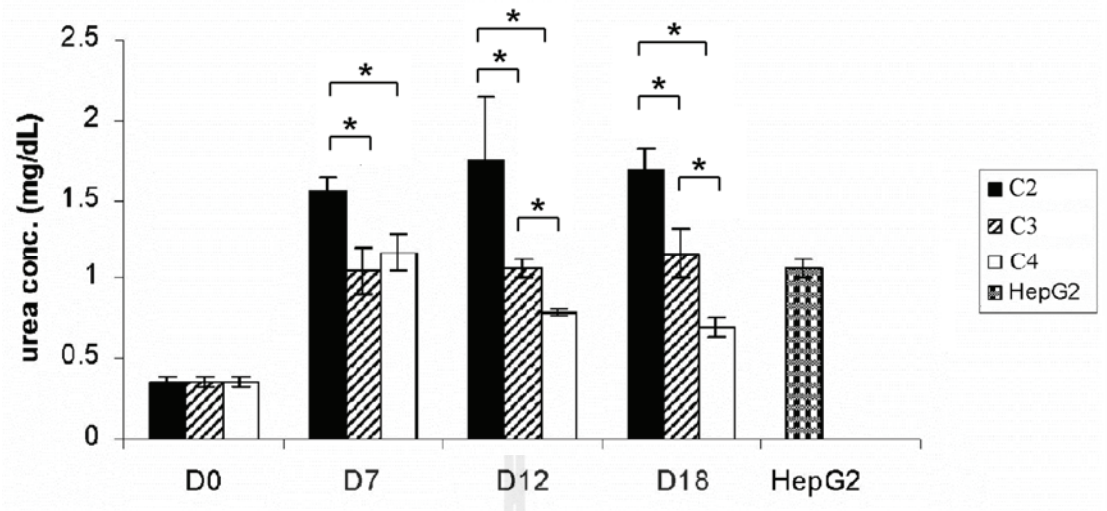


Figure 4.19 Urea production by MSCDHC from C2-4. Urea concentrations were determined by colorimetric assay at different time points during differentiation. HepG2 was used as positive control. All data are presented as mean ± SD (n = 3). *P< 0.05

4.5 Effect of *Gynura procumbens* on hepatogenic differentiation of WJ-MSCs

It was evidence that ethanolic leaves extract of *Gynura procumbens* or paetumpung not only restored blood glucose level of streptozotocin-induced type 2 diabetic rats but also improved the liver function in previous study of Dr. Wilairat Leeanansaksiri's Laboratory. From this result, we hypothesized that this medicinal plant extract has potency to rescue liver damage as well as providing the effect on hepatocytes regeneration. To determine whether the ethanolic leaves extract of *G. procumbens* can facilitate WJ-MSCs differentiation into hepatic lineage *in vitro*, this medicinal plant extract at 200 ng/mL was supplemented into hepatogenic induction medium at various time points of differentiation as day 1-7, day 8-13, day 8-18, and day 1-18. To clearly observe the effect of this extract on supportive hepatic differentiation *in vitro*, induction medium used in stage 2 was adjusted by decreasing cytokines concentrations down to one fold. Also, we excluded the hepatogenic cytokines and added this extract at 200 ng/mL only into the induction medium to confirm their effects on hepatic differentiation of WJ-MSCs. Moreover, hepatogenic induction medium without additional this medicinal plant extract was used as a control to compare the effect of this extract on the cells. Here, WJ-MSCs cultured from conventional medium C2 were used to investigate the effect of *G. procumbens* leaves extract on hepatogenic differentiation *in vitro*. After accomplishment of MSCDHC differentiation, the MSCDHC cells were subjected to hepatocytes characterization in both phenotypes and functions levels including cell morphology, hepatic-specific gene and protein expressions, glycogen storage, low density lipoprotein (LDL) uptake, albumin secretion, and urea production.

4.5.1 Cell morphological changing

Cell morphological changing of MSCDHC was determined at the end of differentiation period on day 18. Undifferentiated cells had fibroblastic cell feature like common phenotype of MSCs throughout the culture period (Figure 4.20A). Also, the treated cells with hepatogenic induction medium which contained *G. procumbens* leaves extract (200 ng/mL) alone could not differentiate into hepatocyte-like cells as shown by the appearance of fibroblastic phenotype like MSCs after complete induction (Figure 4.20B). Therefore, we excluded the treated cells with *G. procumbens* leaves extract alone (without additional the hepatogenic cytokines) for further hepatocytes characterization. On the contrary, all treated cells with hepatogenic induction medium which contained the hepatogenic cytokines with or without additional this extract could differentiate into hepatic lineage as shown by the appearance of hepatocyte-like cells morphology at the end of induction. Upon induction, these cells still maintained fibroblastic cell phenotype throughout first stage of induction. Upon entry into stage 2, these cells gradually changed morphology from fibroblastic shape into epithelial shape from day 10 onward (data not shown). On day 18, about 80-90% of treated cells with or without additional this medicinal plant extract showed cell morphology similar to hepatocytes as polygonal epithelioid shape (Figure 4.20C-G). These findings reveal that *G. procumbens* leaves extract did not have negative effect on hepatogenic differentiation of WJ-MSCs at least in cell phenotype. The difference in cell morphology could not be observed among treated cells with this extract at various time points of differentiation period. However, these results indicate that the extract could support hepatogenic differentiation of WJ-MSCs and yield normal hepatocytes morphology after complete induction.

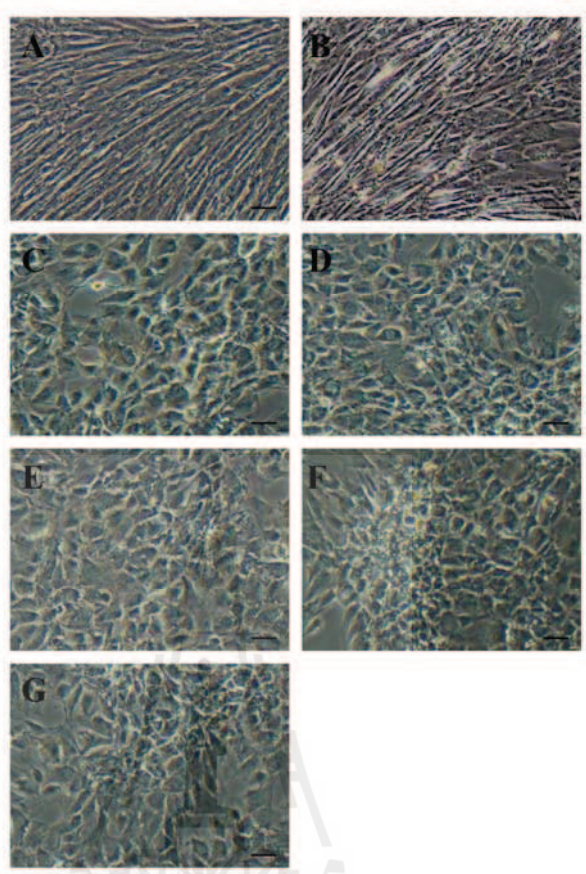


Figure 4.20 Cell morphology of MSCDHC in induction medium with or without additional *G. procumbens* leaves extract (200 ng/mL) at various time points of differentiation period. Undifferentiated cells were used as negative control (A). Treated cells with *G. procumbens* leaves extract alone (without additional the hepatogenic cytokines) were shown as picture B. Treated cells with hepatogenic induction medium were further supplemented with this extract during day 1-7 (C), day 8-13 (D), day 8-18 (E), and day 1-18 (F) to observe morphological changing of MSCDHC at the end of differentiation on day 18. MSCDHC cells without adding the extract were used as a control (G). Magnification x200. Scale bar = 50 μm.

4.5.2 Hepatic-lineage gene and protein expressions

To determine hepatic lineage marker expressions of MSCDHC after complete induction, the expression profiles at the mRNA level were investigated by RT-PCR analysis. At the end of induction on day 18, all treated cells with hepatogenic induction medium expressed mature hepatic markers such as albumin (*ALB*), glucose-6-phosphatase (*G6P*), and transthyretin (*TTR*) at the mRNA level. Although these markers were also observed in undifferentiated cells, the expression of α-1-antitrypsin (*AAT*) was strong detected in MSCDHC with or without additional *G. procumbens* leaves extract only. Additionally, the expression of key hepatic transcription factor as hepatocyte nuclear factor 4 alpha (*HNF4α*) could be detected in all *G. procumbens* treated cells at 200 ng/mL with low level whereas undifferentiated cells did not totally express this marker. From these data imply that *G. procumbens* leaves extract could drive WJ-MSCs into hepatic lineage. The low expression level may be affected by using induction medium with decreasing cytokines concentrations down to one fold from original formula used in previous experiment. Focusing on the effect of this extract on hepatogenic differentiation, no difference in hepatic marker expressions were observed among treated cells with or without additional *G. procumbens* leaves extract at various time points of differentiation period (Figure 4.21).

To confirm *in vitro* hepatogenic differentiation of WJ-MSCs, the expression of mature hepatic markers, albumin (ALB) and cytokeratin 18 (CK-18), were further analyzed at protein level by immunocytochemical staining of MSCDHC on day 18. Undifferentiated cells did not express both ALB (Figure 4.22) and CK-18 (Figure 4.23) at protein level. Like the mRNA expression, more than 80% of all

G. procumbens treated cells could express both ALB and CK-18 at the end of induction. Moreover, we could not discriminate the difference of these protein expressions among treated cells with or without additional this extract. Taken together, these results reveal that the ethanolic leaves extract of *G. procumbens* can support hepatogenic differentiation of WJ-MSCs as analyzed by both cell morphology and hepatic lineage marker expressions.

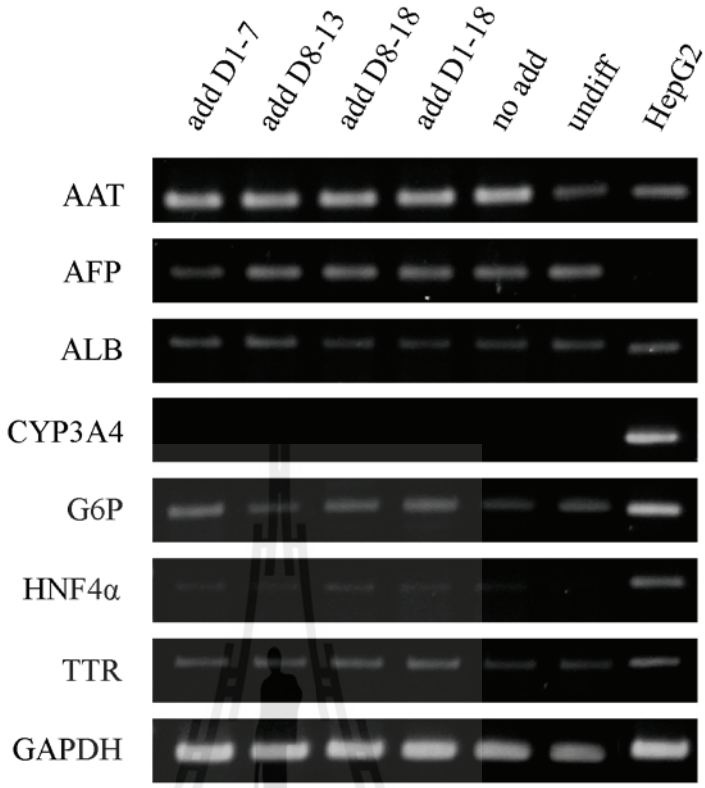


Figure 4.21 Hepatic marker expressions of MSCDHC in induction medium with or without additional *G. procumbens* leaves extract (200 ng/mL) at various time points of differentiation period. Undifferentiated cells were used as negative control. Treated cells with hepatogenic induction medium were further supplemented with this extract during day 1-7, day 8-13, day 8-18, and day 1-18. Treated cells without adding this extract were used as a control. Total RNA was isolated from the cells to observe the hepatogenic mRNA expression profiles of MSCDHC at the end of differentiation on day 18. HepG2 was used as positive control. *GAPDH* mRNA expression was used as internal control.

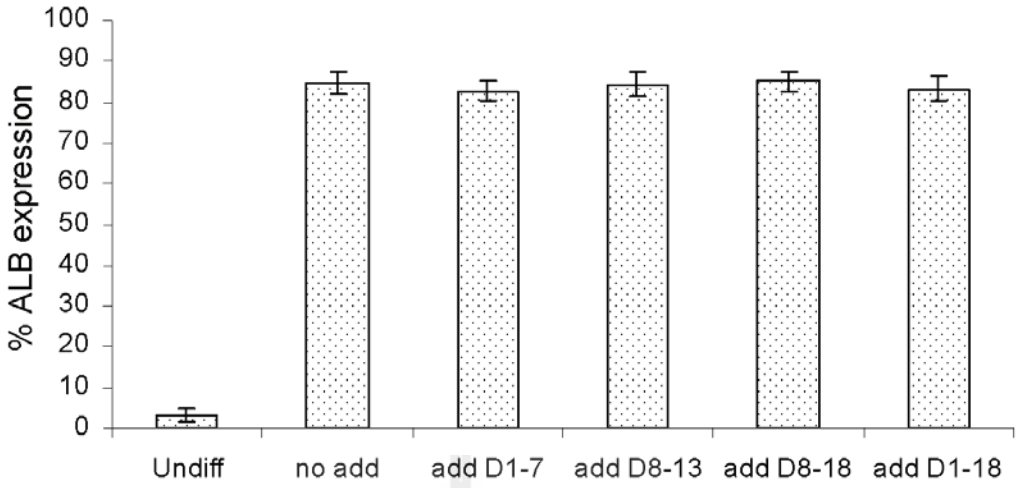


Figure 4.22 Hepatic marker expressions (ALB) of MSCDHC in induction medium with or without additional *G. procumbens* leaves extract (200 ng/mL) at various time points of differentiation period. Undifferentiated cells were used as negative control. Treated cells with hepatogenic induction medium were further supplemented with this extract during day 1-7, day 8-13, day 8-18, and day 1-18. Treated cells without additional extract were used as a control. Percentages of ALB expressions were calculated by counting the total numbers of positive stained cells relative to the total numbers of cell nuclei. All data are presented as mean ± SD (n = 3).

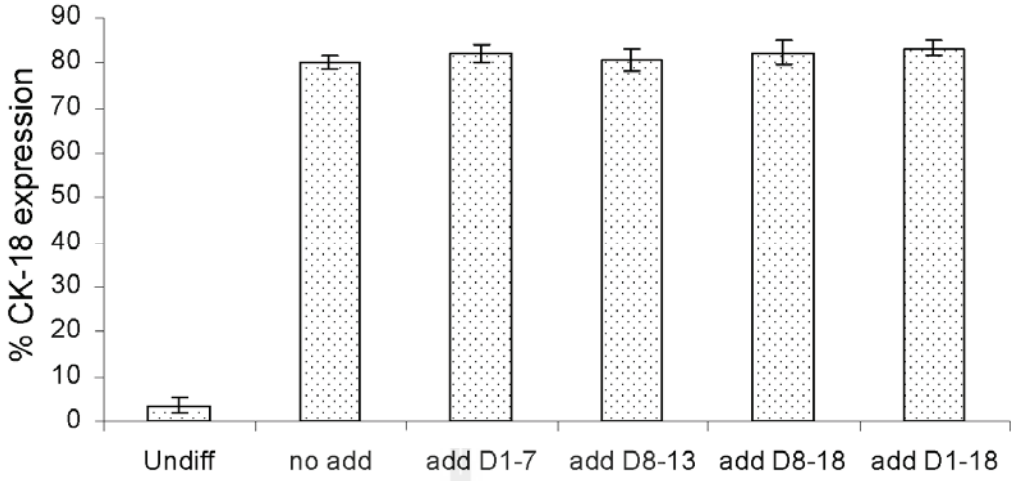


Figure 4.23 Hepatic marker expressions (CK-18) of MSCDHC in induction medium with or without additional *G. procumbens* leaves extract (200 ng/mL) at various time points of differentiation period. Undifferentiated cells were used as negative control. Treated cells with hepatogenic induction medium were further supplemented with this extract during day 1-7, day 8-13, day 8-18, and day 1-18. Treated cells without additional extract were used as a control. Percentages of CK-18 expressions were calculated by counting the total numbers of positive stained cells relative to the total numbers of cell nuclei. All data are presented as mean ± SD (n = 3).

4.5.3 Biological functions of MSCs-derived hepatocyte-like cells

To determine whether MSCDHC are acquired characteristics of functional hepatocytes after complete induction, hepatic functions were evaluated in these cells at the end of induction on day 18. PAS staining was performed to determine glycogen storage capacity of hepatocytes. In comparison to undifferentiated cells, treated cells with or without additional this extract displayed positive for PAS staining which indicated the presence of glycogen inside the cells on day 18 of differentiation (Figure 4.24). Cellular uptake of low-density lipoprotein (LDL) is a one characteristic of functional hepatocytes. At the end of induction, all treated cells could uptake LDL into the cells whereas undifferentiated cells did not perform this ability (Figure 4.25). Although treated cells with or without additional this extract acquired the characteristics of functional hepatocytes like glycogen storage and LDL uptake, the supportive effect of this extract still unclear. Furthermore, the other tests were also investigated to clarify the benefit of this extract. Regarding determination of albumin secretion, undifferentiated cells did not secrete detectable level of albumin even low mRNA expression was detected by RT-PCR analysis. Conversely, treated cells with or without additional this extract could secrete albumin after complete induction for 18 days. Interestingly, treated cells with additional *G. procumbens* leaves extract on day 1-7 showed significant higher level of albumin secretion than those observed in treated cells with induction medium alone (without *G. procumbens* leaves extract) (Figure 4.26). In comparison to HepG2, all MSCDHC treated cells showed higher capacity to secrete albumin than HepG2 did. This finding confirms that the extract supports albumin secretion capacity of MSCDHC. According to urea production, all treated cells with additional this extract in different duration produce

more urea than cells differentiated in induction medium alone. Like albumin secretion, treated cells with this extract supplementation on day 1-7 showed significant higher level of urea production than those observed in cells without adding this extract (Figure 4.27). Taken together, these results demonstrate that this medicinal plant extract provided supportive hepatogenic differentiation of WJ-MSCs, especially adding on day 1-7.

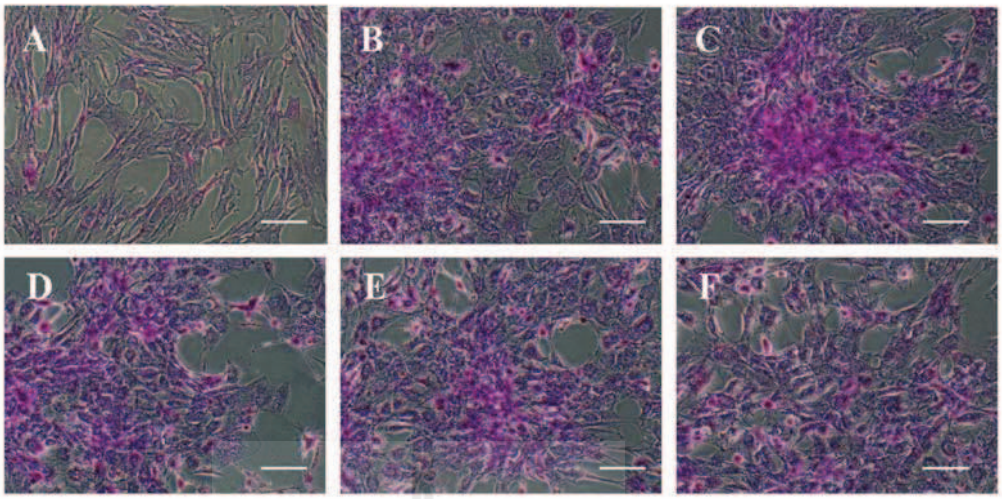


Figure 4.24 Periodic acid–schiff (PAS) staining of MSCDHC in induction medium with or without adding *G. procumbens* leaves extract (200 ng/mL) at various time points of differentiation period. Undifferentiated cells were used as negative control (A). Treated cells with hepatogenic induction medium were further supplemented with this extract during day 1-7 (B), day 8-13 (C), day 8-18 (D), and day 1-18 (E). Treated cells without additional this extract were used as a control (F). Positive staining refers to purple color of glycogen granules in the cells at the end of differentiation on day 18. Magnification x100. Scale bar = 100 μm.

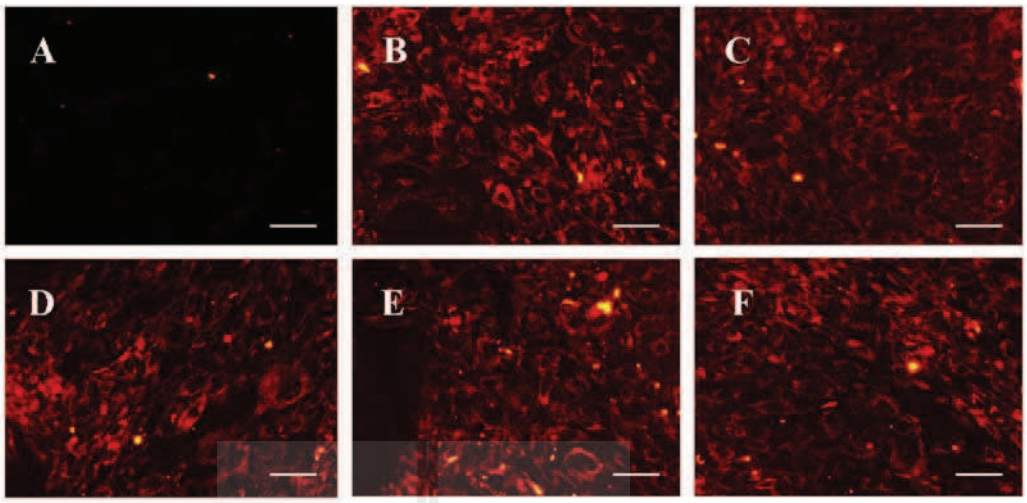


Figure 4.25 Low-density lipoprotein (LDL) uptake capacity of MSCDHC in induction medium with or without adding *G. procumbens* leaves extract (200 ng/mL) at various time points of differentiation period. Undifferentiated cells were used as negative control (A). Treated cells with hepatogenic induction medium were further supplemented with this extract during day 1-7 (B), day 8-13 (C), day 8-18 (D), and day 1-18 (E). Treated cells without additional this extract were used as a control (F). LDL granules were detected by red color of LDL conjugated to fluorochromes at the end of differentiation on day 18. Magnification x100. Scale bar = 100 µm.

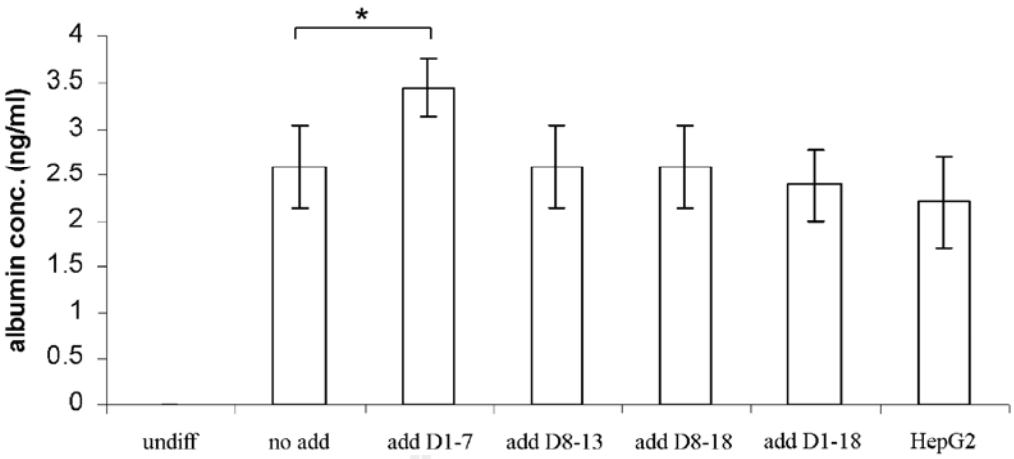


Figure 4.26 Albumin secretion of MSCDHC in induction medium with or without additional *G. procumbens* leaves extract (200 ng/mL) at various time points of differentiation period. Undifferentiated cells were used as negative control. Treated cells with hepatogenic induction medium were further supplemented with this extract during day 1-7, day 8-13, day 8-18, and day 1-18. Treated cells without additional this extract were used as a control. Albumin concentrations were determined by ELISA at the end of differentiation on day 18. HepG2 was used as positive control. All data are presented as mean ± SD (n = 3). *P< 0.05

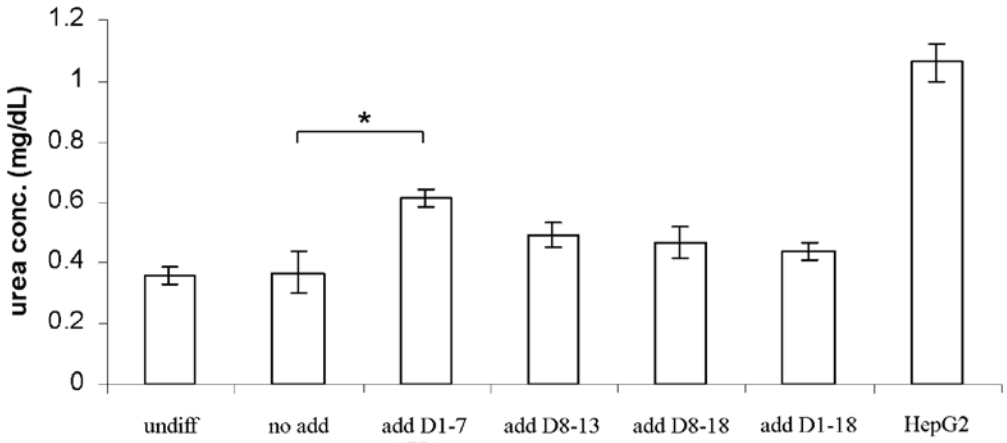


Figure 4.27 Urea production of MSCDHC in induction medium with or without additional *G. procumbens* leaves extract (200 ng/mL) at various time points of differentiation period. Undifferentiated cells were used as negative control. Treated cells with hepatogenic induction medium were further supplemented with this extract during day 1-7, day 8-13, day 8-18, and day 1-18. Treated cells without additional this extract were used as a control. Urea concentrations were determined by colorimetric assay at the end of differentiation on day 18. HepG2 was used as positive control. All data are presented as mean ± SD (n = 3). *P< 0.05

CHAPTER V

DISCUSSION

5.1 The effect of ESCM on enhancing proliferation of WJ-MCSs

To date, umbilical cord Wharton's jelly is interesting as a new source of MSCs. This source shows superior advantages over the other sources such as easy collection with non-invasive procedure and enrichment with MSCs. Umbilical cord Wharton's jelly is derived from the extraembryonic mesoderm which is more primitive origin than other adult tissues. The WJ-MSCs have common MSCs characteristics including self-renewal potential, low immunogenic property, and paracrine factor secretions. Interestingly, increasing evidences have been revealed that WJ-MSCs shared common pluripotency markers of humans ES cells including mutilineages differentiation potential into all three germ layers (Troyer and Weiss, 2008). These benefits make WJ-MSCs to be a promising candidate for using as cell-based therapy. In clinical use, a large number of cells approximately 2-5 x 10^6 cells/kilogram body weights of patients are required for effective therapy. Thus, *in vitro* expansion is still needed to up-scale the cells in a short period of time for effective clinical applications. Typically, MSCs can grow in a common used culture medium as DMEM supplemented with 10% FBS. However, it has been reported that this medium failed to support MSCs proliferation in a long-term culture (Nekanti, Rao, Bahirvani, Jan, Totey, and Ta, 2010). In this study, we also demonstrated that this medium is unable to accelerate the cells even in a short period of time in a normal

oxygen tension as 20% O_2 and 5% CO_2. Additionally, FBS is not suitable for clinical use because it serves as undesirable source of contamination by pathogens and xenogeneic proteins. Thus, other supplementation factors are required for replacement of using FBS. Several studies have been investigated alternative sources of new growth factor for MSCs expansion *in vitro*. One study applied a complex medium which had component based on culture medium for human ES cells to culture WJ-MSCs *in vitro*. They observed high expansion capacity of the cells while still maintenance of stem cells properties after expansion with this medium (Fong et al., 2010). These findings indicate that the complex medium is a richer source of several factors needed for supporting WJ-MSCs proliferation. At present, the effect of embryonic stem cells conditioned medium (ESCM) on proliferation rate of MSCs has not been investigated. Here, we are the first group that investigated whether ESCM can be used as culture medium for WJ-MSCs *in vitro* under hypoxic condition. We found that ESCM-based medium effectively enhanced WJ-MSCs proliferation approximately 204.66 ± 10.39 folds for C3 and 424.88 ± 14.62 folds for C4 within 12 days or around 2 weeks under hypoxic condition. These results reveal that EGF contains potent enhancement effect on WJ-MSCs proliferation when synergize the effect with ESCM. On the contrary, conventional medium C2 could expand the cells only 113.77 ± 7.89 folds in the same atmosphere condition. These findings were consistent with shorter mean population doubling time (PDT) of the expanded cells when compared with C3 and C4. Among different media of C2-4 under hypoxic condition, WJ-MSCs cultured in C2 used longest PDT as 41.10 ± 0.90 h and 43.30 ± 0.89 h for early and late passages, respectively. Similarity, Nekanti et al. (2010) also observed longer doubling time of WJ-MSCs cultured by DMEM-LG as

approximately 63 h under normoxic condition. In consistency, our results showed that DMEM-LG under normoxic condition (C1) could not support WJ-MSCs both phenotype and proliferation rate. In addition, the expanded cells from C2 (DMEM-LG under hypoxic condition) exhibited shorter doubling time than WJ-MSCs cultured in C1 under normoxic condition although media in C1 and C2 are the same. Taken together, these findings reflect the influence of hypoxic condition which provides enhancing effect on WJ-MSCs proliferation *in vitro*. Previous study investigated the effect of hypoxic condition on gene expression profiles and growth kinetics of WJ-MSCs. They found the upregulation of several stem cells markers and early expression of mesodermal/endothelial related genes including improving proliferation of the cells under hypoxic condition (Nekanti, Dastidar, Venugopal, Totey, and Ta, 2010). These data imply that low oxygen tension or hypoxic condition is more suitable for WJ-MSCs expansion than typical atmosphere of 20% oxygen tension.

Among different conditions, WJ-MSCs cultured in C3 and C4 had fast doubling time ranging from 37.13 to 39.00 h and 33.57 to 35.70 h, respectively. Similarity, Fong et al. (2010) also found the shortest PDT around 24 h in the expanded cells from complex medium. This medium contains specific component used for human ES cells culture such as FGF, ITS, and β–mercaptoethanol. Although complex medium seems to support extensive cell proliferation, this medium still retains high proportion of 20% FBS which is not appropriate for therapeutic applications. In this study, we used Knockout-DMEM (KO-DMEM) supplemented with Knockout-serum replacement for ES cells culture in order to minimize exposure to animal serum and other components. Thus, ESCM obtained from this system serves as serum-free, chemically defined conditioned medium which less contaminates with undesirable xenogeneic

substances. Taken together, ESCM serves as more suitable medium for WJ-MSCs expansion for therapeutic purposes. It has been reported that KO-DMEM is the best culture medium for *in vitro* expansion of WJ-MSCs. These expanded cells had faster PDT approximately 25 h in early passages while they still preserved the stemness properties even after extensive expansion (Nekanti et al., 2010). The faster doubling time may be caused by KO-DMEM medium itself and FBS in its component. Here, we agree with previous study that KO-DMEM optimizes for WJ-MSCs cultivation including supportive cell proliferation better than DMEM-LG. Interestingly, we observed that high seeding density impacts on proliferation and morphological changing of the expanded cells from ESCM after long term culture. These evidences were shown by aggregation of the cells like spheroid shape after plating from P8 onward. Similarly, this phenomenon also presented in human skin-derived mesenchymal stem cells after feeding with serum-free basal medium supplemented with FGF-2, EGF, B27, and LIF (Riekstina, Muceniece, Cakstina, Muiznieks, and Ancans, 2008). Toma et al. (2005) explained these findings that are characteristics of stem cells in early stage under serum-free culture condition. Another study found that amniotic epithelium cells isolated from human amnion layer had capacity to form spheroid body in high density culture condition (Miki, Lehmann, Cai, Stolz, and Strom, 2005). Additionally, the cells in spheroid could express stem cell markers that have been detected in human ES cells. Taken together, this feature seems to be a one characteristic of pluripotent stem cells under high cell density and serum-free culture condition.

ESCM provided greater proliferation capacity of WJ-MSCs as well as preserving common MSCs characteristics including cells morphology, cell surface

marker expressions, and mesodermal lineages differentiation potential. Moreover, the expanded cells from ESCM could preserve pluripotency properties as evidences by stemness marker expressions and normal cell cycle distribution. In addition, the expressions of stemness genes including *Oct-4* and *Nanog* could not be detected in late passages cells cultured from conventional medium C2, in contrast, with those cultured from ESCM-based medium C3 and C4. These findings indicate that conventional medium (DMEM-LG supplemented with 10% FBS) could not support stemness genes expression of WJ-MSCs after extensive *in vitro* expansion but ESCM as C3 and C4 could. *Oct-4* and *Nanog* are transcription factors required for maintenance the pluripotency and self-renewal of human ES cells (Chambers and Tomlinson, 2009). It has been reported that ectopic overexpression of *Nanog* and *Oct-4* improved both cell proliferation and stemness properties of BM-MSCs (Liu et al., 2009). In addition to our results, these transcription factors exhibit as a repertoire of pluripotent markers required for maintenance the stemness fate of stem cells both human ES cells and MSCs. EGF may be a one factor which involved not only increasing proliferation but also preserving stemness properties of the cells. Several studies revealed that EGF facilitates MSC proliferation without inducing differentiation into any specific lineages (Tamama, Fan, Griffith, Blair, and Wells, 2006). In addition to our results, EGF serves as additional growth factor not only for supporting *in vitro* MSCs expansion but also preserving their stemness fate. In conclusion, our results reveal the benefit of ESCM in terms of facilitating WJ-MSCs expansion as well as preserving common MSCs characteristics both cellular and molecular levels. ESCM may serve as remarkable medium for short term *in vitro* expansion in order to up-scale WJ-MSCs that is required for therapeutic value.

Additionally, the cost of cell culture will be saved by reusing conditioned medium from human ES cells as culture medium for WJ-MSCs.

5.2 ESCM is a rich source of cytokines, chemokines, and growth factors

Our results reveal that ESCM is a rich source of protein factors secreted by undifferentiated H9 human ES cells culture including C5/C5a, G-CSF, GM-CSF, GROα, sICAM-1, IL-6, IL-8, MCP-1, MIF, and Serpin E1. These proteins are cytokines, chemokines, and growth factors which some of them involved in various biological activities of MSCs. MIF, MCP-1, and GROα are members of the chemokine family which play crucial roles in several functions such as regulated inflammation, development, wound healing including angiogenesis. Several studies have been reported that MIF and MCP-1 play roles in regulating MSCs migration. MCP-1 had ability to trigger MSCs migration into the lesions both *in vitro* and *in vivo* studies in conditions of ischemic cerebral tissues and breast tumor (Li and Jiang, 2011; Rice and Scolding, 2010). Increasing evidences revealed the important functions of MCP-1 and MSCs for BM-MSCs homing into the site of injury in ischemic myocardium and facilitating the repairing process (Wu and Zhao, 2012). In addition to inducing MSCs migration, MCP-1 provided a trend to affect MSCs proliferation at 50 ng/mL and 100 ng/mL (Rice and Scolding, 2010). Conversely, it has evidence that MIF had ability to inhibit MSCs migration into the sites of inflammation (Barrilleaux et al., 2009). However, previous studies found that MIF involved in regulating proliferation and preventing senescence in murine embryonic fibroblasts (MEFs) under hypoxic condition (Welford et al., 2006). MIF has been

shown to inhibit activity of p53 tumor suppressor gene which resulted in proliferation and extension of MEFs life span (Fingerle-Rowson et al., 2003; Hudson et al., 1999). GROα is a chemokine that plays crucial roles in inflammation and tumorigenesis. Accumulating data revealed that GROα involved in tumor development and invasion. These effects were hypothesized that associated with the presence of p53 mutation (Ogata et al., 2010). Although GROα exhibits as an indicator of tumor development, it has been reported that GROα also plays a role in proliferation and migration of human oligodendrocyte based on *in vitro* study (Filipovic, Jakovcevski, and Zecevic, 2003). From these data, we hypothesized that the chemokines presented in ESCM may provide some effect on WJ-MSCs at least in parts of cells survival and proliferation.

G-CSF and GM-CSF are growth factors which most widely used in clinical for mobilizing stem cells particularly haematopoietic progenitor cells from bone marrow into the peripheral blood circulation (Gertz, 2010). These growth factors have been used as cocktail medium for promoting haematopoietic progenitor cells expansion *in vitro* (Liu, Liu, Fan, Ma, and Cui, 2006), but the effect on other progenitor cells has not been reported. G-CSF and GM-CSF have not been detected as secreted factors by human ES cells but they actually observed at low levels in conditioned medium from human dermal fibroblast including human MSCs (Chen, Tredget, Wu, and Wu, 2008). This finding may mirror the importance of human fibroblast feeder for maintenance pluripotency properties of undifferentiated human ES cells by secreting a variety factors including G-CSF and GM-CSF. In MSCs, previous studies found that MSCs could produce haematopoietic growth factors required for supporting proliferation of haematopoietic stem cells in their microenvironment as paracrine mechanism (Li and Wu, 2011). However, it has not been reported about the role of these factors in MSCs.

IL-6 and IL-8 also detected in ESCM. These proinflammatory cytokines involve in regulated biological activities of immune cells and inflammation. Interestingly, it has evidence that IL-6 plays a role in maintenance undifferentiated state of MSCs. Song et al. (2006) reported that IL-6 expression was downregulated during MSCs differentiation and this expression was upregulated upon de-differentiation MSCs to undifferentiated state. Moreover, subsequent study also revealed that IL-6 is essential for enhancing BM-MSC proliferation as well as maintaining undifferentiated state of the cells via ERK1/2 pathway (Pricola, Kuhn, Haleem-Smith, Song, and Tuan, 2009). Similarity, human placental mesenchymal stem cells showed increasing proliferation in responding to low concentrations of proinflammatory cytokines including IL-6 and IL-8 (Li et al., 2007). Thus, IL-6 and IL-8 components in ESCM seem to provide additional supportive effect on increasing proliferation of WJ-MSCs as well as maintaining the cells in undifferentiated state in the same time. In addition to chemokines, cytokines, and growth factors, a soluble form of cell adhesion molecule as sICAM-1 was also detected in ESCM. ICAM-1 is a member of the immunoglobulin superfamily of adhesion molecules which are presented on various cell types such as endothelial cells. ICAM-1 exhibits as leukocyte adhesion receptor on endothelial cells which can trigger leukocytes into the site of inflammation (Hubbard and Rothlein, 2000). In ES cells, this protein has been shown as a marker for defining undifferentiated state of ES cells (Cui et al., 2004). Ren et al. (2010) demonstrated that ICAM-1 and VCAM-1 were also expressed on MSC and mediating immunosuppression on T cells as cell-to-cell interactions. At present, however, the role of their soluble form (sICAM-1) is still unknown. Serpin E1 or also known as PAI-1 is a serine proteinase inhibitor which plays a role in several biological

processes such as regulated hemostasis, wound healing, atherosclerosis, metabolic disturbances, and tumor angiogenesis (Lijnen, 2005). It has been reported that PAI-1 exhibited as a migration-related protein which their expression involved in regulating the migration capacity of MSCs (Li et al., 2009). Like MSCs, this protein was also produced by ES cells but the role of them has not been investigated (LaFramboise et al., 2010). Altogether, both sICAM-1 and Serpin E1 are unlikely relevance in regulating cell proliferation of WJ-MSCs.

This study defined protein factors in ESCM which may contribute to supporting WJ-MSCs proliferation. In addition, other proteins outside of this cytokines array kit may provide additional effect on cell proliferation. One candidate factor is bFGF. The bFGF is a member of the FGF family which has several functions on the cells such as proliferation, differentiation, survival, and motility (Kashiwakura and Takahashi, 2005). It has been reported that human foreskin fibroblast feeders support undifferentiated state of human ES cells by their endogenous bFGF synthesis (Park et al., 2011). In addition, Levenstein et al. (2006) found that exogenous supplementation with high concentrations of bFGF could support self-renewal of human ES cells equivalent to using fibroblast feeders or fibroblast conditioned medium. These results confirm that bFGF plays an important role in stabilizing undifferentiated state of human ES cells. In MSCs, it has evidence that exogenous supplementation with bFGF into basal medium could promote MSCs proliferation *in vitro* (Bianchi et al., 2003). Based on these data, we believed that bFGF is a one crucial factor in ESCM involved in WJ-MSCs proliferation. Some of these factors presented in ESCM as MIF, PAI-1, sICAM-1, IL-6, and IL-8 have also been detected in conditioned medium of undifferentiated H1/H9 (Bendall et al., 2009) and Hsf1 human ES cells lines

(LaFramboise et al., 2010). Interestingly, undifferentiated H1 and H9 human ES cells lines could produce other proteins such as bFGF and TGFβ1 which have been identified as growth factors for inducing MSCs proliferation. Jian et al. (2006) reported that TGFβ1 induced BM-MSCs proliferation through a novel mechanism of cross-talk between the TGF-β and Wnt signaling pathways. Additionally, the combination of TGF-β, PDGF, and bFGF was shown to support BM-MSCs growth in a serum-free medium up to 5 passages (Ng et al., 2008). Based on these data, we believed that bFGF and TGFβ1 also present in ESCM similar to those observed in conditioned medium from previous study because ESCM obtained from culture medium of the same H9 human ES cells lines. Thus, these factors may partly drive the proliferation of WJ-MSCs in addition to other proteins. These data suggest that undifferentiated human ES cells can secrete a variety of cytokines, chemokines, and growth factors into their microenvironment. These factors can further stimulate behaviors of other cells via paracrine signaling. Taken together, ESCM provides a rich source of several factors for WJ-MSCs expansion which is also able to preserve stem cells properties of the expanded cells. In addition, the expanded WJ-MSCs cells may serve as abundant stem cells source for effective therapeutic applications in the future.

5.3 WJ-MCSs serve as a new source of functional hepatocyte-like cells

In addition to expansion and characterization of WJ-MSCs cultured from various conditions, the expanded cells from C2-4 were further investigated whether they have broader capacity to differentiate into other cell lineages from their origin.

Based on mouse and human liver developments, we applied a new cocktail of cytokines-based induction medium to induce *in vitro* hepatogenic differentiation of WJ-MSCs. In this work, we succeeded to generate MSCDHC from expanded WJ-MSCs of C2-4 cultures. In early stage of differentiation, FGFs that are expressed in cardiac mesoderm play a role in induction ventral foregut endoderm to initiate early liver development. Afterward, HGF which serves as a crucial factor for hepatogenesis plays a role in inducing rapid hepatoblast proliferation after liver bud formation. In late stage, OSM that is secreted by haematopoietic cells involves in maturation fate of fetal hepatocytes (Chen and Zeng, 2011). Thus, OSM is commonly used in maturation step in combination with other factors. In addition to these factors, it has been reported that dexamethasone is importance in maintenance the expression of liver-enriched transcription factors required for liver specific genes transcription (Sidhu, Liu, and Omiecinski, 2004). Thus, we also used dexamethasone in combination with OSM to induce more mature hepatocytes differentiation. After induction, all the expanded cells from C2-4 could differentiate into hepatic lineage which had evidences by morphological changing, hepatic-specific genes, and proteins expressions including acquiring hepatic functions such as glycogen storage, LDL uptake, albumin secretion, and urea production. These results suggest that our hepatogenic induction medium had effective impact on hepatic lineage differentiation of WJ-MSCs. In other words, the expanded WJ-MSCs from all 3 conditions (C2-4) could differentiate into functional hepatocyte-like cells. Notably, the expanded cells from ESCM-based medium (C3 and C4) seem to have more potent hepatic functions such as glycogen storage and albumin secretion than those from conventional medium (C2) after complete induction. These findings may be caused by hepatoma-derived

growth factor (HDGF) which has been found in conditioned medium of undifferentiated H1 and H9 human ES cells lines (Bendall et al., 2009). It has been reported that HDGF involved in regulation of fetal hepatocytes proliferation during liver development (Enomoto et al., 2002). Moreover, Enomoto et al. (2009) have shown the relation between HDGF expression and liver regeneration in mice model. They suggested that HDGF may stimulate liver regeneration as an autocrine factor. Based on these knowledge, we hypothesized that HDGF may present in ESCM similar to those observed in conditioned medium from previous study because ESCM obtained from culture medium of the same H9 human ES cells line. Additionally, this factor may partly drive the cells to readily differentiate into liver lineage. Interestingly, low effective of urea detoxification was observed in MSCDHC from C3 and C4 as compared to those obtained from C2. These results indicate that ESCM could support hepatic lineage differentiation in WJ-MSCs but the hepatocyte-like cells derived from this culture had incomplete function at least in a part of urea detoxification. Although the overall gene expressions of mature hepatic markers were detected in MSCDHC from all conditions, the expression of immature marker *AFP* was still detected even at late stage of differentiation. These data suggest that MSCDHC still retained immature feature like hepatoblasts or hepatic progenitor cells instead of mature hepatocytes. Similarity, previous studies demonstrated that human placenta-derived multipotent cells (Chien et al., 2006), umbilical cord matrix stem cells (Campard et al., 2008) and mesenchymal stromal cells derived from umbilical cord Wharton's jelly did not yet fully differentiate into mature hepatocytes based on the presence of *AFP* expression throughout the differentiation period (Zhang et al.,

2009). Based on this knowledge, MSCs isolated from primitive origins trend to be reserved immature property even they have been driven toward hepatic lineage.

Hepatocyte-like cells derived from MSCs serve as a promising source for cell-based therapy in some condition of liver disease. Banas et al. (2009) successfully differentiated human AT-MSCs into hepatocyte-like cells under appropriate condition *in vitro*. Furthermore, the differentiated cells could restore the liver function of acute liver failure mice by decreasing serum ALT, AST, and ammonia levels within 24 h of post-transplantation. Similarity, the hepatocyte-like cells clusters derived from human AT-MSCs had hepatic characteristics after exposure to hepatogenic inducer not only *in vitro* but also *in vivo*. The improvement of serum albumin and total bilirubin levels were observed after transplantation of the cell clusters into NOD-SCID mice with chronic liver injury (Okura et al., 2010). Recent study demonstrated the reducing liver fibrosis effect on CCl_4-induced liver fibrosis rats after intravenous injection with rat bone marrow MSC-derived hepatocyte-like cells. This study revealed that the transplanted cells may induce host immune response to rescue the disease by increasing expression of IL-10 which could further decrease the accumulation of liver fibrosis (Zhao et al., 2012). In addition, hepatic lineage cells derived from pretreated mice MSCs with injured liver tissue provided promising therapeutic potential in mice with CCl_4-induced liver fibrosis. This result was confirmed by reducing liver fibrotic area and improving serum levels of bilirubin and ALP in transplanted group (Mohsin et al., 2011). Interestingly, another study compared the therapeutic effect of autologous transplantation of undifferentiated BM-MSCs and bone marrow MSCs-derived hepatocyte-like cells in patients with liver cirrhosis. The improvement of liver function was observed in patients after transplantation with both

undifferentiated and differentiated cells such as increasing prothrombin and serum albumin levels including decreasing bilirubin and MELD score. Additionally, they did not observe significant difference in clinical improvement between two groups of patients (El-Ansary et al., 2011). In consistency with previous study, Amer et al. (2011) demonstrated the safety and short term therapeutic effect of autologous transplantation with BM-derived hepatocyte-like cells on patients with end-stage liver failure. Clinical improvement was verified by Child score, MELD score, fatigue scale, performance status, and serum albumin level. Taken together, these data revealed the treatable effect of MSCs-derived hepatocyte-like cells on the patients with liver disease even in a short term. Here, we established a new cytokines cocktail medium that was ability to generate functional hepatocyte-like cells from WJ-MSCs. Thus, WJ-MSCs may offer an alternative source of hepatocyte-like cells which can apply for liver disease treatment. Moreover, we also demonstrate that ESCM may provide some effect on the cultured cells to readily differentiate toward hepatic lineage. This advantage may be good for hepatocyte-like cells generation serving for further use in clinical treatment.

5.4 *Gynura procumbens* provides supportive effect on hepatogenic differentiation of WJ-MCSs

G. procumbens is a medicinal plant used to treat a wide range of disorders including gastric ulcer, inflammation, rheumatism, viral diseases of skin, kidney diseases, rashes, fever, migraine, constipation, and cancer (Perry and Metzger, 1980). Although treatable effect of the plant is still not exist in hepatic disease, it has evidence that the ethanolic extract of *G. procumbens* leaves not only restored blood

glucose level of streptozotocin-induced type 2 diabetic rats but also improved the liver function (previous study of Dr. Wilairat Leeanansaksiri's Laboratory). Based on this result, we interested to evaluate the additional effect of this medicinal plant extract on hepatogenic differentiation capacity of WJ-MSCs. We found that this extract could facilitate WJ-MSCs differentiation into hepatic lineage after complete induction. This achievement was verified by both cellular and molecular levels including cell phenotypes, glycogen storage, LDL uptake, albumin secretion, urea production, hepatic-specific gene, and protein expressions. This supportive effect gave the highest impact on hepatic lineage differentiation when use the plant extract as a supplementary factor with the induction medium at first stage of induction on day 1-7. These results imply that *G. procumbens* leaves extract provided the most impact on the cells to prepare itself for transitional phase into maturation step. In addition, we found that this extract alone (without additional hepatogenic cytokines) could not induce hepatogenic differentiation of WJ-MSCs. This finding reflects the valuable of this extract as supplementary factor to accelerate WJ-MSCs differentiation into hepatic lineage. Although this extract alone could not exert total effect for initiation of WJ-MSCs differentiation into hepatic lineage, this extract provided cost-saving by adding them as supplementary factor to compensate using high concentration of several cytokines in the induction medium.

The mechanisms underlying this effect are completely unknown. It has been identified that sterol and sterol glycosides are the active chemical constituents of *G. procumbens* leaves (Sadikun, Aminah, Ismail, and Ibrahim, 1996). We hypothesized that these compounds may involve in promoting hepatogenic differentiation due to the fact that they share a basic chemical structure of steroid

group similar to glucocorticoid derived from dexamethasone (The Free Medical Dictionary, 2012). Dexamethasone is a synthetic glucocorticoid hormone which commonly used in combination with other hepatic growth factors to stimulate hepatic maturation. This compound is essential for liver enzymes stimulation in gluconeogenesis which is a crucial function of hepatocytes (Lavon and Benvenisty, 2005). Thus, we hypothesized that this related chemical structure may trigger the same response in participating hepatic maturation as the same manner of dexamethasone did. Additionally, Rosidah et al. (2008) reported that phenolic compounds presented in the leaves of this plant provided substantial antioxidant activity. Recent study revealed the anti-ulcerogenic activity of *G. procumbens* leaves extract in rat model with gastric mucosal injury. They purposed that anti-ulcerogenic effect may be rendered by antioxidant and anti-inflammatory activities of its active chemical constituents in this extract (Mahmood, 2010). Although phenolic compounds presented in *G. procumbens* leaves possess several properties, we cannot link the relation between antioxidant and anti-inflammatory activities in term of promoting hepatogenic differentiation in WJ-MSCs. Thus, future works are needed to characterize the active compounds of *G. procumbens* leaves extract and further experiments are required to investigate the role of those compounds in hepatic lineage differentiation of WJ-MSCs. Here, we are the first group that reported the additional benefit of *G. procumbens* leaves extract in enhancing hepatogenic differentiation capacity of WJ-MSCs. These promising results may open a new insight of using *G. procumbens* leaves extract as supplementary factor for inducing WJ-MSCs differentiation into hepatic lineage. These data may provide some evidences to further

explore the effect of *G. procumbens* on clinical application in liver disease as regenerative medicine in the future.

CHAPTER VI

CONCLUSION

This study aims to investigate the benefit of ESCM for supporting WJ-MSCs expansion *in vitro* in comparison with the most commonly used culture medium for MSCs as DMEM-LG supplemented with 10% FBS under various conditions. Furthermore, the expanded cells from each condition were further determined *in vitro* liver-lineage differentiation potential using our new cytokine-based cocktail induction medium with or without additional medicinal plant extract from *G. procumbens*. The achievement of these goals may serve as an exploration of several benefits. Firstly, ESCM may serves as a new expansion medium for WJ-MSCs that can save the cost of cell culture by reusing the discarded medium from ES culture system. Secondly, WJ-MSCs may serve as a new source of functional hepatocyte-like cells which can be applied in liver disease as cell-based therapy in future. Thirdly, *G. procumbens* leaves extract may serve as a new supplementary factor for promoting WJ-MSCs differentiation into hepatic lineage. This experiment may open a new insight of further investigation the effect of *G. procumbens* on liver disease treatment in the future.

6.1 The effect of 4 culture conditions on enhancing proliferation of WJ-MCSs

In comparison of 4 conditions (C1-C4), our results provide evidences of acceleration effect of these 4 conditions on the proliferation of WJ-MSCs ranging from highest to lowest as C4 > C3 > C2 > C1. The folds increasing of expansion are 424.88 ± 14.62, 204.66 ± 10.39, 113.77 ± 7.89 folds for C4, C3, and C2, respectively. We also found that the C1 is not suitable for use as culture medium for WJ-MSCs, not only for proliferation but also maintain the stemness of the cells. The population doubling time (PDT) analysis of WJ-MSCs cultured in C2-C4 revealed the shortest time to longest time as C4 < C3 < C2 as about 33-35 h, 37-39 h, and 41-43 h, respectively. These data indicate that ESCM with or without additional EGF provided fold expansion of WJ-MSCs greater than using conventional medium throughout various time points of culture periods. Although ESCM-based medium extensively expanded WJ-MSCs *in vitro*, the expanded cells both at early and late passages also maintained common characteristics of MSCs as evidences by cells morphology, cell surface marker expressions and mesodermal lineages differentiation potential. Moreover, they could preserve the stemness features including stem cell markers. Notably, the expression of pluripotency markers, *Oct-4* and *Nanog,* could not be detected in late passages cells grown in conventional medium (C2) in contrast with those cultured in ESCM-based medium (C3 and C4). Taken together, these findings indicate that ESCM (C3 and C4) had more superior advantages than conventional medium (C2) including supporting cells proliferation and maintaining the stemness properties of WJ-MSCs. Thus, ESCM can use as effective expansion medium for WJ-MSCs and save budget by reusing the discarded ES medium from ES culture system. The expanded WJ-MSCs are also able to maintain their stemness which provides therapeutic value in disease treatments and regenerative medicine.

6.2 ESCM is a rich source of cytokines, chemokines, and growth factors

Our results reveal that ESCM is a rich source of protein factors which are secreted by undifferentiated H9 human ES cells culture. These proteins included cytokines, chemokines, and growth factors which some of them involve in various biological activities of MSCs. Among 36 cytokines of interest, ten proteins were detected at significant higher levels in ESCM compared to unconditioned medium including C5/C5a, G-CSF, GM-CSF, GROα, IL-6, IL-8, MCP-1, MIF, sICAM-1, and Serpin E1. Among these proteins, IL-6, IL-8, and MCP-1 seem to be candidate factors involved in promoting WJ-MSCs proliferation which has been reported by previous studies. However, other 7 proteins may also synergize the acceleration effect with IL-6, IL-8, and MCP-1 on WJ-MSCs proliferation and stemness preservation. In addition, we cannot rule out the effect of other protein factors which did not contain in this cytokines array kit such as bFGF and TGFβ1. We believed that bFGF in our conditioned medium can promote WJ-MSCs proliferation as well. Taken together, ESCM enriches with cytokines, chemokines, and growth factors which some of them have potential to enhance WJ-MSCs proliferation *in vitro*. This experiment confirms the benefit of ESCM as a valuable source of growth factors for supportive WJ-MSCs proliferation and stemness preservation.

6.3 WJ-MCSs serve as a new source of functional hepatocyte-like cells

Our results reveal the achievement of WJ-MSCs differentiation into functional hepatocyte-like cells *in vitro* by using our new cocktail of cytokine-based induction

medium. The expanded cells from C2-4 could differentiate into hepatic lineage which had evidences by morphological changing, hepatic-specific genes, and proteins expressions including acquiring hepatic functions such as glycogen storage, LDL uptake, albumin secretion, and urea production. Interestingly, the expanded cells from ESCM-based medium (C3 and C4) had a trend to achieve hepatic functions after complete induction such as glycogen storage and albumin secretion greater than those obtained from conventional medium (C2). These data imply that ESCM provided some effect on the cultured cells to readily differentiate toward hepatic lineage. Thus, the hepatocyte-like cells derived from this cultured had more potential to acquire hepatic functions than those from the other medium after complete induction. Taken together, ESCM can reuse as supportive medium for WJ-MSCs not only for *in vitro* expansion but also providing some effect on the cells to differentiate into hepatic lineage. Additionally, WJ-MSCs may offer an alternative source of hepatocyte-like cells which can apply for liver disease treatment in the future.

6.4 *Gynura procumbens* provides supportive effect on hepatogenic differentiation of WJ-MCSs

This study investigates the effect of *G. procumbens* leaves extract on hepatogenic differentiation capacity of WJ-MSCs. Our findings indicate that *G. procumbens* leaves extract can facilitate WJ-MSCs differentiation into hepatic lineage, especially when supplementation with the induction medium at first stage of induction on day 1-7. These accomplishments were clarified by both cellular and molecular levels including cell morphology, glycogen storage, LDL uptake, albumin secretion, urea production, hepatic-specific gene, and protein expressions. Although

this extract alone (without additional the hepatogenic cytokines) could not exert total effect for initiation of WJ-MSCs differentiation into hepatic lineage, this extract provided cost-saving by adding them as supplementary factor to compensate using high concentration of several cytokines in the induction medium. The mechanisms underlying the effect of the extract on this hepatogenic differentiation are completely unknown, but we hypothesized that the active chemical constituents in this extract as sterol and sterol glycosides may be involved. Based on sharing basic chemical structure in steroid group, we purposed that sterol and sterol glycosides may trigger the same activity in the cells as the same manner of dexamethasone did. Dexamethasone is a synthetic member of the glucocorticoid class of steroid drugs which used to treat many inflammatory and autoimmune conditions as well as commonly used in combination with other hepatic growth factors to stimulate hepatic maturation. Thus, future works are needed to characterize the active compounds of *G. procumbens* leaves extract and further experiments are required to investigate the role of these compounds in hepatic lineage differentiation of WJ-MSCs. This study opens a new insight in using of *G. procumbens* leaves extract as supplementary factor for promoting WJ-MSCs differentiation into hepatic lineage. Moreover, our results provide some evidences to further explore the effect of *G. procumbens* on liver diseases treatment as regenerative medicine in the future.

REFERENCES

REFERENCES

Abdel-Aziz, M. T., Atta, H. M., Mahfouz, S., Fouad, H. H., Roshdy, N. K., Ahmed, H. H., Rashed, L. A., Sabry, D., Hassouna, A. A. and Hasan, N. M. (2007). Therapeutic potential of bone marrow-derived mesenchymal stem cells on experimental liver fibrosis. **Clin Biochem**. 40(12): 893-899.

Akowuah, G. A., Sadikun, A. and Mariam, A. (2002). Flavonoid identification and hypoglycaemic studies of butanol fraction from *G. procumbens*. **Pharm Biol**. 40: 405-410.

Alfaro, M. P., Pagni, M., Vincent, A., Atkinson, J., Hill, M. F., Cates, J., Davidson, J. M., Rottman, J., Lee, E. and Young, P. P. (2008). The Wnt modulator sFRP2 enhances mesenchymal stem cell engraftment, granulation tissue formation and myocardial repair. **Proc Natl Acad Sci U S A**. 105(47): 18366-18371.

Altekruse, S. F., McGlynn, K. A. and Reichman, M. E. (2009). Hepatocellular carcinoma incidence, mortality, and survival trends in the United States from 1975 to 2005. **J Clin Oncol**. 27(9): 1485-1491.

Amer, M. E., El-Sayed, S. Z., El-Kheir, W. A., Gabr, H., Gomaa, A. A., El-Noomani, N. and Heqazy, M. (2011). Clinical and laboratory evaluation of patients with end-stage liver cell failure injected with bone marrow-derived hepatocyte-like cells. **Eur J Gastroenterol Hepatol**. 23(10): 936-941.

American Association for Clinical Chemistry. (2011). **Liver disease**. [On-line]. Available: http://labtestsonline.org/understanding/conditions/liver-disease/.

Antoine, M., Wirz, W., Tag, C. G., Gressner, A. M., Marvituna, M., Wycislo, M., Hellerbrand, C. and Kiefer, P. (2007). Expression and function of fibroblast growth factor (FGF) 9 in hepatic stellate cells and its role in toxic liver injury. **Biochem Biophys Res Commun**. 361(2): 335-341.

Asakura, A., Komaki, M. and Rudnicki, M. (2001). Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation. **Differentiation**. 68(4-5): 245-253.

Ashton, B. A., Allen, T. D., Howlett, C. R., Eaglesom, C. C., Hattori, A. and Owen, M. (1980). Formation of bone and cartilage by marrow stromal cells in diffusion chambers *in vivo*. **Clin Orthop Relat Res**. (151): 294-307.

Bab, I., Ashton, B. A., Gazit, D., Marx, G., Williamson, M. C. and Owen, M. E. (1986). Kinetics and differentiation of marrow stromal cells in diffusion chambers in vivo. **J Cell Sci**. 84: 139-151.

Baek, S. H., Kioussi, C., Briata, P., Wang, D., Nguyen, H. D., Ohgi, K. A., Glass, C. K., Wynshaw Boris, A., Rose, D. W. and Rosenfeld, M. G. (2003). Regulated subset of G1 growth-control genes in response to derepression by the Wnt pathway. **Proc Natl Acad Sci U S A**. 100(6): 3245-3250.

Baharvand, H., Hashemi, S. M., Kazemi, A. S. and Farrokhi, A. (2006). Differentiation of human embryonic stem cells into hepatocytes in 2D and 3D culture systems in vitro. **Int J Dev Biol**. 50(7): 645-652.

Baksh, D., Boland, G. M. and Tuan, R. S. (2007). Cross-talk between Wnt signaling pathways in human mesenchymal stem cells leads to functional antagonism during osteogenic differentiation. **J Cell Biochem**. 101(5): 1109-1124.

Baksh, D. and Tuan, R. S. (2007). Canonical and non-canonical Wnts differentially affect the development potential of primary isolate of human bone marrow mesenchymal stem cells. **J Cell Physiol**. 212(3): 817-826.

Baksh, D., Yao, R. and Tuan, R. S. (2007). Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow. **Stem Cells**. 25(6): 1384-1392.

Banas, A., Teratani, T., Yamamoto, Y., Tokuhara, M., Takeshita, F., Osaki, M., Kato, T., Okochi, H. and Ochiya, T. (2009). Rapid hepatic fate specification of adipose-derived stem cells and their therapeutic potential for liver failure. **J Gastroenterol Hepatol**. 24(1): 70-77.

Banas, A., Teratani, T., Yamamoto, Y., Tokuhara, M., Takeshita, F., Quinn, G., Okochi, H. and Ochiya, T. (2007). Adipose tissue-derived mesenchymal stem cells as a source of human hepatocytes. **Hepatology**. 46(1): 219-228.

Barrilleaux, B. L., Phinney, D. G., Fischer-Valuck, B. W., Russell, K. C., Wang, G., Prockop, D. J. and O'Connor, K. C. (2009). Small-molecule antagonist of macrophage migration inhibitory factor enhances migratory response of mesenchymal stem cells to bronchial epithelial cells. **Tissue Eng Part A**. 15(9): 2335-2346.

Battula, V. L., Bareiss, P. M., Treml, S., Conrad, S., Albert, I., Hojak, S., Abele, H., Schewe, B., Just, L., Skutella, T. and Buhring, H. J. (2007). Human placenta and bone marrow derived MSC cultured in serum-free, b-FGF-containing medium express cell surface frizzled-9 and SSEA-4 and give rise to multilineage differentiation. **Differentiation**. 75(4): 279-291.

Bendall, S. C., Hughes, C., Campbell, J. L., Stewart, M. H., Pittock, P., Liu, S., Bonneil, E., Thibault, P., Bhatia, M. and Lajoie, G. A. (2009). An enhanced mass spectrometry approach reveals human embryonic stem cell growth factors in culture. **Mol Cell Proteomics**. 8(3): 421-432.

Bianchi, G., Banfi, A., Mastrogiacomo, M., Notaro, R., Luzzatto, L., Cancedda, R. and Quarto, R. (2003). *Ex vivo* enrichment of mesenchymal cell progenitors by fibroblast growth factor 2. **Exp Cell Res**. 287(1): 98-105.

BMJ Evidence Centre. (2011). **Cirrhosis, epidemiology**. [On-line]. Available: http://bestpractice.bmj.com/bestpractice/monograph/278/basics/epidemiology.html.

Bosetti, C., Levi, F., Boffetta, P., Lucchini, F., Negri, E. and La Vecchia, C. (2008). Trends in mortality from hepatocellular carcinoma in Europe, 1980-2004. **Hepatology**. 48(1): 137-145.

Cai, J., Zhao, Y., Liu, Y., Ye, F., Song, Z., Qin, H., Meng, S., Chen, Y., Zhou, R., Song, X., Guo, Y., Ding, M. and Deng, H. (2007). Directed differentiation of human embryonic stem cells into functional hepatic cells. **Hepatology**. 45(5): 1229-1239.

Campagnoli, C., Roberts, I. A., Kumar, S., Bennett, P. R., Bellantuono, I. and Fisk, N. M. (2001). Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. **Blood**. 98(8): 2396-2402.

Campard, D., Lysy, P. A., Najimi, M. and Sokal, E. M. (2008). Native umbilical cord matrix stem cells express hepatic markers and differentiate into hepatocyte-like cells. **Gastroenterology**. 134(3): 833-848.

Cao, H., Yang, J., Yu, J., Pan, Q., Li, J., Zhou, P., Li, Y., Pan, X., Li, J., Wang, Y. and Li, L. (2012). Therapeutic potential of transplanted placental mesenchymal

stem cells in treating Chinese miniature pigs with acute liver failure. **BMC Med**. 10(1): 56.

Caplan, A. I. (1991). Mesenchymal stem cells. **J Orthop Res**. 9(5): 641-650.

Caplan, A. I. (2009). Why are MSCs therapeutic? New data: new insight. **J Pathol**. 217(2): 318-324.

Castro-Malaspina, H., Gay, R. E., Resnick, G., Kapoor, N., Meyers, P., Chiarieri, D., McKenzie, S., Broxmeyer, H. E. and Moore, M. A. (1980). Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny. **Blood**. 56(2): 289-301.

Center of Epidemiological Information, Bureau of Epidemiology, Ministry of Public Health, Thailand. (2011). **National Disease Surveillance R506, hepatitis total cases**. [On-line]. Available: http://www.boe.moph.go.th/boedb/ surdata/ disease.php?dcontent=old&ds=86.

Chambers, I. and Tomlinson, S. R. (2009). The transcriptional foundation of pluripotency. **Development**. 136(14): 2311-2322.

Chao, K. C., Chao, K. F., Fu, Y. S. and Liu, S. H. (2008). Islet-like clusters derived from mesenchymal stem cells in Wharton's Jelly of the human umbilical cord for transplantation to control type 1 diabetes. **PLoS One**. 3(1): e1451.

Chase, L. G., Lakshmipathy, U., Solchaga, L. A., Rao, M. S. and Vemuri, M. C. (2010). A novel serum-free medium for the expansion of human mesenchymal stem cells. **Stem Cell Res Ther**. 1(1): 8.

Chen, C. W., Montelatici, E., Crisan, M., Corselli, M., Huard, J., Lazzari, L. and Pe'ault, B. (2009). Perivascular multi-lineage progenitor cells in human

organs: regenerative units, cytokine sources or both? **Cytokine Growth Factor Rev**. 20(5-6): 429-434.

Chen, L., Tredget, E. E., Wu, P. Y. and Wu, Y. (2008). Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. **PLoS One**. 3(4): e1886.

Chen, M. Y., Lie, P. C., Li, Z. L. and Wei, X. (2009). Endothelial differentiation of Wharton's jelly-derived mesenchymal stem cells in comparison with bone marrow-derived mesenchymal stem cells. **Exp Hematol**. 37(5): 629-640.

Chen, X. and Zeng, F. (2011). Directed hepatic differentiation from embryonic stem cells. **Protein Cell**. 2(3): 180-188.

Chen, Y., Xiang, L. X., Shao, J. Z., Pan, R. L., Wang, Y. X., Dong, X. J. and Zhang, G. R. (2010). Recruitment of endogenous bone marrow mesenchymal stem cells towards injured liver. **J Cell Mol Med**. 14(6B): 1494-1508.

Cheng, Z., Ou, L., Zhou, X., Li, F., Jia, X., Zhang, Y., Liu, X., Li, Y., Ward, C. A., Melo, L. G. and Kong, D. (2008). Targeted migration of mesenchymal stem cells modified with CXCR4 gene to infarcted myocardium improves cardiac performance. **Mol Ther**. 16(3): 571-579.

Chiambaretta, F., De Graeve, F., Turet, G., Marceau, G., Gain, P., Dastugue, B., Rigal, D. and Sapin, V. (2004). Cell and tissue specific expression of human Kruppel-like transcription factors in human ocular surface. **Mol Vis**. 10: 901-909.

Chien, C. C., Yen, B. L., Lee, F. K., Lai, T. H., Chen, Y. C., Chan, S. H. and Huang, H. I. (2006). *In vitro* differentiation of human placenta-derived multipotent cells into hepatocyte-like cells. **Stem Cells**. 24(7): 1759-1768.

Chivu, M., Dima, S. O., Stancu, C. I., Dobrea, C., Uscatescu, V., Necula, L. G., Bleotu, C., Tanase, C., Albulescu, R., Ardeleanu, C. and Popescu, I. (2009). *In vitro* hepatic differentiation of human bone marrow mesenchymal stem cells under differential exposure to liver-specific factors. **Transl Res**. 154(3): 122-132.

Chong, P. P., Selvaratnam, L., Abbas, A. A. and Kamarul, T. (2012). Human peripheral blood derived mesenchymal stem cells demonstrate similar characteristics and chondrogenic differentiation potential to bone marrow derived mesenchymal stem cells. **J Orthop Res**. 30(4): 634-642.

Crisan, M., Yap, S., Casteilla, L., Chen, C.-W., Corselli, M., Park, T. S., Andriolo, G., Sun, B., Zheng, B., Zhang, L., Norotte, C., Teng, P. N., Traas, J., Schugar, R., Deasy, B. M., Badylak, S., Buhring, H. J., Giacobino, J. P., Lazzari, L., Huard, J. and Pe'ault, B. (2008). A perivascular origin for mesenchymal stem cells in multiple human organs. **Cell Stem Cell**. 3(3): 301-313.

Cui, L., Johkura, K., Yue, F., Ogiwara, N., Okouchi, Y., Asanuma, K. and Sasaki, K. (2004). Spatial distribution and initial changes of SSEA-1 and other cell adhesion-related molecules on mouse embryonic stem cells before and during differentiation. **J Histochem Cytochem**. 52(11): 1447-1457.

da Silva Meirelles, L., Chagastelles, P. C. and Nardi, N. B. (2006). Mesenchymal stem cells reside in virtually all post-natal organs and tissues. **J Cell Sci**. 119(Pt 11): 2204-2213.

Darr, H., Mayshar, Y. and Benvenisty, N. (2006). Overexpression of NANOG in human ES cells enables feeder-free growth while inducing primitive ectoderm features. **Development**. 133(6): 1193-1201.

De Becker, A., Van Hummelen, P., Bakkus, M., Vande Broek, I., De Wever, J., De Waele, M. and Van Riet, I. (2007). Migration of culture-expanded human mesenchymal stem cells through bone marrow endothelium is regulated by matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-3. **Haematologica**. 92(4): 440-449.

De Boer, J., Wang, H. J. and Van Blitterswijk, C. (2004). Effects of Wnt signaling on proliferation and differentiation of human mesenchymal stem cells. **Tissue Eng**. 10(3-4): 393-401.

Deng, X., Chen, Y. X., Zhang, X., Zhang, J. P., Yin, C., Yue, H. Y., Lin, Y., Han Z. G. and Xie, W. F. (2008). Hepatic stellate cells modulate the differentiation of bone marrow mesenchymal stem cells into hepatocyte-like cells. **J Cell Physiol**. 217(1): 138-144.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans R., Keating, A., Prockop, D. J. and Horwitz, E. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. **Cytotherapy**. 8(4): 315-317.

Dong, X. J., Zhang, H., Pan, R. L., Xiang, L. X. and Shao, J. Z. (2010). Identification of cytokines involved in hepatic differentiation of mBM-MSCs under liver-injury conditions. **World J Gastroenterol**. 16(26): 3267-3278.

Dos Santos, F., Andrade, P. Z., Boura, J. S., Abecasis, M. M., da Silva, C. L. and Cabral, J. M. (2010). *Ex vivo* expansion of human mesenchymal stem cells: a more effective cell proliferation kinetics and metabolism under hypoxia. **J Cell Physiol**. 223(1): 27-35.

Doucet, C., Ernou, I., Zhang, Y., Llense, J. R., Begot, L., Holy, X. and Lataillade, J. J. (2005). Platelet lysates promote mesenchymal stem cell expansion: a safety substitute for animal serum in cell-based therapy applications. **J Cell Physiol**. 205(2): 228-236.

Eisenmann, D. M. (2005). **Wnt signaling**. [On-line]. Available: http://www.wormbook.org/chapters/www_wntsignaling/wntsignaling.html.

El-Ansary, M., Abdel-Aziz, I., Mogawer, S., Abdel-Hamid, S., Hammam, O., Teaema, S. and Wahdan, M. (2012). Phase II trial: undifferentiated versus differentiated autologous mesenchymal stem cells transplantation in egyptian patients with HCV induced liver cirrhosis. **Stem Cell Rev**. 8(3): 972-981.

Enomoto, H., Nakamura, H., Liu, W., Yoshida, K., Okuda, Y., Imanishi, H., Saito, M., Shimomura, S., Hada, T. and Nishiguchi, S. (2009). Hepatoma-derived growth factor is induced in liver regeneration. **Hepatol Res**. 39(10): 988-997.

Enomoto, H., Yoshida, K., Kishima, Y., Kinoshita, T., Yamamoto, M., Everett, A. D., Miyajima, A. and Nakamura, H. (2002). Hepatoma-derived growth factor is highly expressed in developing liver and promotes fetal hepatocyte proliferation. **Hepatology**. 36(6): 1519-1527.

Filipovic, R., Jakovcevski, I. and Zecevic, N. (2003). GRO-alpha and CXCR2 in the human fetal brain and multiple sclerosis lesions. **Dev Neurosci**. 25(2-4): 279-290.

Fingerle-Rowson, G., Petrenko, O., Metz, C. N., Forsthuber, T. G., Mitchell, R., Huss, R., Moll, U., Muller, W. and Bucala, R. (2003). The p53-dependent effects of macrophage migration inhibitory factor revealed by gene targeting. **Proc Natl Acad Sci U S A**. 100(16): 9354-9359.

Fong, C. Y., Subramanian, A., Biswas, A., Gauthaman, K., Srikanth, P., Hande, M. P. and Bongso, A. (2010). Derivation efficiency, cell proliferation, freeze-thaw survival, stem-cell properties and differentiation of human Wharton's jelly stem cells. **Reprod Biomed Online**. 21(3): 391-401.

Fox, J. M., Chamberlain, G., Ashton, B. A. and Middleton, J. (2007). Recent advances into the understanding of mesenchymal stem cell trafficking. **Br J Haematol**. 137(6): 491-502.

Friedenstein, A. J., Gorskaja, J. F. and Kulagina, N. N. (1976). Fibroblast precursors in normal and irradiated mouse hematopoietic organs. **Exp Hematol**. 4(5): 267-274.

Gertz, M. A. (2010). Current status of stem cell mobilization. **Br J Haematol**. 150(6): 647-662.

Globinmed. (2012). ***Gynura procumbens*** **(Lour.) Merr**. [On-line]. Available: http://www.globinmed.com/index.php?option=com_content&view=article&id=62750:gynura-procumbens-lour-merr&catid=371:g.html.

Gregory, C. A., Singh, H., Perry, A. S. and Prockop, D. J. (2003). The Wnt signaling inhibitor dickkopf-1 is required for reentry into the cell cycle of human adult stem cells from bone marrow. **J Biol Chem**. 278(30): 28067-28078.

Gu, J., Shi, X., Zhang, Y. and Ding, Y. (2009). Heterotypic interactions in the preservation of morphology and functionality of porcine hepatocytes by bone marrow mesenchymal stem cells in vitro. **J Cell Physiol**. 219(1): 100-108.

Guillot, P. V., Gotherstrom, C., Chan, J., Kurata, H. and Fisk, N. M. (2007). Human first-trimester fetal MSC express pluripotency markers and grow faster and have longer telomeres than adult MSC. **Stem Cells**. 25(3): 646-654.

Haack-Sorensen, M., Friis, T., Mathiasen, A. B., Jorgensen, E., Hansen, L., Dickmeiss, E., Ekblond, A. and Kastrup, J. (2013). Direct intramyocardial mesenchymal stromal cell injections in patients with severe refractory angina - one year follow-up. **Cell Transplant**. 22(3): 521-528.

Han, J. C., Zhang, K. L., Chen, X. Y., Jiang, H. F., Kong, Q. Y., Sun, Y., Wu, M. L., Huang, L., Li, H. and Liu, J. (2007). Expression of seven gastric cancer-associated genes and its relevance for Wnt, NF-kappaB and Stat3 signaling. **APMIS**. 115(12): 1331-1343.

Hass, R., Kasper, C., Bohm, S. and Jacobs, R. (2011). Different populations and sources of human mesenchymal stem cells (MSC): A comparison of adult and neonatal tissue-derived MSC. **Cell Commun Signal**. 9: 12.

Higashiyama, R., Inagaki, Y., Hong, Y. Y., Kushida, M., Nakao, S., Niioka, M., Watanabe, T., Okano, H., Matsuzaki, Y., Shiota, G. and Okazaki, I. (2007). Bone marrow-derived cells express matrix metalloproteinases and contribute to regression of liver fibrosis in mice. **Hepatology**. 45(1): 213-222.

Hocking, A. M. and Gibran, N. S. (2010). Mesenchymal stem cells: paracrine signaling and differentiation during cutaneous wound repair. **Exp Cell Res**. 316(14): 2213-2219.

Hoe, S. Z., Lee, C. N., Mok, S. L., Kamaruddin, M. Y. and Lam, S. K. (2011). *Gynura procumbens* Merr. decreases blood pressure in rats by vasodilatation via inhibition of calcium channels. **Clinics (Sao Paulo)**. 66(1): 143-150.

Honczarenko, M., Le, Y., Swierkowski, M., Ghiran, I., Glodek, A. M. and Silberstein, L. E. (2006). Human bone marrow stromal cells express a distinct set of biologically functional chemokine receptors. **Stem Cells**. 24(4): 1030-1041.

Hubbard, A. K. and Rothlein, R. (2000). Intercellular adhesion molecule-1 (ICAM-1) expression and cell signaling cascades. **Free Radic Biol Med**. 28(9): 1379-1386.

Hudson, J. D., Shoaibi, M. A., Maestro, R., Carnero, A., Hannon, G. J. and Beach, D. H. (1999). A proinflammatory cytokine inhibits *p53* tumor suppressor activity. **J Exp Med**. 190(10): 1375-1382.

In't Anker, P. S., Scherjon, S. A., Kleijburg-van der Keur, C., de Groot-Swings, G. M., Claas, F. H., Fibbe, W. E. and Kanhai, H. H. (2004). Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. **Stem Cells**. 22(7): 1338-1345.

Ishkitiev, N., Yaegaki, K., Calenic, B., Nakahara, T., Ishikawa, H., Mitiev, V. and Haapasalo, M. (2010). Deciduous and permanent dental pulp mesenchymal cells acquire hepatic morphologic and functional features *in vitro*. **J Endod**. 36(3): 469-474.

Iskander, M. N., Song, Y., Coupar, I. M. and Jiratchariyakul, W. (2002). Antiinflammatory screening of the medicinal plant *Gynura procumbens*. **Plant Foods Hum Nutr**. 57(3-4): 233-244.

Jian, H., Shen, X., Liu, I., Semenov, M., He, X. and Wang, X. F. (2006). Smad3-dependent nuclear translocation of beta-catenin is required for TGF-beta1-induced proliferation of bone marrow-derived adult human mesenchymal stem cells. **Genes Dev**. 20(6): 666-674.

Jiang, R., Han, Z., Zhuo, G., Qu, X., Li, X., Wang, X., Shao, Y., Yang, S. and Han, Z. C. (2011). Transplantation of placenta-derived mesenchymal stem cells in type 2 diabetes: a pilot study. **Front Med**. 5(1): 94-100.

Jin, S. Z., Liu, B. R., Xu, J., Gao, F. L., Hu, Z. J., Wang, X. H., Pei, F. H., Hong, Y., Hu, H. Y. and Han, M. Z. (2012). *Ex vivo*-expanded bone marrow stem cells home to the liver and ameliorate functional recovery in a mouse model of acute hepatic injury. **Hepatobiliary Pancreat Dis Int**. 11(1): 66-73.

Jung, K. H., Shin, H. P., Lee, S., Lim, Y. J., Hwang, S. H., Han, H., Park, H. K., Chung, J. H. and Yim, S. V. (2009). Effect of human umbilical cord blood-derived mesenchymal stem cells in a cirrhotic rat model. **Liver Int**. 29(6): 898-909.

Kashiwakura, I. and Takahashi, T. A. (2005). Fibroblast growth factor and *ex vivo* expansion of hematopoietic progenitor cells. **Leuk Lymphoma**. 46(3): 329-333.

Kazemnejad, S., Allameh, A., Soleimani, M., Gharehbaghian, A., Mohammadi, Y., Amirizadeh, N. and Jazayery, M. (2009). Biochemical and molecular characterization of hepatocyte-like cells derived from human bone marrow mesenchymal stem cells on a novel three-dimensional biocompatible nanofibrous scaffold. **J Gastroenterol Hepatol**. 24(2): 278-287.

Kern, S., Eichler, H., Stoeve, J., Kluter, H. and Bieback, K. (2006). Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. **Stem Cells**. 24(5): 1294-1301.

Kharaziha, P., Hellstrom, P. M., Noorinayer, B., Farzaneh, F., Aghajani, K., Jafari, F., Telkabadi, M., Atashi, A., Honardoost, M., Zali, M. R. and Soleimani, M. (2009). Improvement of liver function in liver cirrhosis patients after autologous mesenchymal stem cell injection: a phase I-II clinical trial. **Eur J Gastroenterol Hepatol**. 21(10): 1199-1205.

Kiel, M. J. and Morrison, S. J. (2008). Uncertainty in the niches that maintain haematopoietic stem cells. **Nat Rev Immunol**. 8(4): 290-301.

Kim, S. J., Park, K. C., Lee, J. U., Kim, K. J. and Kim, D. G. (2011). Therapeutic potential of adipose tissue-derived stem cells for liver failure according to the transplantation routes. **J Korean Surg Soc**. 81(3): 176-186.

Kitaori, T., Ito, H., Schwarz, E. M., Tsutsumi, R., Yoshitomi, H., Oishi, S., Nakano, M., Fujii, N., Nagasawa, T. and Nakamura, T. (2009). Stromal cell-derived factor 1/CXCR4 signaling is critical for the recruitment of mesenchymal stem cells to the fracture site during skeletal repair in a mouse model. **Arthritis Rheum**. 60(3): 813-823.

Kladchareon, N., Treeprasertsuk, S., Mahachai, V., Wilairatana, P. and Kullavanijaya, P. (2004). The prevalence of nonalcoholic steatohepatitis in Thai patients with non-HBV, non-HCV chronic hepatitis. **J Med Assoc Thai**. 87(2): S29-34.

Kohler, B. A., Ward, E., McCarthy, B. J., Schymura, M. J., Ries, L. A., Eheman, C., Jemal, A., Anderson, R. N., Ajani, U. A. and Edwards, B. K. (2011). Annual report to the nation on the status of cancer, 1975-2007, featuring tumors of the brain and other nervous system. **J Natl Cancer Inst**. 103(9): 714-736.

LaFramboise, W. A., Petrosko, P., Krill-Burger, J. M., Morris, D. R., McCoy, A. R., Scalise, D., Malehorn, D. E., Guthrie, R. D., Becich, M. J. and Dhir, R. (2010). Proteins secreted by embryonic stem cells activate cardiomyocytes through ligand binding pathways. **J Proteomics**. 73(5): 992-1003.

Lavon, N. and Benvenisty, N. (2005). Study of hepatocyte differentiation using embryonic stem cells. **J Cell Biochem**. 96(6): 1193-1202.

Lazebnik, L. B., Gusein-Zade, M. G., Kniazev, O. V., Efremov, L. I. and Ruchkina, I. N. (2010). Pharmacoeconomic benefits of patients with ulcerative colitis treatment with help of mesenchymal stem cells. **Eksp Klin Gastroenterol**. 3: 51-59.

Lee, O. K., Kuo, T. K., Chen, W. M., Lee, K. D., Hsieh, S. L. and Chen, T. H. (2004). Isolation of multipotent mesenchymal stem cells from umbilical cord blood. **Blood**. 103(5): 1669-1675.

Lertpipopmetha, K. and Auewarakul, C. U. (2011). High incidence of hepatitis B infection-associated cirrhosis and hepatocellular carcinoma in the Southeast Asian patients with portal vein thrombosis. **BMC Gastroenterol**. 11: 66.

Levenstein, M. E., Ludwig, T. E., Xu, R. H., Llanas, R. A., VanDenHeuvel-Kramer, K., Manning, D. and Thomson, J. A. (2006). Basic fibroblast growth factor support of human embryonic stem cell self-renewal. **Stem Cells**. 24(3): 568-574.

Li, D., Wang, G. Y., Dong, B. H., Zhang, Y. C., Wang, Y. X. and Sun, B. C. (2007). Biological characteristics of human placental mesenchymal stem cells and their proliferative response to various cytokines. **Cells Tissues Organs**. 186(3): 169-179.

Li, G., Zhang, X. A., Wang, H., Wang, X., Meng, C. L., Chan, C. Y., Yew, D. T., Tsang, K. S., Li, K., Tsai, S. N., Ngai, S. M., Han, Z. C., Lin, M. C., He, M. L. and Kung, H. F. (2009). Comparative proteomic analysis of mesenchymal stem cells derived from human bone marrow, umbilical cord, and placenta: implication in the migration. **Proteomics**. 9(1): 20-30.

Li, J., Tao, R., Wu, W., Cao, H., Xin, J., Guo, J., Jiang, L., Gao, C., Demetriou, A. A., Farkas, D. L. and Li, L. (2010). 3D PLGA scaffolds improve differentiation and function of bone marrow mesenchymal stem cell-derived hepatocytes. **Stem Cells Dev**. 19(9): 1427-1436.

Li, J., Zhang, L., Xin, J., Jiang, L., Zhang, T., Jin, L., Li, J., Zhou, P., Hao, S., Cao, H. and Li, L. (2012). Immediate intraportal transplantation of human bone marrow mesenchymal stem cells prevents death from fulminant hepatic failure in pigs. **Hepatology**. 56(3): 1044-1052.

Li, L. and Jiang, J. (2011). Regulatory factors of mesenchymal stem cell migration into injured tissues and their signal transduction mechanisms. **Front Med**. 5(1): 33-39.

Li, T. and Wu, Y. (2011). Paracrine molecules of mesenchymal stem cells for hematopoietic stem cell niche. **Bone Marrow Res**. 2011, 353878.

Liang, F., Wang, Y. F., Nan, X., Yue, H. M., Xu, Y. X., Shi, S. S., Li, R. and Pei, X. T. (2005). *In vitro* differentiation of human bone marrow-derived mesenchymal stem cells into blood vessel endothelial cells. **Zhongguo Yi Xue Ke Xue Yuan Xue Bao**. 27(6): 665-669.

Liao, D., Gong, P., Li, X., Tan, Z. and Yuan, Q. (2010). Co-culture with Schwann cells is an effective way for adipose-derived stem cells neural transdifferentiation. **Arch Med Sci**. 6(2): 145-151.

Lijnen, H. R. (2005). Pleiotropic functions of plasminogen activator inhibitor-1. **J Thromb Haemost**. 3(1): 35-45.

Lin, G. L. and Hankenson, K. D. (2011). Integration of BMP, Wnt, and notch signaling pathways in osteoblast differentiation. **J Cell Biochem**. 112(12): 3491-3501.

Lin, N., Lin, J., Bo, L., Weidong, P., Chen, S. and Xu, R. (2010). Differentiation of bone marrow-derived mesenchymal stem cells into hepatocyte-like cells in an alginate scaffold. **Cell Prolif**. 43(5): 427-434.

Liu, T. M., Wu, Y. N., Guo, X. M., Hui, J. H., Lee, E. H. and Lim, B. (2009). Effects of ectopic *Nanog* and *Oct4* overexpression on mesenchymal stem cells. **Stem Cells Dev**. 18(7): 1013-1022.

Liu, Y., Liu, T., Fan, X., Ma, X. and Cui, Z. (2006). *Ex vivo* expansion of hematopoietic stem cells derived from umbilical cord blood in rotating wall vessel. **J Biotechnol**. 124(3): 592-601.

Lu, D., Chen, B., Liang, Z., Deng, W., Jiang, Y., Li, S., Xu, J., Wu, Q., Zhang, Z., Xie, B. and Chen, S. (2011). Comparison of bone marrow mesenchymal stem cells with bone marrow-derived mononuclear cells for treatment of diabetic critical limb ischemia and foot ulcer: a double-blind, randomized, controlled trial. **Diabetes Res Clin Pract**. 92(1): 26-36.

Luster, A. D., Alon, R. and von Andrian, U. H. (2005). Immune cell migration in inflammation: present and future therapeutic targets. **Nat Immunol**. 6(12): 1182-1190.

Mahmood, A. A., Mariod, A. A., Al-Bayaty, F. and Abdel-Wahab, S. I. (2010). Anti-ulcerogenic activity of *Gynura procumbens* leaf extract against experimentally-induced gastric lesions in rats. **J Med Plants Res**. 4(8): 685-691.

Mansilla, E., Marin, G. H., Sturla, F., Drago, H. E., Gil, M. A., Salas, E., Gardiner, M. C., Piccinelli, G., Bossi, S., Salas, E., Petrelli, L., Lorio, G. and Ramos, C. A. (2005). Human mesenchymal stem cells are tolerized by mice and improve skin and spinal cord injuries. **Transplant Proc**. 37(1): 292-294.

Maw, S. S., Mon, M. M. and Oo, Z. K. (2011). Study on antioxidant and antitumor activities of some herbal extracts. **WASET**. 75: 450-455.

Meirelles Lda, S., Fontes, A. M., Covas, D. T. and Caplan, A. I. (2009). Mechanisms involved in the therapeutic properties of mesenchymal stem cells. **Cytokine Growth Factor Rev**. 20(5-6): 419-427.

Miki, T., Lehmann, T., Cai, H., Stolz, D. B. and Strom, S. C. (2005). Stem cell characteristics of amniotic epithelial cells. **Stem Cells**. 23(10): 1549-1559.

Mohamadnejad, M., Alimoghaddam, K., Mohyeddin-Bonab, M., Bagheri, M., Bashtar, M., Ghanaati, H., Baharvand, H., Ghavamzadeh, A. and Malekzadeh, R. (2007). Phase 1 trial of autologous bone marrow mesenchymal stem cell transplantation in patients with decompensated liver cirrhosis. **Arch Iran Med**. 10(4): 459-466.

Mohsin, S., Shams, S., Ali Nasir, G., Khan, M., Javaid Awan, S., Khan, S. N. and Riazuddin, S. (2011). Enhanced hepatic differentiation of mesenchymal stem cells after pretreatment with injured liver tissue. **Differentiation**. 81(1): 42-48.

Mouiseddine, M., Francois, S., Semont, A., Sache, A., Allenet, B., Mathieu, N., Frick, J., Thierry, D. and Chapel, A. (2007). Human mesenchymal stem cells home specifically to radiation-injured tissues in a non-obese diabetes/severe combined immunodeficiency mouse model. **Br J Radiol**. 80 Spec No 1: S49-55.

Nekanti, U., Dastidar, S., Venugopal, P., Totey, S. and Ta, M. (2010). Increased proliferation and analysis of differential gene expression in human Wharton's jelly-derived mesenchymal stromal cells under hypoxia. **Int J Biol Sci**. 6(5): 499-512.

Nekanti, U., Rao, V. B., Bahirvani, A. G., Jan, M., Totey, S. and Ta, M. (2010). Long-term expansion and pluripotent marker array analysis of Wharton's jelly-derived mesenchymal stem cells. **Stem Cells Dev**. 19(1): 117-130.

Newman, R. E., Yoo, D., LeRoux, M. A. and Danilkovitch-Miagkova, A. (2009). Treatment of inflammatory diseases with mesenchymal stem cells. **Inflamm Allergy Drug Targets**. 8(2): 110-123.

Ng, F., Boucher, S., Koh, S., Sastry, K. S., Chase, L., Lakshmipathy, U., Choong, C., Yang, Z., Vemuri, M. C., Rao, M. S. and Tanavde, V. (2008). PDGF, TGF-beta, and FGF signaling is important for differentiation and growth of mesenchymal stem cells (MSCs): transcriptional profiling can identify markers and signaling pathways important in differentiation of MSCs into adipogenic, chondrogenic, and osteogenic lineages. **Blood**. 112(2): 295-307.

Ogata, H., Sekikawa, A., Yamagishi, H., Ichikawa, K., Tomita, S., Imura, J., Ito, Y., Fujita, M., Tsubaki, M., Kato, H., Fujimori, T. and Fukui, H. (2010). GROalpha promotes invasion of colorectal cancer cells. **Oncol Rep**. 24(6): 1479-1486.

Okura, H., Komoda, H., Saga, A., Kakuta-Yamamoto, A., Hamada, Y., Fumimoto, Y., Lee, C. M., Ichinose, A., Sawa, Y. and Matsuyama, A. (2010). Properties of hepatocyte-like cell clusters from human adipose tissue-derived mesenchymal stem cells. **Tissue Eng Part C Methods**. 16(4): 761-770.

Okura, H., Saga, A., Fumimoto, Y., Soeda, M., Moriyama, M., Moriyama, H., Nagai, K., Lee, C. M., Yamashita, S., Ichinose, A., Hayakawa, T. and Matsuyama, A. (2011). Transplantation of human adipose tissue-derived multilineage progenitor cells reduces serum cholesterol in hyperlipidemic Watanabe rabbits. **Tissue Eng Part C Methods**. 17(2): 145-154.

Organ Procurement and Transplantation Network (OPTN) and Scientific Registry of Transplant Recipients (SRTR). (2011). **OPTN / SRTR 2010 Annual data report**. [On-line]. Available: http://www.srtr.org/annual_reports/2010/.

Ozaki, Y., Nishimura, M., Sekiya, K., Suehiro, F., Kanawa, M., Nikawa, H., Hamada, T. and Kato, Y. (2007). Comprehensive analysis of chemotactic factors for bone marrow mesenchymal stem cells. **Stem Cells Dev**. 16(1): 119-129.

Parekkadan, B., van Poll, D., Megeed, Z., Kobayashi, N., Tilles, A. W., Berthiaume, F. and Yarmush, M. L. (2007). Immunomodulation of activated hepatic stellate cells by mesenchymal stem cells. **Biochem Biophys Res Commun**. 363(2): 247-252.

Parekkadan, B., van Poll, D., Suganuma, K., Carter, E. A., Berthiaume, F., Tilles, A. W. and Yarmush, M. L. (2007). Mesenchymal stem cell-derived molecules reverse fulminant hepatic failure. **PLoS One**. 2(9): e941.

Park, Y., Kim, J. H., Lee, S. J., Choi, I. Y., Park, S. J., Lee, S. R., Sung, H. J., Yoo, Y. D., Geum, D. H., Choi, C. W., Kim, S. H. and Kim, B. S. (2011). Human feeder cells can support the undifferentiated growth of human and mouse embryonic stem cells using their own basic fibroblast growth factors. **Stem Cells Dev**. 20(11): 1901-1910.

Peng, J., Wang, Y., Zhang, L., Zhao, B., Zhao, Z., Chen, J., Guo, Q., Liu, S., Sui, X., Xu, W. and Lu, S. (2011). Human umbilical cord Wharton's jelly-derived mesenchymal stem cells differentiate into a Schwann-cell phenotype and promote neurite outgrowth *in vitro*. **Brain Res Bull**. 84(3): 235-243.

Peng, L., Xie, D. Y., Lin, B. L., Liu, J., Zhu, H. P., Xie, C., Zheng, Y. B. and Gao, Z. L. (2011). Autologous bone marrow mesenchymal stem cell transplantation in liver failure patients caused by hepatitis B: short-term and long-term outcomes. **Hepatology**. 54(3): 820-828.

Pereira, W. C., Khushnooma, I., Madkaikar, M. and Ghosh, K. (2008). Reproducible methodology for the isolation of mesenchymal stem cells from human umbilical cord and its potential for cardiomyocyte generation. **J Tissue Eng Regen Med**. 2(7): 394-399.

Perez-Simon, J. A., Lopez-Villar, O., Andreu, E. J., Rifon, J., Muntion, S., Campelo, M. D., Sa'nchez-Guijo, F. M., Martinez, C., Valcarcel, D. and Canizo, C. D. (2011). Mesenchymal stem cells expanded *in vitro* with human serum for the treatment of acute and chronic graft-versus-host disease: results of a phase I/II clinical trial. **Haematologica**. 96(7): 1072-1076.

Perry, B. C., Zhou, D., Wu, X., Yang, F. C., Byers, M. A., Chu, T. M., Hockema, J. J., Woods, E. J. and Goebel, W. S. (2008). Collection, cryopreservation, and characterization of human dental pulp-derived mesenchymal stem cells for banking and clinical use. **Tissue Eng Part C Methods**. 14(2): 149-156.

Perry, L. M. and Metzger, J. (1980). **Medicinal plants of East and South East Asia: Attributed properties and uses**. Mit Press, Cambridge.

Perz, J. F., Armstrong, G. L., Farrington, L. A., Hutin, Y. J. and Bell, B. P. (2006). The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. **J Hepatol**. 45(4): 529-538.

Piryaei, A., Valojerdi, M. R., Shahsavani, M. and Baharvand, H. (2011). Differentiation of bone marrow-derived mesenchymal stem cells into hepatocyte-like cells on nanofibers and their transplantation into a carbon tetrachloride-induced liver fibrosis model. **Stem Cell Rev**. 7(1): 103-118.

Pountos, I., Georgouli, T., Henshaw, K., Bird, H., Jones, E. and Giannoudis, P. V. (2010). The effect of bone morphogenetic protein-2, bone morphogenetic protein-7, parathyroid hormone, and platelet-derived growth factor on the proliferation and osteogenic differentiation of mesenchymal stem cells derived from osteoporotic bone. **J Orthop Trauma**. 24(9): 552-556.

Pournasr, B., Mohamadnejad, M., Bagheri, M., Aghdami, N., Shahsavani, M., Malekzadeh, R. and Baharvand, H. (2011). *In vitro* differentiation of human bone marrow mesenchymal stem cells into hepatocyte-like cells. **Arch Iran Med**. 14(4): 244-249.

Pricola, K. L., Kuhn, N. Z., Haleem-Smith, H., Song, Y. and Tuan, R. S. (2009). Interleukin-6 maintains bone marrow-derived mesenchymal stem cell stemness by an ERK1/2-dependent mechanism. **J Cell Biochem**. 108(3): 577-588.

Puglisi, M. A., Tesori, V., Lattanzi, W., Piscaglia, A. C., Gasbarrini, G. B., D'Ugo, D. M. and Gasbarrini, A. (2011). Therapeutic implications of mesenchymal stem cells in liver injury. **J Biomed Biotechnol**. 2011: 860578.

Pulavendran, S., Rajam, M., Rose, C. and Mandal, A. B. (2010). Hepatocyte growth factor incorporated chitosan nanoparticles differentiate murine bone marrow mesenchymal stem cell into hepatocytes *in vitro*. **IET Nanobiotechnol**. 4(3): 51-60.

Qihao, Z., Xigu, C., Guanghui, C. and Weiwei, Z. (2007). Spheroid formation and differentiation into hepatocyte-like cells of rat mesenchymal stem cell induced by co-culture with liver cells. **DNA Cell Biol**. 26(7): 497-503.

Qiu, W., Andersen, T. E., Bollerslev, J., Mandrup, S., Abdallah, B. M. and Kassem, M. (2007). Patients with high bone mass phenotype exhibit enhanced osteoblast differentiation and inhibition of adipogenesis of human mesenchymal stem cells. **J Bone Miner Res**. 22(11): 1720-1731.

Rafiq, N., Bai, C., Fang, Y., Srishord, M., McCullough, A., Gramlich, T. and Younossi, Z. M. (2009). Long-term follow-up of patients with nonalcoholic fatty liver. **Clin Gastroenterol Hepatol**. 7(2): 234-238.

Rangsin, R., Mungthin, M., Taamasri, P., Mongklon, S., Aimpun, P., Naaglor, T. and Leelayoova, S. (2009). Incidence and risk factors of *Opisthorchis viverrini* infections in a rural community in Thailand. **Am J Trop Med Hyg**. 81(1): 152-155.

Rattanamongkolgul, S., Wongjitrat, C. and Puapankitcharoen, P. (2010). Prevalence of cirrhosis registered in Nakhon Nayok, Thailand. **J Med Assoc Thai**. 93(2): S87-91.

Rebelatto, C. K., Aguiar, A. M., Moretao, M. P., Senegaglia, A. C., Hansen, P., Barchiki, F., Oliveira, J., Martins, J., Kuligouski, C., Mansur, F., Christofis, A., Amaral, V. F., Brofman, P. S., Goldenberg, S., Nakao, L. S. and Correa, A.

(2008). Dissimilar differentiation of mesenchymal stem cells from bone marrow, umbilical cord blood, and adipose tissue. **Exp Biol Med (Maywood)**. 233(7): 901-913.

Ren, G., Zhao, X., Zhang, L., Zhang, J., L'Huillier, A., Ling, W., Roberts, A. I., Le, A. D., Shi, S., Shao, C. and Shi, Y. (2010). Inflammatory cytokine-induced intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in mesenchymal stem cells are critical for immunosuppression. **J Immunol**. 184(5): 2321-2328.

Rice, C. M. and Scolding, N. J. (2010). Adult human mesenchymal cells proliferate and migrate in response to chemokines expressed in demyelination. **Cell Adh Migr**. 4(2): 235-240.

Riekstina, U., Muceniece, R., Cakstina, I., Muiznieks, I. and Ancans, J. (2008). Characterization of human skin-derived mesenchymal stem cell proliferation rate in different growth conditions. **Cytotechnology**. 58(3): 153-162.

Ries, C., Egea, V., Karow, M., Kolb, H., Jochum, M. and Neth, P. (2007). MMP-2, MT1-MMP, and TIMP-2 are essential for the invasive capacity of human mesenchymal stem cells: differential regulation by inflammatory cytokines. **Blood**. 109(9): 4055-4063.

Robin, C. and Durand, C. (2010). The roles of BMP and IL-3 signaling pathways in the control of hematopoietic stem cells in the mouse embryo. **Int J Dev Biol**. 54(6-7): 1189-1200.

Rojas, M., Xu, J., Woods, C. R., Mora, A. L., Spears, W., Roman, J. and Brigham, K. L. (2005). Bone marrow-derived mesenchymal stem cells in repair of the injured lung. **Am J Respir Cell Mol Biol**. 33(2): 145-152.

Rosidah, Yam, M., Sadikun, A. and Asmawi, M. (2008). Antioxidant potential of *Gynura procumbens.* **Pharm Biol**. 46(9): 616-625.

Rozemuller, H., Prins, H. J., Naaijkens, B., Staal, J., Buhring, H. J. and Martens, A. C. (2010). Prospective isolation of mesenchymal stem cells from multiple mammalian species using cross-reacting anti-human monoclonal antibodies. **Stem Cells Dev**. 19(12): 1911-1921.

Sadikun, A., Idus, A., Ismail, N. and Ibrahim, P. (1996). Sterol and sterol glycosides from the leaves of *Gynura procumbens*, **Nat Prod Sci**. 2: 19-23.

Saulnier, N., Piscaglia, A. C., Puglisi, M. A., Barba, M., Arena, V., Pani, G., Alfleri, S. and Gasbarrini, A. (2010). Molecular mechanisms underlying human adipose tissue-derived stromal cells differentiation into a hepatocyte-like phenotype. **Dig Liver Dis**. 42(12): 895-901.

Schallmoser, K., Bartmann, C., Rohde, E., Reinisch, A., Kashofer, K., Stadelmeyer, E., Drexler, C., Lanzer, G., Linkesch, W. and Strunk, D. (2007). Human platelet lysate can replace fetal bovine serum for clinical-scale expansion of functional mesenchymal stromal cells. **Transfusion**. 47(8): 1436-1446.

Schwartz, J. M. and Carithers R. L. (2011). **Epidemiology and etiologic associations of hepatocellular carcinoma**. [On-line]. Available: http://www.uptodate.com/contents/epidemiology-and-etiologic-association-of-hepatocellular carcinoma.

Sidhu, J. S., Liu, F. and Omiecinski, C. J. (2004). Phenobarbital responsiveness as a uniquely sensitive indicator of hepatocyte differentiation status: requirement of dexamethasone and extracellular matrix in establishing the functional integrity of cultured primary rat hepatocytes. **Exp Cell Res**. 292(2): 252-264.

Singer, N. G. and Caplan, A. I. (2011). Mesenchymal stem cells: mechanisms of inflammation. **Annu Rev Pathol**. 6: 457-478.

Solmesky, L. J., Abekasis, M., Bulvik, S. and Weil, M. (2009). Bone morphogenetic protein signaling is involved in human mesenchymal stem cell survival in serum-free medium. **Stem Cells Dev**. 18(9): 1283-1292.

Soltys, K. A., Soto-Gutierrez, A., Nagaya, M., Baskin, K. M., Deutsch, M., Ito, R., Shneider, B. L., Squires, R., Vockley, J., Guha, C., Roy-Chowdhury, J., Strom, S. C., Platt, J. L. and Fox, I. J. (2010). Barriers to the successful treatment of liver disease by hepatocyte transplantation. **J Hepatol**. 53(4): 769-774.

Song, L., Webb, N. E., Song, Y. and Tuan, R. S. (2006). Identification and functional analysis of candidate genes regulating mesenchymal stem cell self-renewal and multipotency. **Stem Cells**. 24(7): 1707-1718.

Sripa, B., Kaewkes, S., Sithithaworn, P., Mairiang, E., Laha, T., Smout, M., Pairojkul, C., Bhudhisawasdi, V., Tesana, S., Thinkamrop, B., Bethony, J. M., Loukas, A. and Brindley, P. S. (2007). Liver fluke induces cholangiocarcinoma. **PLoS Med**. 4(7): e201.

Stewart, A., Guan, H. and Yang, K. (2010). BMP-3 promotes mesenchymal stem cell proliferation through the TGF-beta/activin signaling pathway. **J Cell Physiol**. 223(3): 658-666.

Stickel, F. and Schuppan, D. (2007). Herbal medicine in the treatment of liver diseases. **Dig Liver Dis**. 39(4): 293-304.

Talens-Visconti, R., Bonora, A., Jover, R., Mirabet, V., Carbonell, F., Castell, J. V. and Go'mez-Lecho'n, M. J. (2006). Hepatogenic differentiation of human

mesenchymal stem cells from adipose tissue in comparison with bone marrow mesenchymal stem cells. **World J Gastroenterol**. 12(36): 5834-5845.

Tamagawa, T., Oi, S., Ishiwata, I., Ishikawa, H. and Nakamura, Y. (2007). Differentiation of mesenchymal cells derived from human amniotic membranes into hepatocyte-like cells *in vitro*. **Hum Cell**. 20(3): 77-84.

Tamama, K., Fan, V. H., Griffith, L. G., Blair, H. C. and Wells, A. (2006). Epidermal growth factor as a candidate for *ex vivo* expansion of bone marrow-derived mesenchymal stem cells. **Stem Cells**. 24(3): 686-695.

Tamama, K., Kawasaki, H. and Wells, A. (2010). Epidermal growth factor (EGF) treatment on multipotential stromal cells (MSCs). Possible enhancement of therapeutic potential of MSC. **J Biomed Biotechnol**. 2010: 795385.

The Free Medical Dictionary. (2012). **Search for steroid**. [On-line]. Available: http://medical-dictionary.thefreedictionary.com/steroid.html.

The Organ Procurement and Transplantation Network. (2012). **View data reports, national data**. [On-line]. Available: http://optn.transplant.hrsa.gov/latestData/rptData.asp.

The Thai Red Cross. (2012). **2011 Annual data report**. [On-line]. Available: http://www.organdonate.in.th/Download/download.html#stat.

The United States National Institutes of Health. (2012). **Search for clinical trials**. [On-line]. Available: http://clinicaltrials.gov/ct2/results?term=mesenchymal+stem+cells&recr=&no_unk=Y.html.

Toma, J. G., McKenzie, I. A., Bagli, D. and Miller, F. D. (2005). Isolation and characterization of multipotent skin-derived precursors from human skin. **Stem Cells**. 23(6): 727-737.

Troyer, D. L. and Weiss, M. L. (2008). Wharton's jelly-derived cells are a primitive stromal cell population. **Stem Cells**. 26(3): 591-599.

Tsai, M. S., Lee, J. L., Chang, Y. J. and Hwang, S. M. (2004). Isolation of human multipotent mesenchymal stem cells from second-trimester amniotic fluid using a novel two-stage culture protocol. **Hum Reprod**. 19(6): 1450-1456.

Tsai, P. C., Fu, T. W., Chen, Y. M., Ko, T. L., Chen, T. H., Shih, Y. H., Hung, S. C. and Fu, Y. S. (2009). The therapeutic potential of human umbilical mesenchymal stem cells from Wharton's jelly in the treatment of rat liver fibrosis. **Liver Transpl**. 15(5): 484-495.

Tsukamoto, T., Mizoshita, T., Mihara, M., Tanaka, H., Takenaka, Y., Yamamura, Y., Nakamura, S., Ushijims, T. and Tatematsu, M. (2005). *Sox2* expression in human stomach adenocarcinomas with gastric and gastric-and-intestinal-mixed phenotypes. **Histopathology**. 46(6): 649-658.

van Poll, D., Parekkadan, B., Cho, C. H., Berthiaume, F., Nahmias, Y., Tilles, A. W. and Yarmush, M. L. (2008). Mesenchymal stem cell-derived molecules directly modulate hepatocellular death and regeneration *in vitro* and *in vivo*. **Hepatology**. 47(5): 1634-1643.

Vernon, G., Baranova, A. and Younossi, Z. M. (2011). Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. **Aliment Pharmacol Ther**. 34(3): 274-285.

Vicente Lopez, M. A., Vazquez Garcia, M. N., Entrena, A., Olmedillas Lopez, S., Garcia-Arranz, M., Garcia-Olmo, D. and Zapata, A. (2011). Low doses of bone morphogenetic protein 4 increase the survival of human adipose-derived

stem cells maintaining their stemness and multipotency. **Stem Cells Dev**. 20(6): 1011-1019.

Visse, R. and Nagase, H. (2003). Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. **Circ Res**. 92(8): 827-839.

Wang, H. S., Hung, S. C., Peng, S. T., Huang, C. C., Wei, H. M., Guo, Y. J., Fu, Y. S., Lai, M. C. and Chen, C. C. (2004). Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. **Stem Cells**. 22(7): 1330-1337.

Wedro, B. (2011). **Liver disease**. [On-line]. Available: http://www.medicinenet.com/liver_disease/page5.htm.

Welford, S. M., Bedogni, B., Gradin, K., Poellinger, L., Broome Powell, M. and Giaccia, A. J. (2006). HIF1alpha delays premature senescence through the activation of MIF. **Genes Dev**. 20(24): 3366-3371.

Willerth, S. M. and Sakiyama-Elbert, S. E. (2008). **Combining stem cells and biomaterial scaffolds for constructing tissues and cell delivery**. [On-line]. Available: http://www.ncbi.nlm.nih.gov/books/NBK27050/.

Williams, A. R., Trachtenberg, B., Velazquez, D. L., McNiece, I., Altman, P., Rouy, D., Mendizabal, A. M., Pattany, P. M., Lopera, G. A., Fishman, J., Zambrano, J. P., Heldman, A. W. and Hare, J. M. (2011). Intramyocardial stem cell injection in patients with ischemic cardiomyopathy: functional recovery and reverse remodeling. **Circ Res**. 108(7): 792-796.

Wu, L. F., Wang, N. N., Liu, Y. S. and Wei, X. (2009). Differentiation of Wharton's jelly primitive stromal cells into insulin-producing cells in comparison with

bone marrow mesenchymal stem cells. **Tissue Eng Part A**. 15(10): 2865-2873.

Wu, Y. and Zhao, R. C. (2012). The role of chemokines in mesenchymal stem cell homing to myocardium. **Stem Cell Rev**. 8(1): 243-250.

Wynn, R. F., Hart, C. A., Corradi-Perini, C., O'Neill, L., Evans, C. A., Wraith, J. E., Fairbairn, L. J. and Bellantuono, I. (2004). A small proportion of mesenchymal stem cells strongly expresses functionally active CXCR4 receptor capable of promoting migration to bone marrow. **Blood**. 104(9): 2643-2645.

Xu, R., Xia, W. J., Rong, X., Ye, X., Shao, Y., Wang, M., Luo, G. P. and Fu, Y. S. (2011). Human platelet lysates promotes the proliferation of mesenchymal stem cells *in vitro*. **Nan Fang Yi Ke Da Xue Xue Bao**. 31(8): 1396-1400.

Yan, Y., Xu, W., Qian, H., Si, Y., Zhu, W., Cao, H., Zhou, H. and Mao, F. (2009). Mesenchymal stem cells from human umbilical cords ameliorate mouse hepatic injury *in vivo*. **Liver Int**. 29(3): 356-365.

Yao, S., Chen, S., Clark, J., Hao, E., Beattie, G. M., Hayek, A. and Ding, S. (2006). Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions. **Proc Natl Acad Sci U S A**. 103(18): 6907-6912.

Zhang, D., Jiang, M. and Miao, D. (2011). Transplanted human amniotic membrane-derived mesenchymal stem cells ameliorate carbon tetrachloride-induced liver cirrhosis in mouse. **PLoS One**. 6(2): e16789.

Zhang, S., Chen, L., Liu, T., Zhang, B., Xiang, D., Wang, Z. and Wang, Y. (2012). Human umbilical cord matrix stem cells efficiently rescue acute liver failure

through paracrine effects rather than hepatic differentiation. **Tissue Eng Part A**. 18(13-14): 1352-1364.

Zhang, Y. N., Lie, P. C. and Wei, X. (2009). Differentiation of mesenchymal stromal cells derived from umbilical cord Wharton's jelly into hepatocyte-like cells. **Cytotherapy**. 11(5): 548-558.

Zhang, Z., Lin, H., Shi, M., Xu, R., Fu, J., Lv, J., Chen, L., Lv, S., Li, Y., Yu, S., Geng, H., Jin, L., Lau, G. K. and Wang, F. S. (2012). Human umbilical cord mesenchymal stem cells improve liver function and ascites in decompensated liver cirrhosis patients. **J Gastroenterol Hepatol**. 27 Suppl 2: 112-120.

Zhao, W., Li, J. J., Cao, D. Y., Li, X., Zhang, L. Y., He, Y., Yue, S. Q., Wang, D. S. and Dou, K. F. (2012). Intravenous injection of mesenchymal stem cells is effective in treating liver fibrosis. **World J Gastroenterol**. 18(10): 1048-1058.

Zheng, Y. B., Gao, Z. L., Xie, C., Zhu, H. P., Peng, L., Chen, J. H. and Chong, Y. T. (2008). Characterization and hepatogenic differentiation of mesenchymal stem cells from human amniotic fluid and human bone marrow: a comparative study. **Cell Biol Int**. 32(11): 1439-1448.

Zorn, A. M. (2008). **Liver development**. [On-line]. Available: http://www.ncbi.nlm.nih.gov/books/NBK27068/.

Zuk, P. A., Zhu, M., Mizuno, H., Huang, J., Futrell, J. W., Katz, A. J., Benhaim, P., Lorenz, H. P. and Hedrick, M. H. (2001). Multilineage cells from human adipose tissue: implications for cell-based therapies. **Tissue Eng**. 7(2): 211-228.

APPENDIX

APPENDIX

SOLUTION PREPARATION

1. Reagent for cell culture

1.1 complete DMEM (cDMEM)

Mix the reagent as follow:

- DMEM (low glucose)	10.00	g
- FBS	100.00	ml
- $NaHCO_3$	3.70	g
- 1000 U/ml Penicillin/1000 μg/ml Streptomycin	10.00	ml
- 2 mg/ml Amphotericin B	0.20	ml

Add sterile ultra-pure water to bring a volume up to 1000 ml and adjust pH to 7.4. Sterilize by filter sterile.

1.2 PBS

Mix the reagent as follow:

- NaCl	8.00	g
- Na_2HPO_4	1.44	g
- KCl	0.20	g
- KH_2PO_4	0.24	g

Add sterile ultra-pure water to bring a volume up to 1000 ml and adjust pH to 7.4. Sterilize by autoclaved at 121ºC for 15 minutes and store at room temperature.

1.3 0.25% Trypsin/EDTA

Mix the reagent as follow:

- Trypsin	0.25	g
- EDTA	0.04	g

Add sterile PBS to bring a volume up to 100 ml.

2. Reagent for mesodermal-lineages differentiation

2.1 1% Alcian blue

Mix the reagent as follow:

- Alcian blue	1.00	g
- 0.1 N HCL	100.00	ml

Mix well. Filter into the reagent bottle and filter before use.

2.2 2% Alizarin Red S

Mix the reagent as follow:

- Alizarin Red S	2.00	g
- Distilled water	100.00	ml

Mix well and adjust the pH to 4.2. Filter into the reagent bottle and filter before use.

2.3 Oil Red O

Mix the reagent as follow:

- Oil Red O	0.30	g
- 99% Isopropanol	100.00	ml

Mix well. Filter into the reagent bottle and filter before use.

2.4 4% Paraformaldehyde

Dissolve 4 g. of paraformaldehyde in 90 ml. of distilled water to dissolve and adjust the pH to 7.4. Then, add 10 ml. of 10x PBS (for a final concentration of 4 g. in 100 ml. of 1x PBS). Filter sterile through a 0.22 μm filter and store at 4ºC.

3. Reagent for agarose gel electrophoresis

3.1 5x TBE buffer

Mix the reagent as follow:

- Tris base	53.00	g
- Boric acid	27.50	g
- 0.5 M EDTA (pH 8.0)	20.00	ml

Add sterile ultra-pure water to bring a volume up to 1000 ml.

3.2 6x DNA loading dye

Mix 0.025 g. of bromophenol blue, 0.025 g. of xylene cyanol and 3 ml. of 100% glycerol in distilled water to a 10 ml. final volume and store at 4ºC.

CURRICULUM VITAE

Name	Miss Patcharee Prasajak
Date of Birth	4 December 1980
Place of Birth	Prachinburi, Thailand
Education	
1999 – 2003	B.Sc. (Medical Technology), Mahidol University, Thailand
2003 – 2006	M.Sc. (Microbiology), Mahidol University, Thailand
2007 – present	Ph.D. candidate, Suranaree University of Technology, Thailand
Experience	
2006 – 2007	Medical Technologist, International Emerging Infections Program, (CDC), Nakhon Phanom, Thailand
Award	
	International Society for Stem Cell Research (ISSCR) travel award in ISSCR 7th Annual Meeting, Barcelona, Spain 2009
Poster presentation	
	Characterization of human wharton's jelly-derived mesenchymal stem cells proliferation rate under hypoxic condition. The International Society for Stem Cell Research (ISSCR) 7th Annual Meeting, Centre Convencions Internacional Barcelona, Barcelona, Spain; July 8-11 2009