EFFECT OF SODIUM BUTYRATE ON HEPATOGENIC TRANSDIFFERENTIATION OF HUMAN WHARTON'S JELLY-DERIVED MESENCHYMAL STEM CELLS

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ผลของโซเดียมบิวทิเรทต่อการเหนี่ยวนำเซลล์ต้นกำเนิดมีเซนไคม์จากเนื้อเยื่อวาร์ตันเจลลี่มนุษย์เป็นเซลล์ตับ

วชิระ พันธ์ทา

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

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วชิระ พันธ์ทา : ผลของโซเดียมบิวทิเรทต่อการเหนี่ยวนำเซลล์ต้นกำเนิดมีเซนไคม์จากเนื้อเยื่อวาร์ตันเจลลี่มนุษย์เป็นเซลล์ตับ (EFFECT OF SODIUM BUTYRATE ON HEPATOGENIC TRANSDIFFERENTIATION OF HUMAN WHARTON'S JELLY-DERIVED MESENCHYMAL STEM CELLS) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร. รังสรรค์ พาลพ่าย, 104 หน้า.

เซลล์ต้นกำเนิดมีเซนไคม์เป็นเซลล์ที่เกิดจากเนื้อเยื่อชั้นมีโซเดิร์ม ที่มีคุณสมบัติสามารถเพิ่มจำนวน และพัฒนาไปเป็นเซลล์ของเนื้อเยื่อชั้นมีโซเดิร์มได้ทุกชนิด อีกทั้งยังสามารถพัฒนาไปเป็นเซลล์ของเนื้อเยื่อประสาทชั้นเอ็กโตเดิร์ม และเซลล์ของเนื้อเยื่อชั้นเอ็นโดเดิร์มบางชนิดได้ รวมทั้งเซลล์ตับ ปัจจุบันเซลล์ต้นกำเนิดมีเซนไคม์จากเนื้อเยื่อวาร์ตันเจลลี่ที่เก็บได้จากสายสะดือมนุษย์ (Human Wharton's jelly-derived MSCs, hWJ-MSCs) ถือได้ว่าเป็นแหล่งที่น่าสนใจสำหรับนำมารักษาโรคด้วยวิธีเซลล์บำบัด แม้ว่านักวิจัยหลายคนสามารถเหนี่ยวนำ hWJ-MSCs ไปเป็นเซลล์คล้ายเซลล์ตับได้ แต่ด้วยสารเคมีที่มีราคาแพงและระยะเวลาการเหนี่ยวนำที่นาน เป็นข้อจำกัดของการได้เซลล์คล้ายเซลล์ตับ ดังนั้น การใช้สารเคมีชนิดอื่นที่ถูกกว่าและใช้เวลาสั้น เพื่อช่วยส่งเสริมให้ hWJ-MSCs เปลี่ยนแปลงไปเป็นเซลล์คล้ายเซลล์ตับเป็นสิ่งที่ควรทำ มีงานวิจัยจำนวนมาก พบว่า โซเดียมบิวทิเรท (NaB) สามารถส่งเสริมการเหนี่ยวนำเซลล์ต้นกำเนิดตัวอ่อนไปเป็นเซลล์คล้ายเซลล์ตับได้

วัตถุประสงค์การศึกษานี้ คือ เพื่อหาผลของ NaB ที่ใช้ร่วมกับ Epidermal growth factor (EGF) และ Basic fibroblast growth factor (bFGF) ต่อการเหนี่ยวนำ hWJ-MSCs ไปเป็นเซลล์คล้ายเซลล์ตับของเนื้อเยื่อชั้นเอ็นโดเดิร์ม นอกจากนี้ยังตรวจสอบความเข้มข้นที่เหมาะสมของ NaB ที่ใช้ร่วมกับ EGF และ bFGF ก่อนการเหนี่ยวนำ hWJ-MSCs ไปเป็นเซลล์ตับ โดยใช้ข้อมูลการแสดงออกของยีนที่เกี่ยวข้องกับการพัฒนาไปเป็นเซลล์ในชั้นเอ็นโดเดิร์มด้วยวิธี qPCR และการแสดงออกของโปรตีน SOX17 ด้วยวิธี Immunocytochemistry ทำการเปรียบเทียบการเหนี่ยวนำ hWJ-MSCs ไปเป็นเซลล์คล้ายเซลล์ตับระหว่างกลุ่มน้ำยาเหนี่ยวนำเซลล์ตับที่เติม NaB ช่วงก่อนการเหนี่ยวนำไปเป็นเซลล์ตับร่วมกับ EGF และ bFGF เป็นเวลา 72 ชั่วโมง (Hepatogenic medium + NaB pre-treated) และกลุ่มน้ำยาเหนี่ยวนำเซลล์ตับที่ไม่เติม NaB ช่วงก่อนการเหนี่ยวนำไปเป็นเซลล์ตับร่วมกับ EGF และ bFGF เป็นเวลา 72 ชั่วโมง (Hepatogenic medium - NaB pre-treated) โดยตรวจสอบการเปลี่ยนแปลงไปเป็นเซลล์คล้ายเซลล์ตับด้วยวิธี Immunocytochemistry, qPCR, Periodic acid-Schiff (PAS) reaction และ Urea production assay ของวันที่ 3, 10, 17 และ 24 ของการเหนี่ยวนำ นอกจากนี้ยังตรวจสอบผลของ NaB ต่อการยับยั้งการทำงานของเอนไซม์ Histone deacetylase inhibitor 1

(HDAC1) ด้วยวิธี Immunocytochemistry, Western blot และ qPCR ผลการทดลองพบว่า การเติม NaB 1 mM ในน้ำยา ก่อนการเหนี่ยวไปเป็นเซลล์ตับ (PTM + 1 mM NaB) เป็นความเข้มข้นที่เหมาะสม ในการยับยั้งการทำงานของเอนไซม์ HDAC1 และช่วยส่งเสริมให้ hWJ-MSCs เปลี่ยนแปลงไปเป็นเซลล์ในชั้นเอนโดเดิร์มได้ นอกจากนี้การแสดงออกของยีน *CXCR4, SOX17* และ *GATA6* ยังสูงกว่ากลุ่มความเข้มข้นอื่น อย่างมีนัยยะสำคัญทางสถิติ ($p<0.001$) อีกทั้งการแสดงออกของโปรตีน SOX17 ยังให้ผลสอดคล้องกับการแสดงออกของยีน *SOX17* อีกด้วย ดังนั้นจึงเลือก NaB ความเข้มข้น 1 mM เติมในน้ำยาก่อนการเหนี่ยวนำไปเป็นเซลล์ตับ ร่วมกับ EGF และ bFGF เป็นเวลา 72 ชั่วโมง ก่อนการเหนี่ยวนำ hWJ-MSCs ไปเป็นเซลล์คล้ายเซลล์ตับ ผลการทดลองพบว่า กลุ่ม Hepatogenic medium + NaB pre-treated มีการแสดงออกของยีนและโปรตีนของ early hepatic markers (AFP และ HNF3β), late hepatic markers (CK18 และ ALB) และความสามารถการทำหน้าที่ของเซลล์คล้ายเซลล์ตับที่โตเต็มที่ (การสะสมไกลโคเจนและการผลิตยูเรีย) มากกว่ากลุ่ม Hepatogenic medium - NaB pre-treated อย่างมีนัยยะสำคัญทางสถิติ ($p<0.05$) การทดลองนี้สามารถสรุปได้ว่า การใช้ NaB ร่วมกับ EGF และ bFGF ช่วงก่อนการเหนี่ยวนำไปเป็นเซลล์คล้ายเซลล์ตับ มีประสิทธิภาพในการเหนี่ยวนำ hWJ-MSCs ไปเป็นเซลล์ในชั้นเอนโดเดิร์ม เซลล์ตั้งต้นเซลล์ตับ และเซลล์คล้ายเซลล์ตับที่สามารถทำหน้าที่ได้ ดังนั้น การเหนี่ยวนำ hWJ-MSCs ไปเป็นเซลล์คล้ายเซลล์ตับด้วยน้ำยา Hepatogenic medium + NaB pre-treated สามารถนำมาใช้เป็นวิธีการทางเลือกในการผลิตเซลล์คล้ายเซลล์ตับ เพื่อนำมารักษาโรคด้วยวิธีเซลล์บำบัด และทดสอบประสิทธิภาพของยาในการประยุกต์ใช้งานทางคลินิค

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2559 ลายมือชื่ออาจารย์ที่ปรึกษา_____

WACHIRA PANTA : EFFECT OF SODIUM BUTYRATE ON HEPATOGENIC TRANSDIFFERENTIATION OF HUMAN WHARTON'S JELLY-DERIVED MESENCHYMAL STEM CELLS. THESIS ADVISOR : ASSOC. PROF. RANGSUN PARNPAI, Ph.D., 104 PP.

WHARTON'S JELLY-DERIVED STEM CELLS/HEPATOCYTE-LIKE CELLS/ HEPATOGENIC DIFFERENTIATION/SODIUM BUTYRATE/ HISTONE DEACETYLASE INHIBITORS (HDACi)

Mesenchymal stem cells (MSCs) are multipotent mesoderm-derived cells that can self-renew and differentiate into all mesodermal, and some neuroectodermal and endodermal progenies include hepatocytes. Nowadays, human Wharton's jelly-derived MSCs (hWJ-MSCs) are an attractive source for cell-based therapy. Although, many investigators successfully used hWJ-MSCs to differentiate into hepatocyte-like cells, however, high chemical costs and long period of time for induction are the limitation factors. Therefore, the used of other cheaper chemical and less-time consuming to promote hWJ-MSCs into hepatocyte-like cells are needed. With many evidences, sodium butyrate (NaB) can promote embryonic stem cells (ESCs) differentiation toward hepatocyte-like cells.

The aim of this study was to verify the effect of NaB in combination with Epidermal growth factor (EGF) and Basic fibroblast growth factor (bFGF) on hWJ-MSCs transdifferentiation toward hepatoendodermal lineage. Furthermore, the optimal concentration of NaB in combination with EGF and bFGF right before hepatogenic

transdifferentiation of hWJ-MSCs was evaluated using the qPCR data of endodermal-related genes expression and immunocytochemistry of SOX17 protein expression. The differentiation of hWJ-MSCs into hepatocyte-like cells with hepatogenic medium with NaB-pretreatment (Hepatogenic medium + NaB-pretreated) and hepatogenic medium without NaB pre-treatment (Hepatogenic medium – NaB-pretreated) were also assessed by immunocytochemistry, qPCR, Periodic acid-Schiff (PAS) reaction and urea production assays at day 3, 10, 17 and 24 of differentiation. Besides, the effect of NaB on histone deacetylase 1 (HDAC1) inhibition was also investigated by immunofluorescence, Western blot and qPCR analyses. The results found that 1 mM NaB in pre-treatment medium (PTM + 1 mM NaB) group was found to be the optimum concentration for strong HDAC1 inhibition and promote endodermal transdifferentiation (up-regulated *CXCR4, SOX17* and *GATA6* transcripts and SOX17 protein expression) from hWJ-MSCs. The hepatogenic medium + NaB pre-treated group was found to express hepatoblastic markers (*AFP* and *HNF3β*), hepatic markers (*CK18* and *ALB)*, and functional matured hepatocyte-like cells (glycogen storage and urea production) greater than the Hepatogenic medium – NaB pre-treated group. This experiment can be concluded that combination of NaB with EGF and bFGF in pre-treatment step efficiently induced hWJ-MSCs toward endoderm, hepatoblast and hepatocyte-like cells. Thus, the differentiated hWJ-MSCs with NaB pre-treatment might be conceived as an alternative protocol to produce hepatocyte-like cells for cell-based therapy and drug screening in clinical applications.

School of Biotechnology

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Student's Signature Wachira Panta

Advisor's Signature

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Wachira Panta.

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LIST OF ABBREVIATIONS

ADSCs	=	adipose tissue-derived stem cells
AF-MSCs	=	amniotic fluid-derived mesenchymal stem cells
AFP	=	alpha-fetoprotein
ALB	=	albumin
α-MEM	=	Alpha-Minimum Essential medium
AM-MSCs	=	amniotic membrane-derived mesenchymal stem cells
bFGF	=	basic fibroblast growth factor
BM-MSCs	=	bone marrow-derived mesenchymal stem cells
BSA	=	bovine serum albumin
CD	=	cluster of differentiation
cDNA	=	complementary deoxyribonucleic acid
CK18	=	cytokeratin 18
cm^2	=	square centimeter
CO_2	=	carbon dioxide
CXCR4	=	C-X-C chemokine receptor type 4
^oC	=	degree Celsius
d	=	day
DAPI	=	4', 6-diamidino-2-phenylindole
DE	=	definitive endoderm
DMEM	=	Dulbecco's Modified Eagle's medium
DMSO	=	dimethyl sulfoxide
DPSCs	=	dental pulp-derived stem cells

LIST OF ABBREVIATIONS (Continued)

DTT	=	dithiothreitol
EGF	=	epidermal growth factor
ESCs	=	embryonic stem cells
FBS	=	fetal bovine serum
FGF	=	fibroblast growth factor
FOXA2	=	forkhead box protein A2
GAPDH	=	glyceroldehyde-3-phosphate dehydrogenase
GATA6	=	GATA-binding factor 6
h	=	hour
hBM-MSCs	=	human bone marrow-derived mesenchymal stem cells
HDAC	=	histone deacetylase
HDAC1	=	histone deacetylase 1
HDACi	=	histone deacetylase inhibitors
HGF	=	hepatocyte growth factor
HNF3β	=	hepatocyte nuclear factor-3β
hWJ-MSCs	=	human Wharton's jelly-derived mesenchymal stem cells
IBMX	=	3-isobuutyl-1-methylxanthine
IgG	=	immunoglobulin G
IMDM	=	Iscove's Modified Dulbecco's medium
ISCT	=	International Society for Cellular Therapy
ITS	=	insulin-transferin-selenium
ITS-X	=	insulin-transferin-selenium-ethanolamine
LETFs	=	liver-enriched transcription factors

LIST OF ABBREVIATIONS (Continued)

mRNA	=	messenger RNA
µg	=	microgram
µl	=	microliter
µm	=	micrometer (micron)
µM	=	micromolar
ml	=	milliliter
mm	=	millimeter
min	=	minute
MSCs	=	mesenchymal stem cells
MELD	=	model of end-stage liver disease
MTT	=	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium
NH_4Cl	=	ammonium chloride
ng	=	nanogram
PAS	=	Periodic acid-Schiff
PBS	=	phosphate buffer saline
PBS-T	=	phosphate buffer saline + Tween-20
PFA	=	paraformaldehyde
PL-MSCs	=	placenta-derived mesenchymal stem cells
PTM	=	pre-treated medium
qPCR	=	quantitative polymerase chain reaction
SDS	=	sodium dodecyl sulfate
SOX17	=	sex-determining region Y chromosome (SRY)-box 17
NaB	=	sodium butyrate

LIST OF ABBREVIATIONS (Continued)

OSM	=	oncostatin M
RNA	=	ribonucleic acid
TSA	=	tricostatin A
U	=	units
UC-MSCs	=	umbilical cord-derived mesenchymal stem cells
UCB-MSCs	=	umbilical cord blood-derived mesenchymal stem cells
VPA	=	valproic acid
WJ	=	Wharton's jelly
WJ-MSCs	=	Wharton's jelly-derived mesenchymal stem cells

CHAPTER I

INTRODUCTION

1.1 Background

Liver is an important and largest organ that can produce metabolites and detoxify toxic substances in our body. Hepatocytes are cells in the liver that are gradually being destroyed from the toxic substances which lead to liver failure and death at the end-stage of liver diseases (reviewed in Gordillo, Evans and Gouon-Evans, 2015). The treatments of orthotropic liver organ and cells transplantations have recently been pioneered for these patients. Nevertheless, the inadequate and the shortage of organ donors and low quality cultivation of hepatocytes are the main problems for these kinds of treatments (Zaret and Grompe, 2008; Dhawan et al., 2010). The limitation of these treatments needs some new alternative therapeutic approaches. Presently, stem cell technology has been considered as a promising tool in cell-based therapy. The field of mesenchymal stromal cells (MSCs) has been getting high attention.

The fact that the used of MSCs have less ethical concern and fewer teratomatous formation when compared with pluripotent embryonic stem cells (ESCs) makes them a promising tool. Also, MSCs have shown some attractive qualities; such as plastic attachment, self-renewal, positive expression of unrestricted MSCs markers and tri-mesodermal differentiation such as osteoblasts, chondrocytes and adipocytes

(Dominici et al., 2006). The MSCs can be isolated from both fetus and postnatal tissues such as bone marrow, adipose tissue, amniotic fluid, umbilical cord blood and umbilical cord tissue (Romanov et al., 2005; Troyer and Weiss, 2008; Witkowska-Zimny and Wrobel, 2011). These entire reasons make MSCs became a good candidate for therapeutic purpose in clinical applications.

Umbilical cord Wharton's jelly tissue is the richest and attractive source of MSCs. It can be obtained through noninvasive collection. It also has low immunogenicity when compared to other adult MSC tissue sources (Karahuseyinoglu et al., 2007). The characteristics of Wharton's jelly-derived MSCs (WJ-MSCs) similar to bone marrow-derived MSCs (BM-MSCs) are prototype. They can differentiate into hepatocyte-like cells of endodermal lineage which make them an alternative choice for an extensive hepatocyte-like cells differentiation study (Baksh, Yao and Tuan, 2007; Wang et al., 2004). The derivation of MSC-derived hepatocyte-like cells *in vitro* mimics the embryonic hepatoendodermal development *in vivo* and the ESCs differentiation *in vitro* studies of animal and human development models (reviewed in Gordillo, Evans and Gouon-Evans, 2015). Nowadays, many hepatogenic differentiation protocols of hWJ-MSCs *in vitro* have been successfully achieved (Campard et al., 2008; Zhang et al., 2009; Zhao et al., 2009; Anzalone et al., 2010; Yoon et al., 2010; Prasajak and Leeanansaksiri, 2013; An et al., 2014; Borhani-Haghighi et al., 2015); however, more attractive protocols needed to be developed to obtain homogenous hepatocyte-like cells population with less-time consuming and cheaper chemicals.

Sodium butyrate (NaB) is a small molecule, short chain butyric acid, which in a fatty acid in its sodium salt formed from dietary fibers fermentation of anaerobic

bacteria in alimentary tract (Ghosh et al., 2012). It plays a key role as histone deacetylase inhibitor (HDACi) in anti-tumor drug (Chen, Ghazawi, Bakkar, and Li, 2006), neurological disorder treatments (Bourassa et al., 2016) and stem cell differentiation at physiological concentrations (Vrba, Trtkova and Ulrichova, 2011; Shah et al., 2013). It has been used successfully and consistently in ESCs differentiation to hepatocyte-like cells (Rambhatla et al., 2003; Sharma et al., 2006; Zhou et al., 2007; Mizumoto et al., 2008; Hay et al., 2008; Ren et al., 2010; Zhou et al., 2010; Cao et al., 2010; Yan et al., 2011). Therefore, these evidences made NaB as an appropriate chemical of choices to promote MSC differentiation into hepatocyte-like cells. However, the combination of NaB with growth factors, such as activin A (Mizumoto et al., 2008) or bFGF and BMP4 (Cao et al., 2010) can promote ESCs differentiation into hepatocyte-like cells greater than NaB treatment alone. Therefore, it is important to find out in details of the combination of NaB and growth factors that can promote MSC differentiation into hepatocyte-like cells.

The aim of this study was to verify the approach of hWJ-MSCs differentiation into functional hepatocyte-like cells that expressed endodermal, hepatoblast and hepatocyte-like features. To achieve this goal, the combination of NaB with EGF and bFGF in the pre-treatment step for 72 h before hepatogenic differentiation from hWJ-MSCs with other maturation factors (Campard et al., 2008), such as HGF, bFGF and nicotinamide (in the differentiation step) and OSM, dexamethasone and ITS (in the maturation step) were investigated. This protocol could provide an alternative source of hWJ-MSCs-derived hepatocyte-like cells for stem cell-based therapy in patients with end-stage liver diseases.

1.2 Research objectives

1.2.1 To isolate and characterize hWJ-MSCs from human umbilical cord Wharton's Jelly tissue.

1.2.2 To investigate the effect of NaB on HDACi through HDAC1 gene and protein expressions using immunocytochemistry, Western Blot and RT-qPCR analyses.

1.2.3 To determine the optimum concentration of NaB on cell viability, endodermal and hepatogenic transdifferentiate of hWJ-MSCs *in vitro*.

1.2.4 To characterize HI-hWJMSCs *in vitro* using Immunocytochemistry, qRT-PCR and biochemical function analyses.

1.3 Research hypotheses

1.3.1 Under defined culture system, MSCs could be isolated and expanded from human umbilical cord Wharton's jelly tissue and should exhibit typical properties of MSCs, which are self-replicate, immunophenotyping, as well as their ability to tri-mesodermal lineages differentiation in *in vitro*.

1.3.2 The optimized NaB pre-treatment could promote endodermal-related genes and protein expressions through partially HDAC1 inhibition in the present of EGF and bFGF supplementation and enhances hepatogenic differentiation of hWJ-MSCs in *in vitro*.

1.4 Scope of the study

1.4.1 Under defined culture system, MSCs were isolated and expanded from human umbilical cord Wharton's jelly tissue. MSC signatures from this tissue were examined by the expression profile of surface antigens (e.g. CD34, CD73, CD90 and CD105). Tri-mesodermal lineages differentiation of hWJ-MSCs was performed by the differentiated osteoblast, chondrocyte and adipocyte.

1.4.2 The optimized NaB pre-treatment were checked from cell viability and endodermal-related genes (*CXCR4, SOX17,* and *GATA6*) and SOX17 protein expressions through partially HDAC1 inhibition in the present of EGF and bFGF supplementation before hepatogenic differentiation. The differentiated hWJ-MSCs with NaB pre-treatment can be differentiated into hepatoblast-like cells (AFP and HNF3β) and hepatocyte-like cells (CK18 and ALB) greater than the differentiated hWJ-MSCs without NaB pre-treatment. Besides, functional mature hepatic properties were evaluated by Periodic acid-Schiff (PAS) reaction and urea production assays.

CHAPTER II

LITERATURE REVIEW

2.1 Liver

The liver is the largest internal gland and consists of four poorly defined lobes of the human body. It has providing many important metabolic, exocrine and endocrine functions, such as bile production, dietary compound metabolism, detoxification, blood glucose levels regulation through glycogen storage, blood homeostasis control by blood clotting factors and serum protein albumin (ALB) secretion. The structural and functional unit of the liver is the hepatic lobule, which consists of polygonal-shaped hepatic cord of liver cells arranged around a central vein that is a terminal branch of a hepatic vein. Branches of hepatic artery, portal vein, together with a bile duct (Fig. 2.1A), form the classic portal triad found in the portal spaces (reviewed in Zorn and Wells, 2007; Gordillo, Evan and Gouon-Evans, 2015).

2.1.1 Cell types and their organization of the hepatic lobule

The mature liver contains multiple cell types, including hepatocytes and cholangiocytes are derived from the embryonic endoderm, whereas the endothelial, stellate and Kupffer cells are of mesodermal origin (Fig. 2.1B).

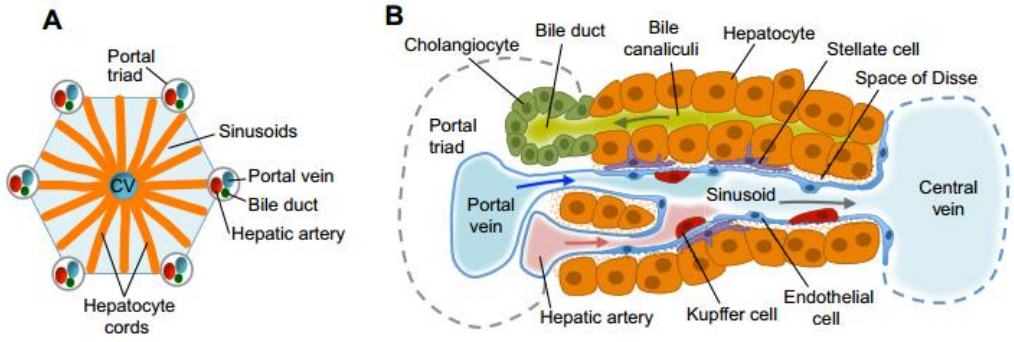

Figure 2.1 Liver structure and cell types. (Gordillo, Evan and Gouon-Evans, 2015)

(A) Structure of the hepatic lobule and portal triad.

(B) Organization of the hepatic lobule and cell types in the mature liver.

2.1.1.1 Hepatocytes and cholangiocytes

Hepatocytes are the functional metabolic, exocrine and endocrine cells of the hepatic lobules that accounting for approximately 80% of the mass of the adult organs. A hepatocyte has two distinct domains: basolateral and apical domains. The basolateral domain with microvilli was separated from blood circulating of hepatic sinusoid by space of Disse, which enables an exchange metabolites and toxins between blood and hepatocyte. Representation of the bile pole of hepatocyte, an apical domain, secreted bile out into the bile canaliculi (grooves in the cell surface) that flow through the intra hepatic bile duct (IHBD) to the extra hepatic bile duct (EHBD) and stored into the gall bladder before release into the duodenum (reviwed in Zorn and Wells, 2007; Gordillo, Evan and Gouon-Evans, 2015).

2.1.1.2 Endothelial and Kupffer cells

In the liver, endothelial and Kupffer cells line the hepatic sinusoid. Endothelial cells have a fenestrated cytoplasm associated with a discontinuous basal lamina. Kupffer cells are the differentiated phagocytic cell derived from monocytes (reviewed in Gordillo, Evan and Gouon-Evans, 2015). During liver organogenesis, endothelial cells are important for hepatic specification and hepatoblast expansion from murine endoderm cells and ESC-derived endoderm cells (Han et al., 2011). No evidence of Kupffer cells for hepatic differentiation; however, they play an important role in liver regeneration during chronic injury by influencing the invasive behavior of liver progenitor cells (Boulter et al., 2012).

2.1.1.3 Stellate cells

Stellate (Ito) cells are the major mesenchymal component in the liver that located in the space of Disse. They are the major vitamin A reservoir when quiescent state, but when injury or infection occurred, they were activated into α-smooth muscle actin (αSMA)-expressing myofibroblast-like cells differentiation that extracellular matrix (ECM) deposits capacity and directing temporary fibrotic scar formation (Puche et al., 2013).

2.2 Liver diseases and their therapies

2.2.1 Liver diseases

Liver or hepatic diseases are describing all the potential problems because the liver performance to fail theirs designated functions. More than 75% of liver tissue needs to be affected before a decrease in function occurs. They occurred from many causes, such as infection and inflammation, alcoholism, genetic defects and tissue injury from toxic drugs or compounds. Common symptoms of liver diseases include weakness, fatigue, weight loss, nausea, vomiting and jaundice. However, since there are a variety of liver diseases, the symptoms tend to be specific for that illness until late-stage liver disease and liver failure occurs (Wedro, 2011). The stages of liver diseases are classified by the American Association for Clinical Chemistry (AACC) of the US groups in 2011. Initially, the higher dosage and time exposure of toxins and drugs including alcoholic over-uptake, leads to lipid formation in the liver, known as fatty liver, caused by both alcoholic and non-alcoholic cases. Then, the lesions are progressed to inflammation of reversible fibrous scar tissue of the liver, hepatitis. Unfortunately, the advanced fibrous scar tissue results in irreversible regeneration of liver tissue and function, known as cirrhosis. In some case, it could progress to hepatocarcinoma and lead to liver failure and death from other complications (ascites, hepatic encephalopathy, and portal hypertension) at late-stage liver diseases.

2.2.2 Regenerative therapies

As mentioned above, approximately three quarters of liver tissue are destroyed that affect irreversible or difficult reversible of normal physiological liver functions. Normally, treatment of liver diseases depends on the severity of disorders or symptoms. It has not been only relieved with medicine, but also the operation to cure may be used. For instance, ascites fluid and portal hypertension are cured by removed excess fluid and operation with minimize the risk of bleeding. Nevertheless, orthotropic liver transplantation is the final choice of the effective treatment of these diseases (Wedro, 2011). However, the shortage time and the inadequate of liver organ, high costs and life-long requirement for immunosuppression were limited to use of this therapy (Dhawan, Puppi, Hughes, and Mitry, 2010). The field of stem cell therapy has raised great hope for improving the treatment of liver diseases. Due to, they may retain the potential to transdifferentiation from one phenotype to another type, presenting exciting possibilities for cellular therapies (Talèns-Visconti et al., 2006). Therefore, a promising source of stem cells for the end-stage of liver diseases treatment could be challenged.

2.3 Mesenchymal stem cells (MSCs)

The first identification of mesenchymal stem cells (MSCs) occurred from the minor population of bone marrow in 1961 (Friedenstein, et al., 1961). After this discovery occurred, the multipotent differentiation potential of tri-mesodermal lineages (Fig. 2.2) also occurred into osteoblasts, chondrocytes and adipocytes (Pittenger, et al., 1999). Otherwise, MSCs have also been isolated from adipose tissue

(Fraser et al., 2008), dental pulp (Liu et al., 2009), umbilical cord blood (Phuc et al., 2011) and Wharton's jelly of umbilical cord tissue (Wang et al., 2004). In 2006, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) had established the MSCs definition and changed the name from mesenchymal stem cells to mesenchymal stromal cells. Firstly, MSCs have been characterized by their plastic adherent growth and subsequent expansion under appropriate culture conditions. Secondly, they must express their protein surface markers CD73, CD90 and CD105, and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules. Thirdly, they must differentiate into osteoblasts, chondrocytes and adipocytes *in vitro* under appropriate culture conditions (Dominici et al., 2006). With the multipotent characteristics, abundant source of cells, and little ethical controversy, MSCs could be more appropriate source of cell for liver diseases treatment.

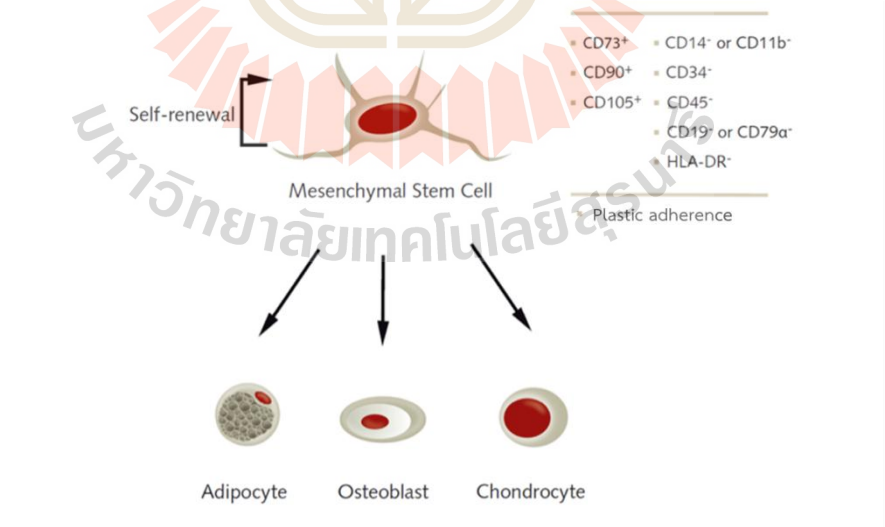

Figure 2.2 Three minimal criteria recommended for identification of MSCs. (Modified from Application note of PromoCell GmbH Company, Germany, 2015).

2.4 Human umbilical cord and human Wharton's jelly-derived stem cells (hWJ-MSCs)

Wharton's jelly (WJ) is proteoglycans and mucopolysaccharides-rich connective tissue that surrounded two arteries and a vein (Fig. 2.3) of umbilical cord of extra-embryonic membrane (Karahuseyinoglu et al., 2007). Naturally, WJ supports the structure of umbilical blood vessels during pregnancy. The cell component is presented by mesenchyme-derived cells, such as fibroblasts, myofibroblasts, smooth muscle cells, and MSCs. Presently, the MSCs from umbilical cord include cells derived from the total umbilical cord or its different sections; perivascular, intervascular, and subamnion zones of WJ and subendothelial layer, but not from umbilical cord lining or inner blood vessel walls (Bongso and Fong, 2013).

Thomas Wharton discovered WJ tissue in 1956. This tissue was considered medical waste with no scientific value. Until McElreavey and collaborates isolated fibroblast-like cells from WJ and characterized them (McElreavey, Irvine, Ennis, and McLean, 1991). These fibroblast-like cells were proved to be MSCs as they expressed CD29, CD44, CD51, CD73, and CD105, lacked expression of CD34 and CD45, and were able to differentiate into cells of the adipogenic and osteogenic lineages (Wang et al., 2004). According to previous report, only 0.001-0.01% of MSCs presented in bone marrow stromal, while for MSCs of umbilical cord tissue it reached 0.2-1.8% (Wegmeyer, et al., 2013). Besides, MSCs of umbilical cord tissue have higher proliferative potential, easily accessible and abundant than MSCs from other sources; adipose tissue (postnatal), placenta and amniotic membrane (neonatal sources) (Li et al., 2014; Shaer, Azarpira, Aghdaie, and Esfandiari, 2014). Additionally, several publications reported the successful *in vitro* differentiation of WJ-derived MSCs (WJ-

MSCs) into various specific cell types beyond their mesodermal lineage, such as glial cells and neurons (Mitchell et al., 2003), dopaminergic neurons (Fu et al., 2006) in ectodermal lineage and insulin-producing cells (Chao et al., 2008; Wang et al., 2011) as well as hepatocyte-like cells (Zhang, Lie, and Wei, 2009) in endoderm lineage. Therefore, these benefits bring up umbilical cord tissue as a potential adult stem cell reservoir for further clinical application in each individual.

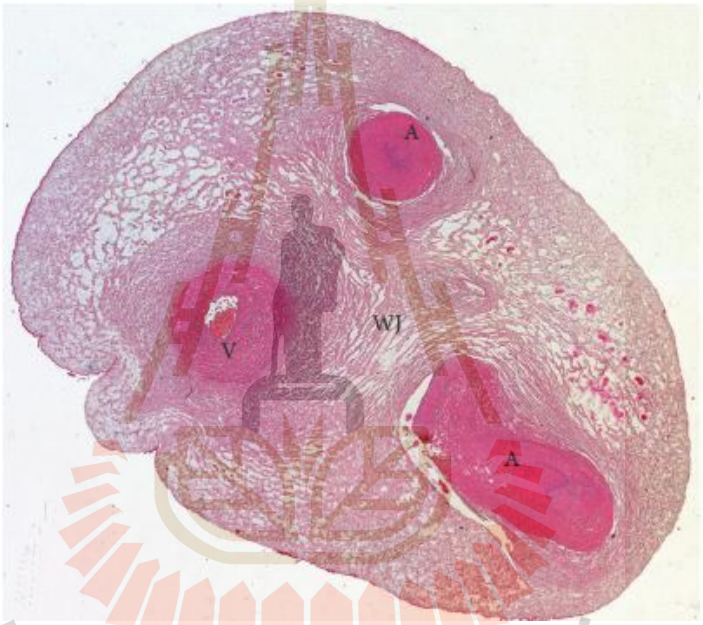

Figure 2.3 Cross section of normal human umbilical cord. A: artery; V: vein; WJ: Wharton's jelly. Hematoxylin and eosin staining, scale bar = 200 μm. (Arutyunyan, Elchaninov, Makarov, and Fatkhudinov, 2016).

2.5 Hepatogenesis and their cell signaling pathway regulation

With highly complexity and organization of liver developmental processes from embryo to adult can be divided into several distinct stages based on molecular and functional properties of human and animal models *in vivo* and ESC-derived hepatocyte-like cells *in vitro* studies. Many evidences of hepatogenesis are evolutionarily conserved and occur through a progressive series of mutual tissue interactions between the embryonic endoderm and nearby mesoderm. Nowadays, liver development duration is identifiable in zebrafish, mouse and human models (**Table 2.1**) (reviewed in Gordillo, Evan and Gouon-Evans, 2015).

Table 2.1 Interspecies comparison of the timeline of liver development (reviewed in Gordillo, Evan and Gouon-Evans, 2015).

Developmental stages	Zebrafish(h)	Mouse (d)	Human (d)
1. Endoderm	6	7.5	< 23
2. Liver diverticulum: hepatoblast specification	18-24	8.5-9.0	23-26
3. Liver bud: hepatoblast migration and proliferation	24-28	9.5	26-32
4. Liver bud outgrowth: proliferation of hepatoblasts and sinusoid expansion	48-120	9.5-14.5	31-56
5. Hepatocyte and cholangiocyte differentiation	24-48	13.5 (**initiation**) to 18.5 (**termination**)	56-58 (**initiation**) to ~210 (**termination**)
6. Gestation duration	**NA**	18-19	266-280

ABBREVIATION: NA: not applicable, h: hour, d: day.

According previous mentioned above, hepatocytes and cholangiocytes are derived from endoderm that emerges from the anterior primitive streak of the gastrulating embryo. Four key major steps model during fetal liver development are summarized following mentioned below (Fig. 2.4) (reviewed in Gordillo, Evan and Gouon-Evans, 2015).

(A) Endoderm specification; activation of Activin/Nodal signaling, is a transforming growth factor β (TGFβ) family, that signal through activin receptors type I and II, and the co-receptor Cripto (Teratocarcinoma-derived growth factor-1; Tdgf1) to drive mesendoderm formation from the embryonic epiblast lineage in *Xenopus* (Hyde and Old, 2000; Xanthos et al., 2001) and zebrafish (Schier and Talbot, 2005; Fan et al., 2007), while in mice uncleaved Nodal precursor in the epiblast activates bone morphogenic protein 4 (BMP4), and then activates Wingles 3 (Wnt3) to maintain high levels of Nodal selectively (Ben-Haim et al., 2006). Bipotential mesendoderm cells segregate into brachyury-expressing (BRY^{+}) mesoderm and definitive endoderm (DE). With low Activin/Nodal doses inducing mesoderm and higher doses inducing endoderm (Zorn and Wells, 2007). The prospective foregut endoderm maintains expression of the pioneer transcription factors forkhead box protein A2 (FOXA2) or hepatocyte nuclear factor-3β (HNF3β) and GATA-binding factor 4/6 (GATA4/6), denoted GATA. Recently, C-X-C chemokine receptor type 4 (CXCR4) can be used to distinguish early visceral and definitive endoderm in both human and mouse ESCs (D'Amour et al., 2005; Yasunaga et al., 2005; Takenaga, Fukumoto, and Hori, 2007; Teo et al., 2011).), as well as in the differentiated hWJ-MSCs into DE cells (Allahbakhshi et al., 2013; An et al., 2014).

(B) Endothelial cells influence during liver morphogenesis; studies of ESC cultures suggest that the DE includes a subset of kinase insert domain receptor-expressing (KDR^+) progenitors that generate and support the development of hepatic cells; they can also give rise to CD31-expressing or platelet endothelial cell adhesion molecule 1-expressing ($PECAM1^+$) endothelial cells.

(C) Hepatic specification from hepato-pancreatic progenitors; the graded activity of Wnt, fibroblast growth factor (FGF) and BMP signaling patterns the endoderm along the anterior-posterior (A-P) axis (Horb and Slack, 2001; Kimura et al., 2007), to generate posterior foregut precursors with hepato-pancreatic potential that can be divided from anterior foregut, deriving lung and thyroid and midgut-hindgut, deriving intestine progenitors. Foregut DE generates a bipotential hepato-pancreatic progenitor; SRY (sex determining region Y chromosome)-box 17-expressing ($SOX17^+$), hematopoietically-expressed homeobox-expressing ($HHEX^+$), and $GATA^+$ that produces both pancreatic and duodenal homeobox 1-expressing ($PDX1^+$) pancreatic progenitors and $HNF1\beta^+$, $HNF4\alpha^+$, $PDX1^-$ hepatoblasts.

Hepatoblast expansion is regulated by interactions with surrounding endothelial and mesenchymal cells in the septum transversum (Shin and Monga, 2013). Wnt signaling promotes the proliferation of hepatoblasts (Goessling et al., 2008; McLin et al., 2007), acting in cross-talk with hepatocyte growth factor (HGF) by the phosphorylation of β-catenin through HGF receptor (HGFR) or tyrosine-protein kinase methionine proto-oncogene (c-MET) or by nuclear translocation and FGF signaling (Berg et al., 2007; Schmidt et al., 1995). Besides, hepatoblasts are marked by the expression of both hepatocyte (alpha-fetoprotein (AFP), albumin (ALB), cytokeratin 18 (CK18)) and cholangiocyte (CK19) transcript and protein

markers. Additionally, delta homolog 1 (DLK1), epithelial cadherin (E-cadherin), epithelial cell adhesion molecule (EpCAM), CD133 or prominin 1 (PROM1) and the hepatocyte-specific antigen (HepPar1) are also expressing in hepatoblasts (Haruna et al., 1996; Terrace et al., 2007; Wauthier et al., 2008).

(D) Hepatic commitment from hepatoblast; hepatoblast differentiation into cholangiocytes and hepatocytes, and the final maturation of these cells, is regulated by a wide array of signaling pathways that display complex cross-regulation. Signaling by HGF, oncostatin M (OSM), and glucocorticoid hormones act in a reinforcing to promote hepatocyte maturation (Shin and Monga, 2013), while cholangiocyte induction activate TGFβ, Notch, BMP, FGF, Wnt, and Hippo/Yap signaling pathway. These pathways also influence the 3D structural organization of the liver and define its zonal characteristics. The hepatoblast is a bipotential progenitor for both cholangiocytes (bile duct cells; $HNF6^{+}$, $SOX9^{+}$, $HNF1\beta^{+}$) and hepatocytes (Prospero homeobox protein 1-expressing; $PROX1^{+}$ and $HNF4\alpha^{+}$). Besides, CCAAT enhancer binding protein (C/EBP)-α, -β are also expressing in mature hepatocyte (reviewed in Snykers et al., 2009). In human ESC (hESC) differentiation cultures, KDR-expressing progenitors for hepatoblast-like cells occur concomitantly with committed hepatic cells and promote their maturation, as indicated by ALB expression (Goldman et al., 2013). Note, in many cases, such as for FoxA and GATA factors, they also continue to be expressed and also function at later stages.

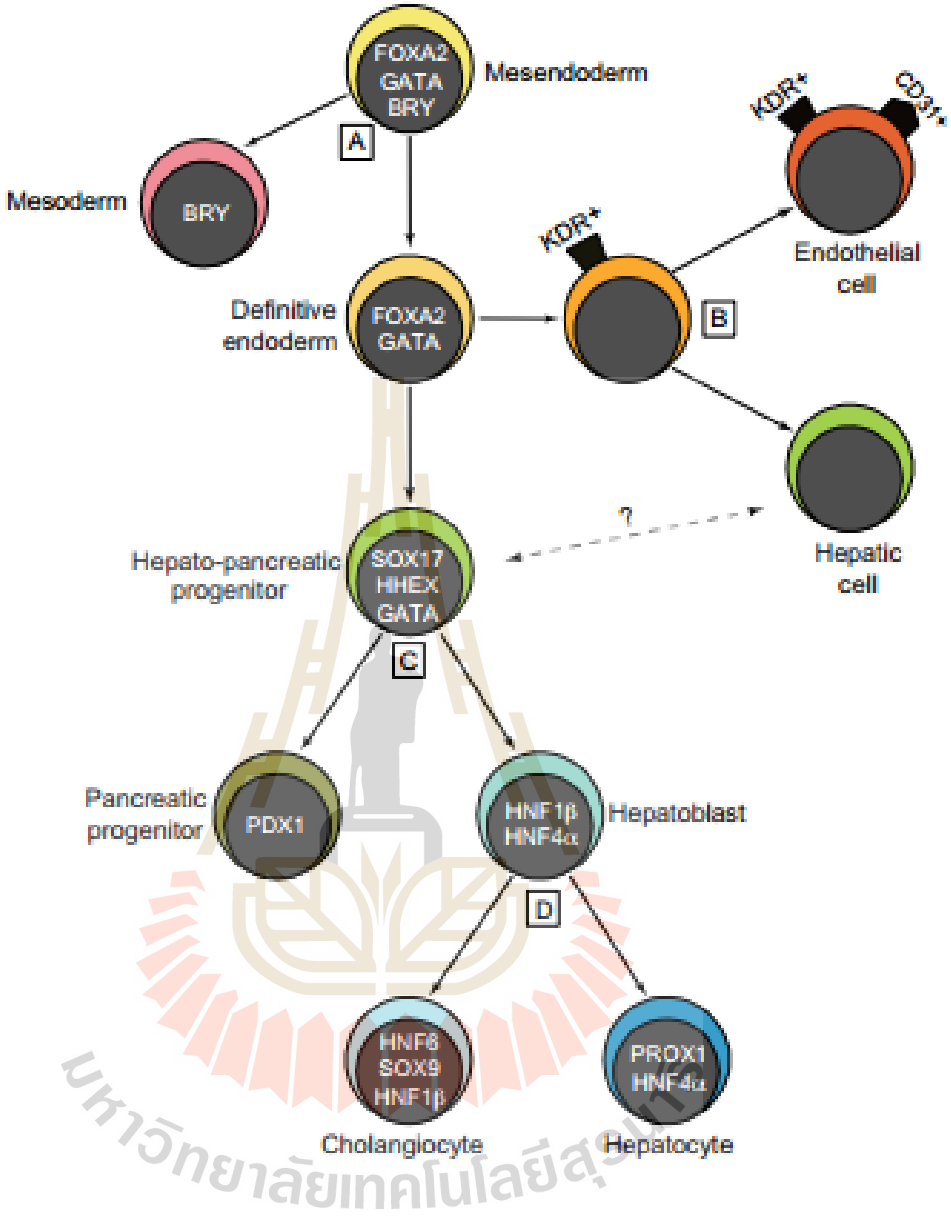

Figure 2.4 Bipotential progenitors progressively generate hepatic lineages. (Gordillo, Evan and Gouon-Evans, 2015)

2.6 Hepatogenic differentiation of human MSC potential

Nowadays, many evidences revealed extra-hepatic MSC-derived hepatocytes are driven by complex processes have been published in recently, based on cellular stimulation with exogenous cytokines, growth factors, hormones, co-culture with fetal or adult hepatocytes, challenging with conditioned media from cultured hepatocytes, two- (2D) or three-dimensional (3D) matrices to favor differentiation (reviewed in Snykers et al., 2009; Anzalone et al., 2010; Ye et al., 2015). Presently, MSCs obtained from various sources, such as human bone marrow-derived MSCs (hBM-MSCs), human umbilical cord blood-derived MSCs (hUCB-MSCs), human adipose tissue-derived stem cells (hADSCs), human placenta-derived MSCs (hPL-MSCs), human amniotic membrane-derived MSCs (hAM-MSCs), human amniotic fluid-derived MSCs (hAF-MSCs), hWJ-MSCs and human dental pulp-derived SCs (hDPSCs), have been demonstrated to process hepatic differentiation potential which allows them to differentiate into hepatocyte-like cells under culture conditions *in vitro* (Lee et al., 2004; Seo et al., 2005; Chien et al., 2006; Talèns-Visconti et al., 2006&2007; Banas et al., 2007; Tamagawa et al., 2007; Zheng et al., 2008; Campard et al., 2008; Zhang, Lie and Wei, 2009; Zhao et al., 2009; Ishkitiev et al., 2010; Pournasr et al., 2011; Lee et al., 2012; Prasajak and Leeanansaksiri; 2013).

The first report of MSC differentiation into functional hepatocyte-like cells was successfully studied by Lee and collaborates in 2004, by using hBM-MSCs and hUCB-MSCs. They cultured into 2-step differentiation paradigm with EGF and bFGF pre-conditioned for 48 h. Then, they induced MSCs into hepatoblast-like cells by using bFGF, HGF and nicotinamide, and following exposed OSM, insulin-

transferrin-selenium (ITS), and dexamethasone in order to differentiate into functional mature hepatocytes for 6 weeks. Nevertheless, previous study showed the pioneer discovered hepatic differentiation cocktail of MSC-like cells (multipotent progenitor cells; MAPCs), they lack expression of CD45 ($CD45^-$), and glycophorin A ($GlyA^-$), acquired the ability to undergo hepatocyte-like cell differentiation by optimized various cytokines, growth factors, hormones and chemicals. However, only FGF4 and HGF can induce MAPCs into hepatocyte-like cells, the differentiated cells were not homogenous hepatic characteristic population (Schwartz et al., 2002). Another report revealed hBM-MSCs also successfully generated hepatocyte-like cell differentiation by cultured in FGF-4 and HGF, and following cultured in the cocktail of HGF, ITS and dexamethasone in medium of differentiation (Pournasr et al., 2011).

Besides, other reports also were successfully acquired functional hepatocyte-like cell-derived MSC differentiation from other sources by some modified from previous protocol, such as hADSCs, hPD-MSCs, and hDPSCs. Firstly, Talèns-Visconti et al. (2006) revealed hADSCs could differentiate into functional hepatocyte-like cells for 21 days with EGF and bFGF pre-induction for 48 h, by demonstrated for the first time that the differentiated hADSCs expressed drug-metabolizing enzymes similar to hBM-MSCs, such as CYP2E1 and CYP3A4. Secondly, Talèns-Visconti et al. (2007) also demonstrated hADSCs could differentiate into hepatocyte-like cells by cultured in both two protocols of some dependently 2-step differentiation paradigms for 14 days after pre-conditioned with EGF and bFGF for 48 h; protocol A, they cultured in serum-free hepatogenic medium supplemented with bFGF, HGF and nicotinamide in both 2 steps, while protocol B they cultured in medium supplemented as same in step-1, and differs at

step-2 by added OSM, ITS and dexamethasone. Thirdly, another group performed after hADSCs exposed FGF1, FGF4 and HGF, and then following exposed OSM and dexamethasone, they could also differentiate toward functional hepatocyte-like cells *in vitro* (Banas et al., 2007). Fourthly, Saulnier et al. (2010) revealed the molecular mechanism underlying the hepatic differentiation of hADSCs by exposed HGF and FGF-4 in the early stage and the mixture of OSM with nicotinamide in the maturation stage. They indicated the transition of molecular pathway occurs during differentiation from mesenchymal to epithelial lineage that is a feature of hepatocytes. Fifthly, Chien and coworkers (2007) showed hPL-MSCs could also differentiate into hepatocyte-like cells by cultured in ITS, linoleic acid, bovine serum albumin, dexamethasone, ascorbic acid, EGF, and platelet-derived growth factor-BB (PDGF-BB) for 16 h, then they following cultured in HGF and FGF-4 of hepatogenic medium until day 28. Sixthly, human umbilical matrix-derived stem cells (hUC-MSCs or hWJ-MSCs) could also differentiate into functional hepatocyte-like cells, revealed by Campard and collaborates (2008). They cultured hUC-MSCs in bFGF, HGF, nicotinamide and dexamethasone at step-1 after pre-conditioned with EGF and bFGF for 48 h, and then they following cultured in OSM, ITS and dexamethasone for 21 days. Finally, Zhao, et al. (2009) and, Prasajak and Leeanansaksiri (2013) performed that hWJ-MSCs could differentiate into more functional hepatocyte-like cells by cultured into 2-step differentiation paradigms by increased concentration of HGF and OSM, respectively, in independently two hepatogenic differentiation protocols without EGF and bFGF pre-treated for 48 h.

Interestingly, previous simple protocol of one-step hepatic differentiation revealed hADSCs could differentiate into hepatocyte-like cells by exposed dimethyl

sulfoxide (DMSO), HGF and OSM *in vitro* (Seo et al., 2005). Besides, hAM-MSCs could also differentiate into functional mature hepatic lineage after treatment with induction medium as 10% FBS, HGF, bFGF, OSM, and dexamethasone (Tamagawa, Oi, Ishiwata, Ishikawa, and Nakamura, 2007). Additionally, hWJ-MSCs were induced with 1% FBS, HGF, and FGF-4; the differentiated cells expressed hepatic specific markers both at gene and protein levels, and functional hepatic characteristics, such as stored glycogen and had ability to uptake LDL (Zhang, Lie, and Wei, 2009).

More recently, Kazemnejad and colleagues (2009) reported the use of 3D biocompatible nanofibrous scaffold to enhance hepatic differentiation of hBM-MSCs. The cells grown on 2D and 3D conditions were stimulated by HGF, OSM, and dexamethasone for 3 weeks. The differentiated cells grown on 3D matrix showed increased expression of hepatic markers and functions with respect to cells differentiated on a 2D culture system. However, other scaffolds also used to enhance hepatogenic differentiation of MSC from murine, such as alginate scaffold (Lin et al., 2010), collagen-coated poly (lactic-co-glycolic acid) (C-PLGA) scaffold (Li et al., 2010), and synthetic extracellular matrix ultraweb nanofibers (Piryaei, Valojerdi, Shahsavani, and Baharvand, 2011).

Another procedure, using co-culture MSC from animals with hepatic stellate cell (HSC) and mature hepatocytes, by assumed that secreting factors from their cells induced hepatic differentiation of MSC (Antoine et al., 2007; Deng et al., 2008; Qihao et al., 2007; Gu, Shi, Zhang, and Ding, 2009). Few years, hWJ-MSCs could differentiate into hepatocyte-like cells by premeabilization of them in the presence of

HepG2 cell extract at the first time (Borhani-Haghighi, Talaei-Khozani, Ayatollahi, and Vojdani, 2015).

The first successful of MSC differentiation toward endoderm-like cells was reported by Allahbakhshi and coworkers (2013). They used hWJ-MSCs exposed with low and high concentrations of activin A (20 and 100 ng/ml) and Wnt3a (25 ng/ml) or combination for 3 days. The differentiated cells could highest express DE (*SOX17* and *CXCR4*) and anterior DE transcripts, (*FOXA2* and *CER*) at low concentration of activin A (20 ng/ml) in combination with Wnt3a for 24 h and followed step-2 they exposed low concentration of activin A (20 ng/ml) for 48 h. Presently, utilizing 3D spheroid formation could efficiently enhance WJ-MSCs into DE cells, by expressing DE transcripts and proteins (CXCR4, SOX17 and FOXA2) (Madhound et al., 2016).

2.7 Histone acetylation of epigenetic modification machinery

Epigenetic modifications of the histone N-terminal tails are subjected to various post-translational modifications, including acetylation, methylation, phosphorylation, ubiquitination, sumoylation, adenosine diphosphate (ADP)-ribosylation and glycosylation (Izzo and Schneider, 2010; Lee, Smith and Shilatifard, 2010). Nevertheless, histone acetylation is the best-understood posttranslational histone modification (reviewed in Snykers et al., 2009). Normally, balancing of histone acetylation of chromatin structure and target gene expression was regulated by two opposing enzyme activities, such as histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Figure 2.5). HATs catalyze the acetylation of core histones through the addition of an acetyl group to the lysine residue (K) on the N-terminus of

histones. These phenomenons, to neutralize their positive charges subsequently leading to a relaxed chromatin structure, which is more accessible to the transcription machinery. Another class of enzymes, HDACs, is expressed in all eukaryotic cells, and their activity is essential for cell proliferation, differentiation and homeostasis, catalyzes deacetylation through the hydrolysis of an acetyl moiety from the K, leading to chromatin condensation and transcriptional repression (reviewed in Schneider et al., 2013; Zwergel, Stazi, Valente and Mai, 2016).

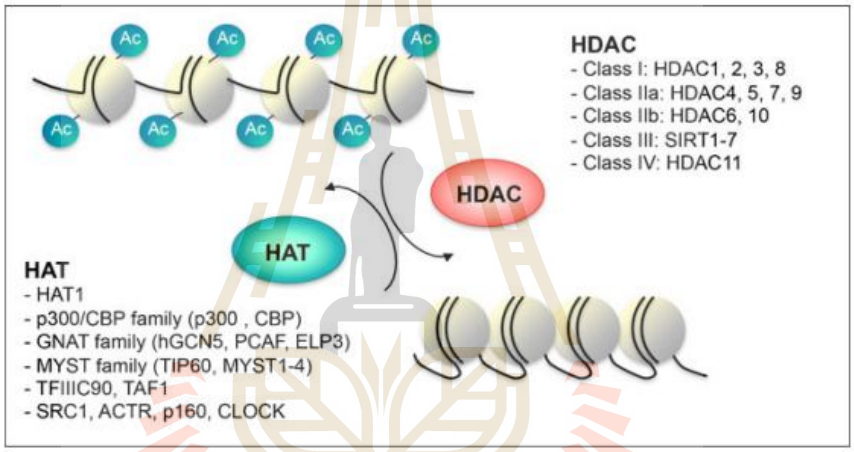

Figure 2.5 Epigenetic modifications of histone tails by histone acetylation through the balance of histone acetyltransferase (HAT) and histone deacetylase (HDAC) regulation and family distinguishes (Schneider et al., 2013).

2.8 Histone deacetylase inhibitors (HDACi)

HDACi possesses usually the following well-known components: a cap group (CAP) allow interacting with the catalytic tunnel border of the enzyme, a polar connection unit (CU) connecting to a hydrophobic spacer (HS) enabling the inhibitor to lie into the aforementioned tunnel, and a Zn-binding group (ZBG) able to complex

the Zn^{2+} at the bottom of the enzyme cavity. To date numerous different CAP, CU, HS and ZBG combinations as HDACi have been either isolated from natural sources or synthesized displaying varying target specificity, pharmacokinetic properties and activity in laboratory and clinical settings (reviewed in Zwergel, Stazi, Valente and Mai, 2016).

Inhibition of HDACs by HDAC inhibitors (HDACi) were used widely in anti-cancer treatments through several structurally distinct HDAC inhibited, most of which divided into four major chemical classes (**Table 2.2**) (reviewed in Smith and Workman, 2009). More recently, clinical studies also using HDACi have been extended to a range of non-oncologic diseases, such as neurological disorders, inflammatory processes and viral infections (reviewed in Delcuve, Khan and Davie, 2012; Zwergel, Stazi, Valente and Mai, 2016).

Table 2.2 HDACi classification and specificity (reviewed in Smith and Workman, 2009).

Class of inhibitors	HDACs inhibited	HDACi names	Stage of clinical development
I. Hydroxamates	Class I, II and IV	SAHA (Vorinostat®)	FDA approved for CTCL
		PXD101 (Belinostat®)	Phrase I
		Tricostatin A (TSA)	None
2. SCFAs	Class I, IIa: 4, 5, 9	Sodium butyrate (NaB)	Phrase II
		Sodium phenylbutyrate	Phrase II
		Valproic acid (VPA)	Phrase II
3. Benzamides	Class I	MS-275	Phrase II
	Class I, IV	MGCD-0103	Phrase II
4. Cyclic tetrapeptides	Class I	Depsipeptide (FK228)	Phrase II
		Apicidin	Preclinical

ABBREVIATION: CTCL: Cutaneous T cell lymphoma: FDA: Food and Drug Administration, SCFAs: short-chain fatty acids, SAHA: Suberoylanilide hydroximc acid.

2.8.1 HDACi on hepatogenic differentiation of hMSCs

Presently, HDACi commonly have been used on hMSC differentiation toward hepatoendodermal lineage, such as TSA (hydroxamates) and VPA (carboxylates) to overcome stem cell fate determination (Snykers et al., 2007; Banas et al., 2007; Zheng et al., 2008; Dong et al., 2013; An et al., 2014).

The successfully differentiated hMSCs into hepatocyte-like cells by exposing with HDACi for 27 days was reported by Snykers and coworkers (2006). They compared hepatogenic differentiation medium cocktail (FGF-4, HGF, ITS and dexamethasone) and sequential (FGF-4, followed by HGF, and followed by a combination of TSA, HGF, ITS and dexamethasone) with or without TSA supplementation from day 6 onward. These results found that the differentiated cells of hepatogenic differentiation medium sequential with TSA supplementation could express highest hepatic phenotypes and functional maturation of hBM-MSCs when compared with other groups. Besides, these hBM-MSCs differentiation toward hepatocyte-like cells could regulate through histone acetylation on histone H4 by TSA-induced HDAC inhibition. Nevertheless, Seo and collaborates (2005) performed firstly the successful of differentiated hADSCs into hepatocyte-like cells by exposing with 0.1% dimethyl sulfoxide (DMSO) in hepatogenic medium (HGF and OSM) from day 10 onward, but the mechanism underlying the hepatic transdifferentiation-inducing effects of DMSO has not yet been fully elucidated, though histone hyperacetylation-inducing effects have been suggested for the action of DMSO (Sarg et al. 2004). hAF-MSCs could also induce to hepatocyte-like cells by exposing TSA with hepatogenic differentiation medium sequential at step-2 (FGF-4 and HGF, and followed by dexamethasone, ITS, and HGF) (Zheng et al., 2008).

Nevertheless, hWJ-MSCs could also differentiate into hepatocyte-like cells by exposing TSA at final step of 4-step hepatogenic differentiation medium (FGF-4, followed by ITS and HGF, followed by dexamethasone, ITS, glucagon and OSM, and followed by dexamethasone, ITS, glucagon and OSM+TSA or DMSO) for 21 days (Yoon et al., 2010). More recently, hBM-MSCs could differentiated into functional mature hepatocyte-like cells by exposing VPA-induced HDAC inhibition for 72 h pre-treatment through regulation of histone acetylation on histones H3 and H4 before culturing in hepatogenic differentiation sequential (FGF-4, followed by HGF, and followed by HGF, OSM and dexamethasone) for 21 days (Dong et al., 2013). Another report, emphasize that VPA-induced HDAC inhibition could also enhance hWJ-MSCs differentiation into hepatocyte-like cells by exposing VPA-induced HDAC inhibition for 6 h pretreatment through mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/ERK) and phosphatidylinositol 3-kinase/Protein Kinase B (PI3K/AKT) signaling pathways of endoderm specification before culturing in hepatogenic differentiation cocktail (HGF, OSM and dexamethasone) for 15 days (An et al., 2014). However, no study MSC differentiation into functional mature hepatocyte-like cells by exposing NaB, so this study we would like to investigate effect of NaB on hepatogenic differentiation of hWJ-MSCs.

2.9 Sodium butyrate (NaB)

NaB is the short-chain fatty acid butyric acid in sodium salt formed (Ghosh et al. 2012). Normally, butyrate is a 4-carbon fatty acid that produced by anaerobic bacterial fermentation of dietary fibers through hydrolysis of ethylbutyrate, and is known to play a key role in the homeostasis of the gastrointestinal tract (Liu et al., 2014).

The first study of NaB was reported by Pace and coworkers (1967) by treating on mammalian cell cultures in vitro, the result showed that it affected morphology, growth rate and gene expression. But at higher concentrations of NaB (10 mM and above) were toxic to the cells. Nowadays, NaB not only used as antitumor agents, but also for non-oncologic uses for years and has been shown to have HDACi activity, such as anti-inflammatory agents, cystic fibrosis treatment and application of neurodegenerative disorders trials (reviewed in Canani, Costanzo and Leone, 2012). Therefore, NaB has been regarded as the epigenetic effects with potential clinical applications in further human medicine.

Recently, NaB has been found to play an important role in stem cell differentiation through HDACi activity at millimolar (mM) range of therapeutic concentrations (Chen et al., 2007; Vrba, Trtkova and Ulrichova, 2011; Shah et al., 2013). Besides, NaB can also specifically induce the generation of hepatic progenitor cells from ESCs (Zhang et al., 2011), osteoblast and inhibit adipogenic differentiation (Rahman et al., 2003; Chen et al., 2007) and smooth muscle cell differentiations from MSCs (Liu et al., 2014), suggesting that NaB might be an effective regulator to promote MSC differentiation into certain terminal cell types.

Previous study, many reports of hepatic differentiation of ESCs found that NaB could promote ESCs differentiation toward hepatocyte-like cells (Rambhatla et al., 2003; Sharma et al., 2006; Zhou et al., 2007; Hay et al., 2008; Ren et al., 2010; Zhou et al., 2010). However, no study the effect of epigenetic modifiers of HDACi NaB on hepatogenic differentiation of MSCs. Therefore, these statements will be increased the effect of HDACi NaB on hepatogenic differentiation through epigenetic modification through histone acetylation.

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals and Reagents

All chemicals and reagents were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA), unless otherwise indicated.

3.2 Human hepatocarcinoma (hHep G2) and NIH3T3 cell culture

hHep G2 and NIH3T3 cell lines were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Life Technologies Inc. Gibco-BRL Division, Grand Island, NY, USA) and 100 µg/ml streptomycin and 100 U/ml penicillin at 37ºC in a humidified atmosphere with 5% CO_2. hHepG2 and NIH3T3 cells were used as a positive control in the experiments.

3.3 Isolation and culture of hWJ-MSCs

A Human umbilical cord was collected and preserved aseptically after full-term delivery from Suranaree University of Technology Hospital, Nakhon Ratchasima with patient's consent. Isolation and cultivation of hWJ-MSCs was performed by tissue explant procedure as previously described (Petsa et al., 2009). Briefly, Wharton's Jelly was cut into small pieces of about 3×3 mm^2 after removing vessels and other parts of

the tissue. The tissues were plated in a six-well plate (SPL Life Sciences, Gyeonggi-do, Korea) and cultured in standard growth medium composed of Alpha-Minimum Essential medium (α-MEM) supplemented with 10% FBS and 100 μg/ml streptomycin and 100 U/ml penicillin and incubated at 37°C with saturated humidity containing 5% CO_2. Culture cells were then incubated at 37ºC in a humidified atmosphere of 5% CO_2 in air for 7-10 days. Medium was replaced every 2 days and, when visible fibroblast-like cells were observed, the remained pieces of tissue explants were removed. The cells were expanded further until passage 3 and the cells were either directly used for experiments or cryopreserved with 10% dimethyl sulfoxide (DMSO, Calbiochem, San Diego, CA, USA) and stored in liquid nitrogen.

3.4 Treatment of NaB

Various concentrations of NaB 0, 1, 2.5 and 5 mM were added in serum-free medium, Iscove's Modified Dulbecco's medium (IMDM) supplemented with 20 ng/ml EGF (Peprotech, Rocky Hill, NJ, USA), 10 ng/ml bFGF (Peprotech) and 100 μg/ml streptomycin and 100 U/ml penicillin of hWJ-MSCs pre-treatment before hepatogenic differentiation, for 72 h at 37ºC in a humidified atmosphere of 5% CO_2 in air. The pre-treated cells with (the PTM + 1, 2.5 and 5 mM NaB groups) and without NaB (the PTM group) were checked for the endodermal-related gene expressions by qPCR and immunocytochemistry for SOX17 expression. The HDAC1 expression was also checked by immunofluorescence, qPCR and Western blot analyses.

3.5 Immunophenotyping

hWJ-MSCs at passage 5 were cultured on 4-well tissue culture dishes (Nunc, Roskilde, Denmark) until reaching 80% confluence. Cells were fixed with 4% paraformaldehyde (PFA) for 30 min. Nonspecific binding was blocked with 10% normal goat serum. Primary antibodies against CD34 (BD biosciences, San Jose, CA, USA), CD73 (Millipore, Massachusetts, USA), CD90 (Santa Cruz Biotechnology, Dallas, TX, USA), and CD105 (Santa Cruz Biotechnology) were added and incubated at 4°C overnight. Cells were the incubated with secondary antibodies, Alexa fluor® 488 goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) or Alexa fluor® 488 goat anti-rabbit IgG (Invitrogen). Nuclei were stained with 4, 6-diamino-2-phenylindole (DAPI; Millipore) and the stains were observed under a fluorescence microscope (Nikon Eclipse Ti-S, Japan).

3.6 Multipotency assays

hWJ-MSCs were cultured at the final density of approximately 2×10^4 cells/cm^2 on 6-well culture plates coated with 0.1% gelatin.

hWJ-MSCs were induced to osteogenic differentiation by culture in the culture medium with reduced FBS to 5% and supplemented with 100 nM dexamethasone, 0.2 mM L-ascorbate-2-phosphate, and 10 mM β-glycerophosphate. The medium was subsequently replaced every 2 days for 3 weeks. Calcium deposits from the cells were then visualized by Alizarin Red staining.

To induce adipogenic differentiation, hWJ-MSCs were cultured in the culture medium with reduced FBS to 5% and supplemented with 10 μg/mL insulin, 100 μM indomethacin, 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine (IBMX). IBMX was removed from the medium after 1 week of culture. The medium was subsequently replaced every 2 days for 3 weeks. Cells were then stained with Oil Red O to observe cells containing oil droplets.

To induce chondrogenic differentiation, hWJ-MSCs were cultured in a completed chondrogenic medium consisting of culture medium with reduced FBS to 2% and supplemented with 1% Insulin-Transferrin-Selenium-Ethanolamine (ITS-X, Invitrogen), 50 μg/mL ascorbate-2-posphate, 40 μg/mL L-proline, 100 μg/mL sodium pyruvate, 100 nM dexamethasone, and 10 ng/mL of TGF-β3 (Prospec, East Brunswick, NJ, USA). The medium was replaced every 2 days for 3 weeks. Glycosaminoglycan production was assessed by Alcian blue staining.

3.7 Hepatogenic differentiation

Approximately 1.5×10^3 cells of hWJ-MSCs were plated on 4-well plates for immunophenotypic examination and 1.5×10^5 cells were plated on 35 mm diameter dish (Corning, Acton, Massachusetts, USA) and 6-well plates for qPCR, glycogen storage and urea production assays, respectively. The plates were coated with 0.1% gelatin, and cultured in standard growth medium until reaching about 80-85% confluence. Hepatogenic differentiation was performed as described (Campard et al.,

2008) with some modifications. Briefly, cells were cultured in IMDM without serum and supplemented with 10 ng/ml bFGF, 20 ng/ml EGF, 100 µg/ml streptomycin and 100 U/ml penicillin and combined with and without the optimized concentrations of NaB for 72 h (in the pre-treatment step) obtained from *Treatment of NaB* mentioned above. Then, cells were induced to become hepatic lineages by 2-step differentiation protocol. Step 1 (in the differentiation step) consisted of IMDM without serum supplemented with 10 ng/ml bFGF, 40 ng/ml hepatocyte growth factor (HGF; Peprotech) and 5 mM nicotinamide for 7 days. Step 2 (in the maturation step) consisted of IMDM without serum supplemented with 10 ng/ml oncostatin M (OSM), 1×10^{-8} M dexamethasone and 1% ITS-X for 14 days (Fig. 3.1). In all steps, media were changed twice weekly.

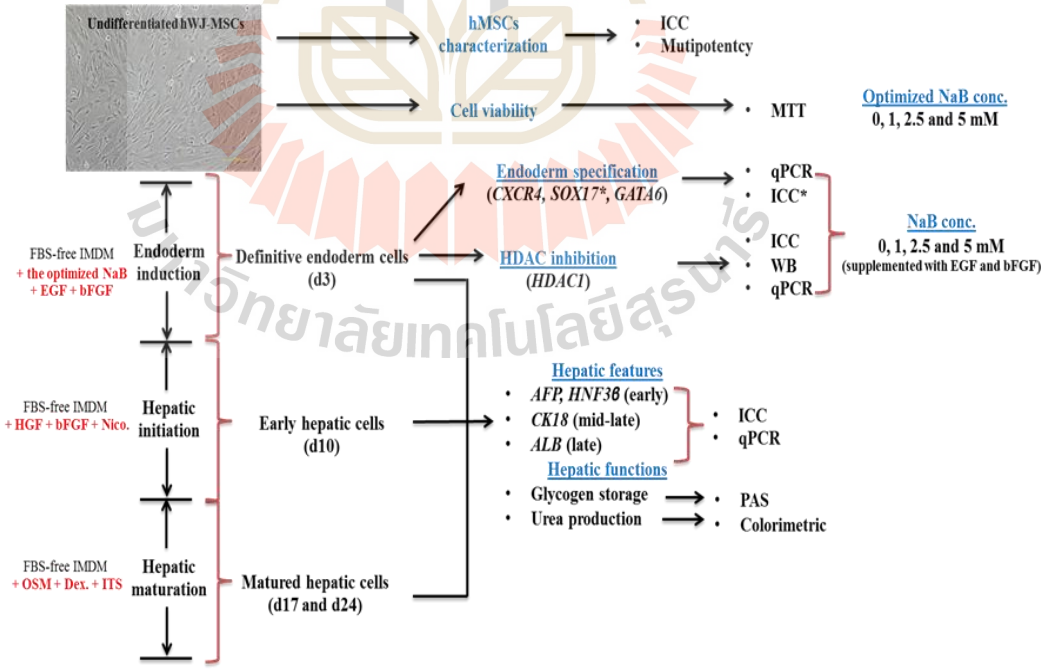

Figure 3.1 Schematic diagram of hepatogenic differentiation protocol of hWJ-MSCs.

3.8 Cytotoxicity test

One thousand hWJ-MSCs were re-plated and cultured in 96-well culture plates (SPL life sciences) in the culture medium for 6 h to allow attachment. The NaB cytotoxicity was assessed by adding NaB to the culture medium at the concentrations of 0, 1, 2.5 and 5 mM, respectively. All cultures were maintained at 37ºC for 72 h in a humidified atmosphere of 5% CO_2 in air. The effects of NaB on cell viability were quantified by the MTT assay (Liu et al., 2014). Briefly, culture medium was replaced by 5 mg/mL MTT solution (Invitrogen) in culture medium and cells were incubated for 2 h. DMSO was then added and incubated at 37°C for 10 min. The absorbance was measured at 540 nm (Microplate reader Sunrise, TECAN, Austria).

3.9 Gene expression analysis

After 3, 10, 17 and 24 days of differentiation, total RNA was isolated from the cells by total RNA extraction kit (RBC Real Genomics, RBC Bioscience, Taipei, Taiwan) according to the manufacturer's instructions. Then, the RNAs were reverse-transcribed in the presence of oligo-dT primer for complementary DNA (cDNA) synthesis by iScript™ Reverse Transcription Supermix for qPCR (BioRad, Hercules, CA, USA). The expression of genes in **Table 3.1** was assessed by using Light Cycler® 480 (Roche Diagnostics, Basel, Switzerland) and KAPA SYBR-Green PCR Master mix (Applied Biosystems, Carlsbad, CA, USA). The primers used are shown in **Table 3.1**. Melting curve analysis was also checked to determine the specificity of the primers. Gene expression was normalized to the reference gene *GAPDH* and

calculated as relative expression compared to control cells. The qPCR were performed in triplicates.

Table 3.1 Primers used for qPCR analysis.

Genes	Primer Sequence	Annealing Temp. (°C)	Size (bp)	References
SOX17	Forward 5'-GGCGCAGCAGAATCCAGA-3'	65	61	[36]
	Reverse 5'- CCACGACTTGCCCAGCAT-3'			
CXCR4	Forward 5'- ACTACACCGAGGAAATGGGCT-3'	60	133	This study
	Reverse 5'- CCCACAATGCCAGTTAAGAAGA-3'			(NM_003467.2)
GATA6	Forward 5'-CCATGACTCCAACTTCCACC-3'	62	214	[37]
	Reverse 5'-ACGGAGGACGTGACTTCGGC-3'			
AFP	Forward 5'-CTTTGGGCTGCTCGCTATGA-3'	60	131	This study
	Reverse 5'-GCATGTTGATTTAACAAGCTGCT-3'			(NM_001134)
HNF3β	Forward 5'- CCTACTCGTACATCTCGCTCATC-3'	65	69	[33]
	Reverse 5'-CGCTCAGCGTCAGCATCTT-3'			
CK18	Forward 5'-TCGCAAATACTGTGGACAATGC-3'	60	171	This study
	Reverse 5'-GCAGTCGTGTGATATTGGTGT-3'			(NM_199187.1)
ALB	Forward 5'-GCACAGAATCCTTGGTGAACAG-3'	65	101	[38]
	Reverse 5'-ATGGAAGGTGAATGTTTCAGCA-3'			
HDAC1	Forward 5'-CCAAGTACCACAGTGATGACTACATT-3'	65	135	This study
	Reverse 5'- AGAACTCAAACAGGCCATCAAA-3'			(NM_004964.2)
GAPDH	Forward 5'-TGCACCACCAACTGCTTAGC-3'	60	87	[38]
	Reverse 5'-GGCATGGACTGTGGTCATGAG-3'			

3.10 Immunofluorescence staining

The cells at day 3, 10, 17 and 24 were fixed with 4% PFA for 30 min, then they were blocked and permeabilized for 2 h at 37°C with 2% bovine serum albumin (BSA), 5% normal goat serum, 3 mM sodium azide and 0.2% triton-X100. The cells

were incubated at 4 ^{o}C overnight with primary antibodies as follow: mouse anti-human α-fetoprotein (AFP) (1:100), mouse anti-human cytokeratin18 (CK18) (1:100; Santa Cruz Biotechnology), rabbit anti-human hepatocyte nuclear factor 3β (HNF3β) (1:100; Santa Cruz Biotechnology) and rabbit anti-human albumin (ALB) (1:200; Abcam, Cambridge, UK). Then, samples were incubated for 2 h with the respective secondary antibodies. Samples were stained with DAPI and observed under a fluorescence microscope. Besides, HDAC1 and SOX17 protein expression for 72 h was also investigated by immunofluorescence staining with rabbit anti-human HDAC1 (1:200; Millipore) and mouse anti-human SOX17 (1:100; Abcam) primary antibodies.

3.11 Western blot analysis

Total protein was extracted from the samples for 72 h post-induction by lysis buffer containing 10% sodium dodecyl sulfate (SDS), 0.1 M dithiothreitol (DTT, Invitrogen), 1% glycerol, 1.2% urea, and 1 M Tris-HCl pH 7.4 and complete protease inhibitor. The total protein concentration was determined by Bradford assay (Bradford, 1976). Twenty micrograms of total protein were separated on 10% SDS-PAGE, followed by electro-transfer to nitrocellulose membrane (BioRad). The membranes were exposed to blocking buffer (5% skim milk in PBS with 0.1% Tween-20 (PBS-T)) and then incubated with either anti-human HDAC1 (dilution 1:500, Millipore) or anti-human β-actin (dilution 1:1,000, Millipore). Membranes were incubated with (goat anti-rabbit or -mouse) secondary antibody conjugated to alkaline phosphatase (dilution 1:20,000) and were then developed by using 5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium (SIGMA FAST™ BCIP/NBT).

3.12 Periodic acid-Schiff staining

The cells were cultured until day 3, 10, 17, and 24 were fixed with 4% PFA and oxidized in 1% periodic acid in PBS^- for 30 min, and washed three times in deionized (DI) water. Then, they were incubated in Schiff's reagent for 15 min. After that they were washed with DI water for 5 min, the cells were then visualized under an inverted microscope.

3.13 Urea production

The cells from each experimental group, hWJ-MSCs, the pre-treatment stage (d3), the early stage (d10), the mid-stage (d17) and the late stage (d24) of the hWJ-MSCs differentiation with and without NaB pre-treatment and hHep G2, as positive cells, were incubated with IMDM containing 5 mM NH_4Cl for 24 h and the supernatants were colorimetrically measured according to the manufacturer's instructions (Quantichrom™ Urea assay kit, Bioassay Systems, CA, USA). Fresh culture media supplemented with 5 mM NH_4Cl was used as a negative control. Samples from separate culture were analyzed in triplicate for each group.

3.14 Statistical analysis

Statistical analysis was performed using the SPSS version 16.0 (SPSS, Inc., USA), and data were expressed as mean±S.D. Differences between the values were determined using the independent sample by using one-way analysis of variance

(ANOVA) with Turkey's HSD Post Hoc Test to compare between two groups. The data were analyzed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). A value of $P<0.05$ was considered to be significant, whereas $P<0.001$ was highly significant difference.

CHAPTER IV

RESULTS

4.1 Isolation and characterization of hWJ-MSCs

hWJ-MSCs were obtained from the isolation of a tissue explant procedure as previously described (Petsa et al., 2009). The migrated cells from the tissue showed fibroblast-like shaped after cultured (Fig. 4.1A) and become 80% confluences in 7 days (Fig. 4.1B). The cells were harvested and expanded for further use.

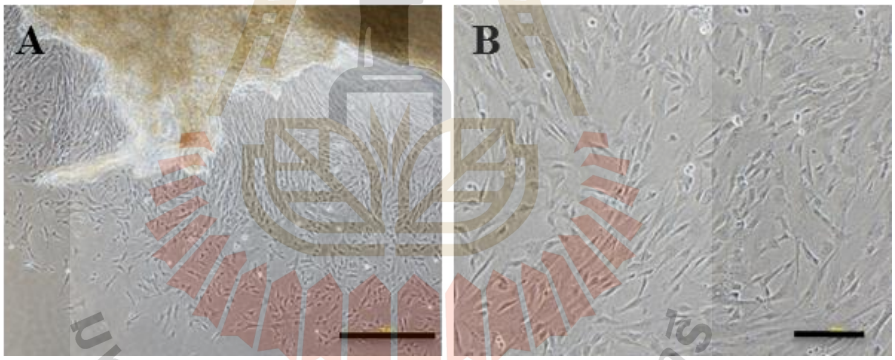

Figure 4.1 Morphology of hWJ-MSCs with typical fibroblast-like morphology. (A) A phase contrast images of hWJ-MSCs expanded form Wharton's Jelly tissue and (B) hWJ-MSCs at 80% confluences. Scale bar: 100 (A) and 50 (B) μm.

The MSC signatures from umbilical cord Wharton's Jelly tissue were characterized at the 4th passage with immunophenotype and multipotency assays. Tri-mesodermal lineages differentiation potentials *in vitro* of hWJ-MSCs were examined after 21 days of induction with Alizarin Red, Alcian Blue and Oil Red O staining for

osteogenic, chondrogenic and adipogenic characterizations, respectively. The differentiated cells positively formed calcium mineralization of osteoblast (Fig 4.2A), proteoglycan matrix of chondroblast (Fig. 4.2B) and intracytoplasmic lipid droplets of adipocyte (Fig. 4.2C). Immunocytochemical analysis showed that hWJ-MSCs positively expressed MSC markers CD73, CD90 and CD105, while the expression of expressed CD34, hematopoietic marker, was not detected (Fig. 4.2D-G). These data demonstrated that the hWJ-MSCs have typical MSC characteristics.

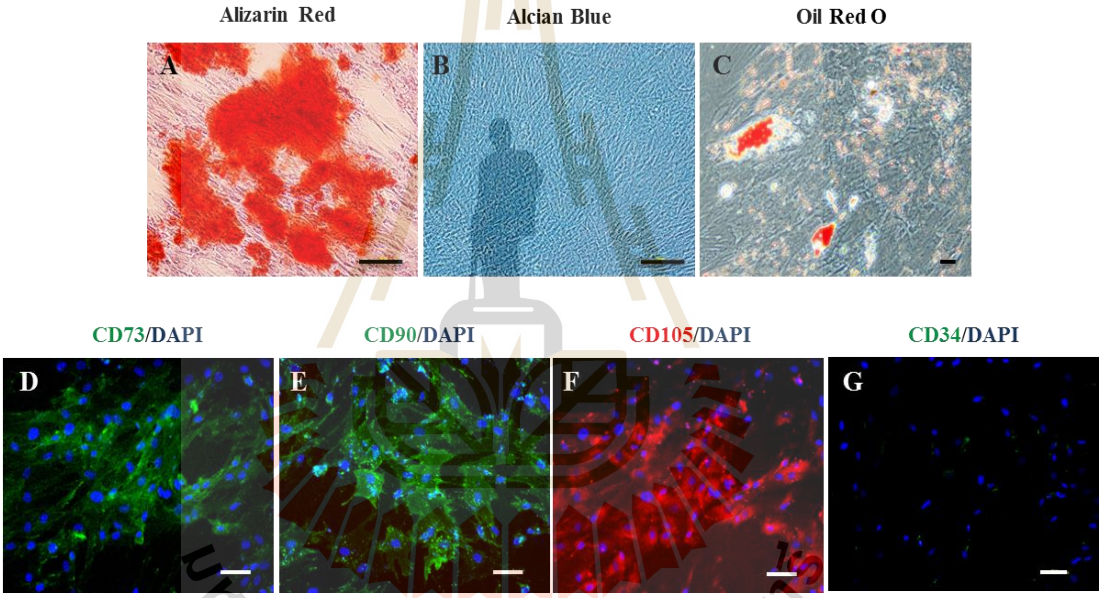

Figure 4.2 Characterization of hWJ-MSCs. **(A-C)** Multilineage differentiation potential of hWJ-MSCs after 21 days, evaluated by **(A)** Alizarin Red (osteogenic), **(B)** Alcian Blue (chondrogenic) and **(C)** Oil Red O (adipogenic) staining. Scale bar: 100 **(A & B)** and 10 **(C)** μm. **(D-G)** Representative images of immunophenotype of hWJ-MSCs **(D)** CD73, **(E)** CD90, **(F)** CD105 and **(G)** CD34. Scale bar: 50 μm.

4.2 Effect of NaB of hWJ-MSCs viability

To examine the cytotoxicity of NaB, hWJ-MSCs were cultured in serum-free medium supplemented with the various concentrations of NaB (0, 1, 2.5 and 5 mM) for 72 h and then they quantified by using MTT assay. The 1 mM NaB supplementation showed significantly higher cell viability (98.39±0.98%) when compared to the 2.5 and 5 mM NaB supplementation groups (81.77±0.53% and 79.01±0.82%), respectively (Fig. 4.3). These data demonstrated that 1 mM NaB can be used for hepatogenic differentiation in pre-treatment step.

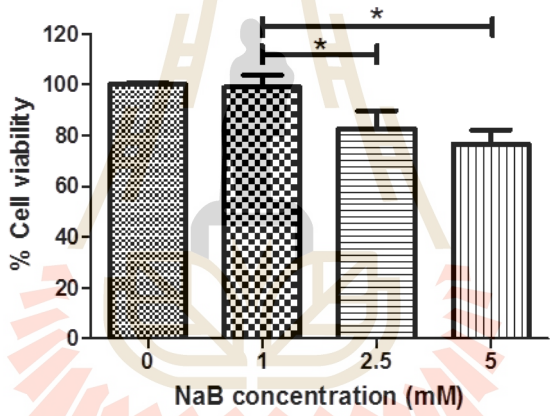

Figure 4.3 Effect of NaB on cytotoxicity of hWJ-MSCs. hWJ-MSCs were cultured with 0-5 mM NaB for 72 h in 96-well plate. Then, the viability was determined by MTT assay. Data were shown as means ± SD. *$p < 0.05$.

4.3 Combination of NaB with EGF and bFGF pre-treatment of hWJ-MSCs differentiation

To investigate whether dose-dependent manner of NaB pre-treatment was probably done through HDAC1 inhibition, the endodermal-related gene expressions were observed. hWJ-MSCs pre-treatment with various concentrations of NaB (0, 1, 2.5 and 5 mM) and supplemented with EGF and bFGF in the pre-treatment medium (PTM) for 72 h before hepatogenic differentiation where check for the endodermal-related gene expression. The mRNA levels of endodermal-related genes, *CXCR4, SOX17* and *GATA6* were quantified by qPCR. As shown in Fig. 4.4A-C, hWJ-MSCs cultured in the PTM + 1 mM NaB group gave rise to the highest expressions fold change of *CXCR4*, *SOX17* and *GATA6* which were statistically significant different when compared to other groups. Similar to the qPCR data, SOX17-positive cells were detected in the PTM + 1 mM NaB group (Fig. 4.4F) greater than the control (hWJ-MSCs) (Fig. 4.4D) and the PTM group (Fig. 4.4E). However, in the PTM + 2.5 and 5 mM NaB groups (Fig. 4.4G-H), SOX17 expressed cells were not detected. All of the result demonstrated that the PTM + 1 mM NaB group up-regulated the endoderm-related genes and SOX17 protein expressions. Accordingly, the PTM + 1 mM NaB group inhibited HDAC1 protein expression shown by immunofluorescent and Westren blot analyses (Fig. 4.5A-E and Fig. 4.5F), respectively. However, the mRNA levels of *HDAC1*expression of the PTM + 1 mM NaB group were partially inhibited but with not significantly difference when compared to the PTM group (Fig. 4.5G). These results demonstrated that the hWJ-MSCs can differentiate into endodermal lineage through HDAC1 inhibition in the PTM + 1 mM NaB pre-treatment. Besides, this

result also confirmed that the PTM + 1 mM NaB pre-treatment can be used for hepatogenic differentiation of hWJ-MSCs.

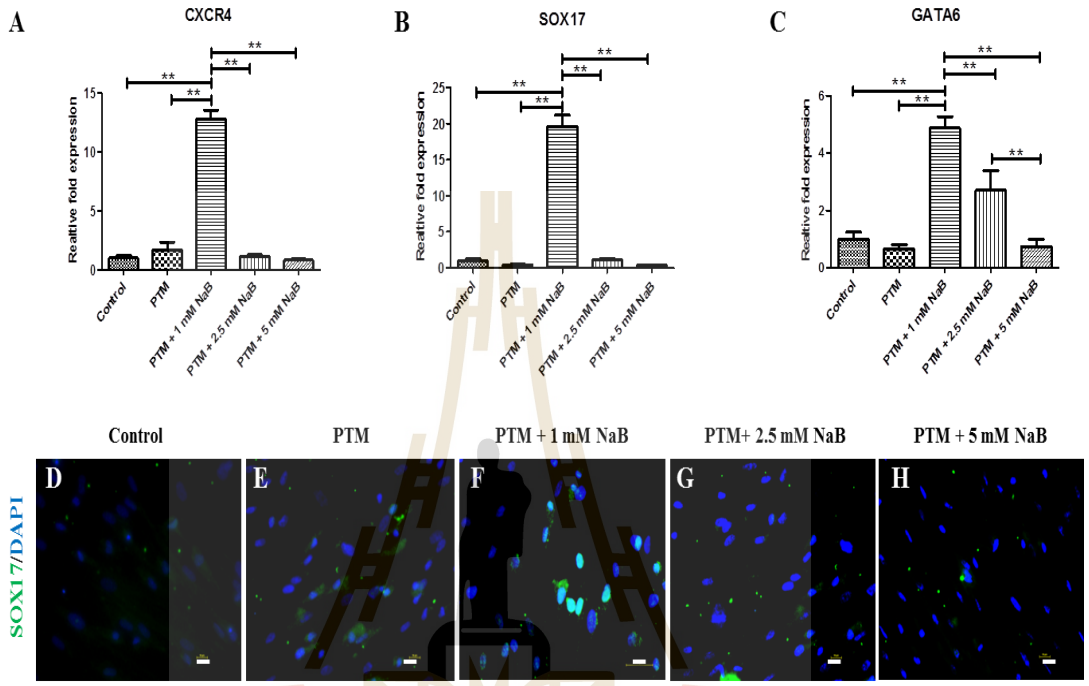

Figure 4.4 Effect of NaB on endodermal differentiation of hWJ-MSCs after NaB treatment. **(A-C)** qPCR analysis of embryonic endodermal gene expressions for **(A)** *CXCR4*, **(B)** *SOX17* and **(C)** *GATA6* after 72 h of pre-treatment. Gene expressions were normalized to corresponding *GAPDH* and calculated by relative fold expression compared to control cells (hWJ-MSCs). The experiments were performed in triplicates. Data were shown as mean ± SD, $^{**}p < 0.001$. **(D-H)** Representative indirect immunofluorescent staining for SOX17 after NaB (0, 1, 2.5 and 5 mM) pre-treatments combined with EGF and bFGF for 72 h. Scale bar: 10 μm.

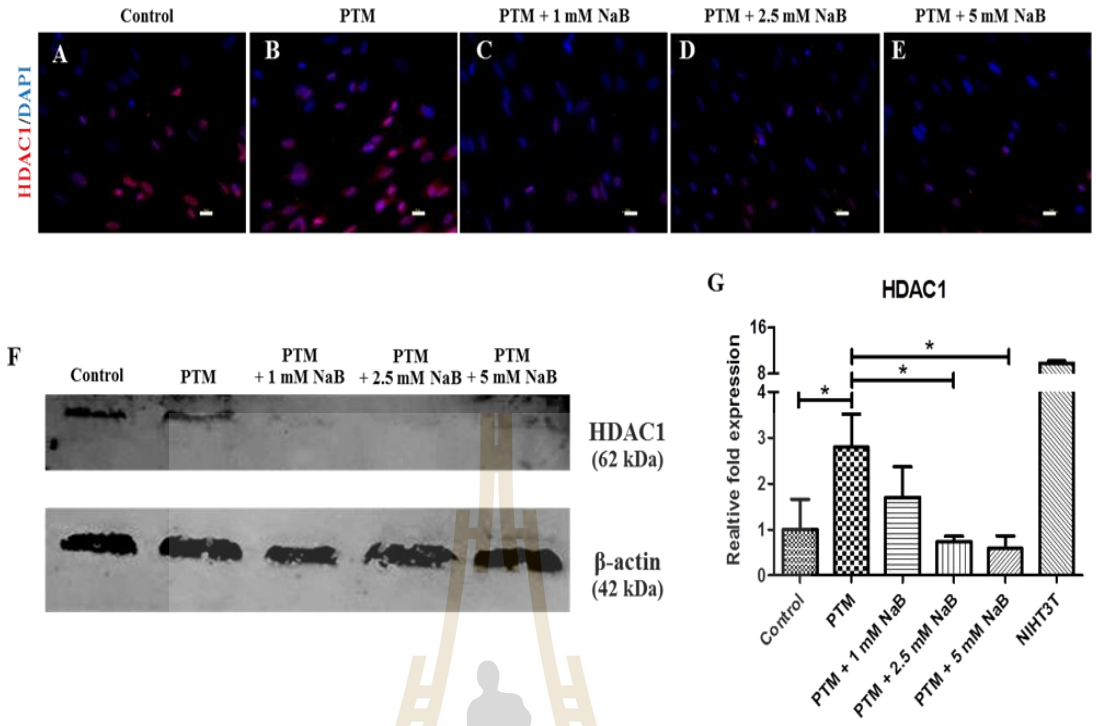

Figure 4.5 Effect of NaB on histone deacetylase 1 (HDAC1) inhibition. **(A-E)** Representative indirect immunofluoresence staining for HDAC1 after various concentrations of NaB (0, 1, 2.5 and 5 mM) pre-treatment combined with EGF and bFGF and control group for 72 h. Scale bar: 10 μm. **(F)** Western blot analysis for the HDAC1 protein levels after pre-treatment for 72 h and β-actin was used as an internal control. **(G)** qPCR analysis for the *HDAC1* gene expressions after 72 h of pre-treatment. Gene expressions were normalized to corresponding *GAPDH* and calculated by relative fold expression compared to control cells (hWJ-MSCs). NIH3T3 cells were used as positive control. The experiments were done triplicates. Data were shown as mean ± SD, $*p < 0.05$.

4.4 Hepatogenic differentiation and characterizations

We next examined the hWJ-MSCs differentiation into hepatocyte-like cells by the optimized NaB concentration of 1 mM pre-treatment combined with EGF and bFGF for 72 h before differentiated to hepatic lineage with 2-step differentiation protocol of Campard and colleagues with some modification. The morphological changes were determined on day 3, 10, 17 and 24 followed the differentiation. Upon differentiation, we observed morphological changed from fibroblast-like cells of hWJ-MSCs into round or polygonal cells of hepatocyte features (S1 Fig., Appendix A). At day 3, the fibroblast-like morphology of hWJ-MSCs did not change much in both groups of the differentiated hWJ-MSCs with and without NaB pre-treatment (Fig. 4.6A-B). On day 10 of differentiation, the cells shape changed into more spindles like (Fig. 4.6C-D) and continuously changed into epithelial round on day 17 (Fig. 4.6E-F) and and polygonal cells on day 24 (Fig. 4.6G-H), in both differentiated hWJ-MSCs with and without NaB pre-treatment groups.

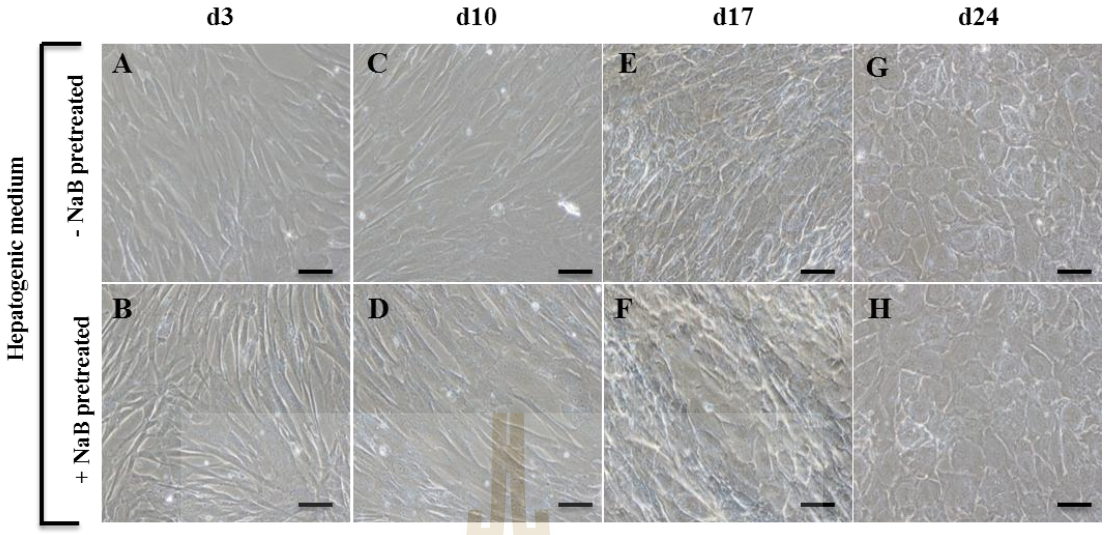

Figure 4.6 Hepatic-like cell features of the differentiated hWJ-MSCs. Morphology of the differentiated hepatocyte-like cells from hWJ-MSCs with and without NaB pre-treatment in EGF and bFGF supplementation at d3 **(A-B)**, 10 **(C-D)**, 17 **(E-F)** and 24 **(G-H)** were observed under a phrase contrast microscope following the differentiation period. Scale bar: 50 μm.

The hepatic markers, AFP, HNF3β, CK18 and ALB were examined by qPCR and immunofluorescent analyses on day 3, 10, 17 and 24 following differentiation. The qPCR data demonstrated that the expression of *AFP*, *HNF3β*, *CK18* and *ALB* increased with time in both differentiated hWJ-MSCs with and without NaB pre-treatment groups during hepatogenic differentiation with different levels (Fig 4.7). During the early stages (within 10 days) of differentiation, much higher transcript levels of *AFP* and *HNF3β* (Fig. 4.7A-B), could be detected in the differentiated hWJ-MSCs with NaB pre-treatment group than the differentiated hWJ-MSCs without NaB pre-treatment and the control groups and gradually decreased on day 17 and 24. However, expression of *HNF3β* transcripts of the differentiated hWJ-MSCs without NaB pre-treatment group was higher on day 17 and gradually decreased on day 24.

But the AFP-positive and HNF3β-positive cells were detected in the differentiated hWJ-MSCs with NaB pre-treatment group greater than the differentiated hWJ-MSCs without NaB pre-treatment group at the late stages of differentiation (Fig. 4.8B-C and E-F). At day 3, 10 and 17 of the differentiation the AFP-positive (S2 Fig., Appendix A) and HNF3β-positive (S3 Fig., Appendix A) cells were gradually detected on day 3 and stronger on day 10 and 17, respectively. Therefore, this result indicated that hepatogenic differentiation in NaB pre-treatment group occurred earlier than in the control (hWJ-MSCs) and NaB non-pre-treatment groups. According to the earlier hepatogenic differentiation markers of protein expressions, day 24 of the differentiation the strongest expression of CK18 and ALB were also detected in both NaB pre-treatment (Fig. 4.8H and K) and NaB non-pre-treatment (Fig. 4.8I and L) groups, respectively. On day 3, 10 and 17 of the differentiation, CK18 (S4 Fig., Appendix A) and ALB (S5 Fig., Appendix A) were also expressed but less on day 3 and 10 and stronger on day 17. The expression of *CK18* (Fig. 4.7C) and *ALB* (Fig. 4.7D) mRNA from the qPCR data indicated that the hWJ-MSCs differentiated into hepatiocyte-like cells. During the early stage (within 10 days) of differentiation, the expression of mRNA levels of *CK18* and *ALB*, were detected higher in the differentiated hWJ-MSCs with NaB pre-treatment group than the differentiated hWJ-MSCs without NaB pre-treatment and the control groups in different levels. On day 17 of differentiation, the differentiated hWJ-MSCs with NaB pre-treatment group much gradually increased in both *CK18* and *ALB* mRNA expression and highest expression levels on day 24 of differentiateion, which were statiscally different when compared to the differentiated hWJ-MSCs without NaB pre-treatment and the control groups.

Therefore, these results demonstrated that hWJ-MSCs differentiated into hepatoblast-like and hepatocyte-like cells after NaB pre-treatment in hepatogenic differentiation.

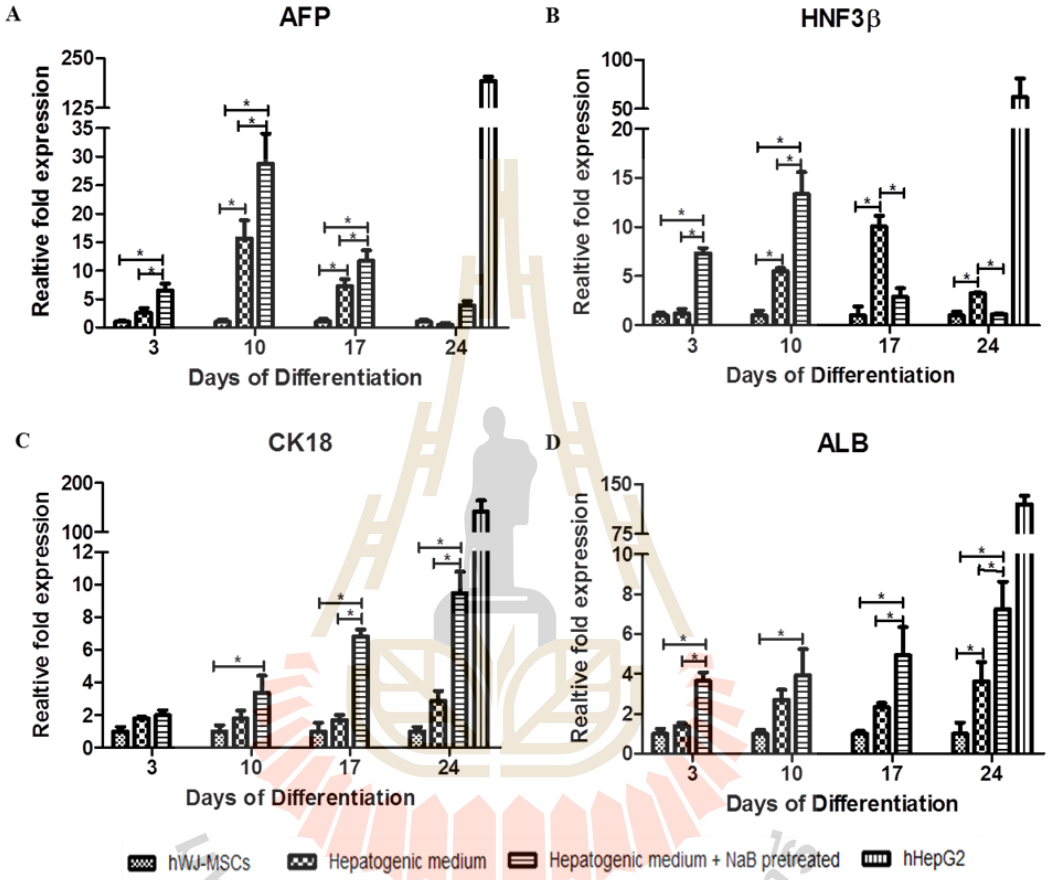

Figure 4.7 Heptic-specific gene expressions of the differentiated hWJ-MSCs with and without NaB pre-treatment in EGF and bFGF supplementation. **(A-D)** qPCR analysis of hepatogenic gene expressions as *AFP*, *HNF3β*, *CK18* and *ALB* after differentiation. Gene expressions were normalized to *GAPDH* and calculated by relative fold expression compared to control cells (hWJ-MSCs). hHepG2 was used as positive control. The experiments were done in triplicates. Data were shown as mean±SD, $*p < 0.05$.

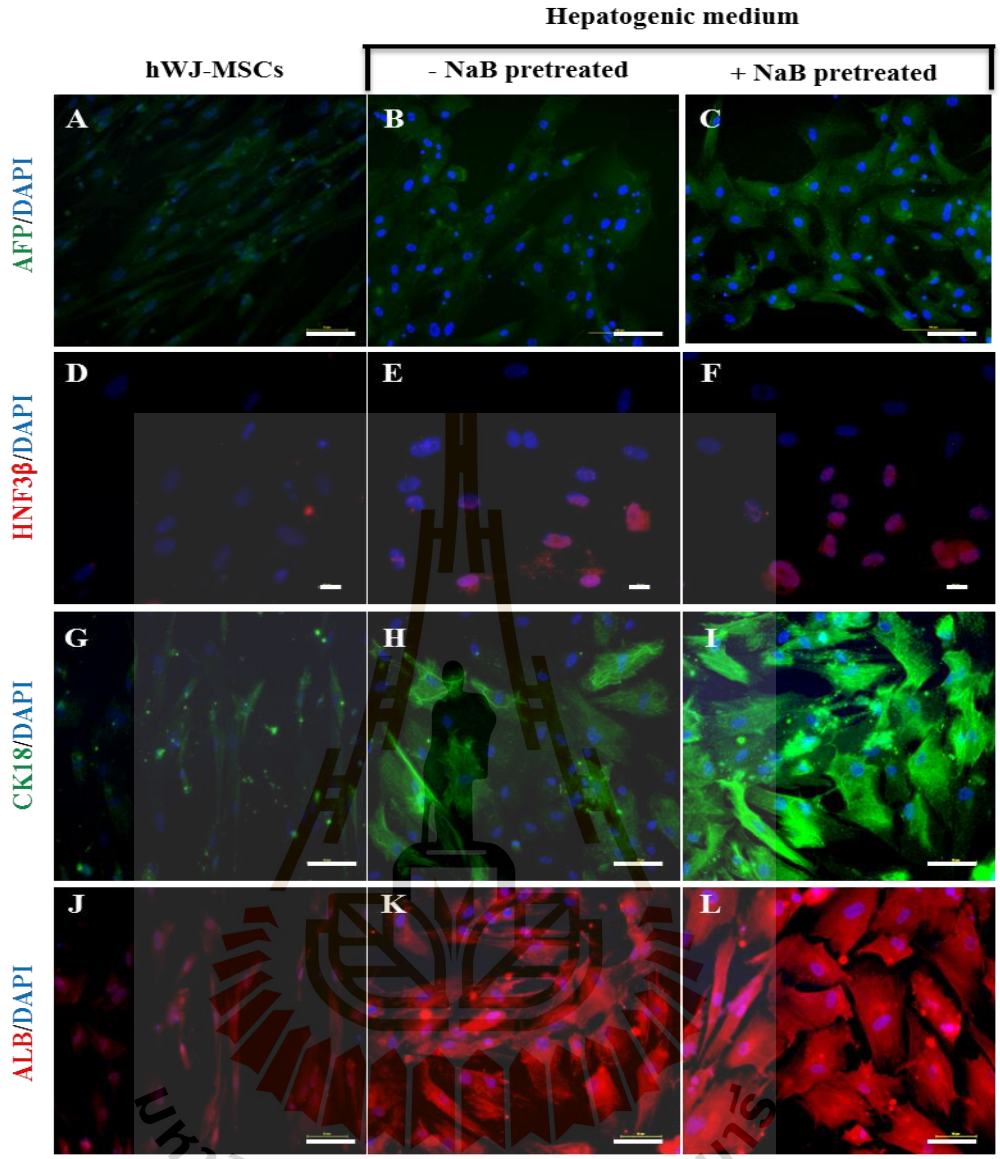

Figure 4.8 Hepatic-specific protein expressions of the differentiated hWJ-MSCs with and without NaB pre-treatment in EGF and bFGF supplementation by immunofluorescent analysis at day 24. The differentiated hWJ-MSCs with and without NaB pre-treatment in EGF and bFGF supplementation were stained with a specific antibody for **(A-C)** AFP, **(D-F)** HNF3β, **(G-I)** CK18 and **(J-L)** ALB, respectively. Scale bar: 50 **(A-C & G-L)** and **10 (D-F)** μm.

Biochemical functions of mature hepatocytes were assessed by the observation of cytoplasmic glycogen granules storage with Periodic acid-Schiff (PAS) staining and urea production assays at day 3, 10, 17 and 24 of differentiation. The results indicated that the undifferentiated hWJ-MSCs group did not show positive signal of PAS staining (Fig. 4.9A), but the differentiated hWJ-MSCs with NaB pre-treatment (Fig. 4.9C) show strong signal of PAS staining when compare to the differentiated hWJ-MSCs without NaB pre-treatment (Fig. 4.9B). This results corresponding to the protein expressions of hepatic markers at day 3, 10 and 17, that they increase the signal of PAS reaction on day 3 and 10, and stronger signal on day 17 in both groups of the differentiated hWJ-MSCs with and without NaB pre-treatment (S6 Fig., Appendix A). Urea secretion products from the metabolic function of ammonia detoxification were investigated at day 3, 10, 17 and 24 (Fig. 4.9D). In both groups of the differentiated hWJ-MSCs with and without NaB pre-treatment could be slightly detected urea at day 3 and 10 significantly difference when compared to control group, but no difference when compare between groups. On day 17 and 24 of differentiation, in both groups of the differentiated hWJ-MSCs with and without NaB pre-treatment gradually increased with significantly difference ($\Delta p<0.05$), and when compared to the control group, they were also difference ($^{*}p<0.05$). Therefore, this result indicated that hWJ-MSCs cultured in hepatogenic medium with 1 mM NaB pre-treatment for 72 h can differentiated into matured hepatocyte-like cells with their functions greater than the cell grown in hepatogenic medium without NaB pre-treatment.

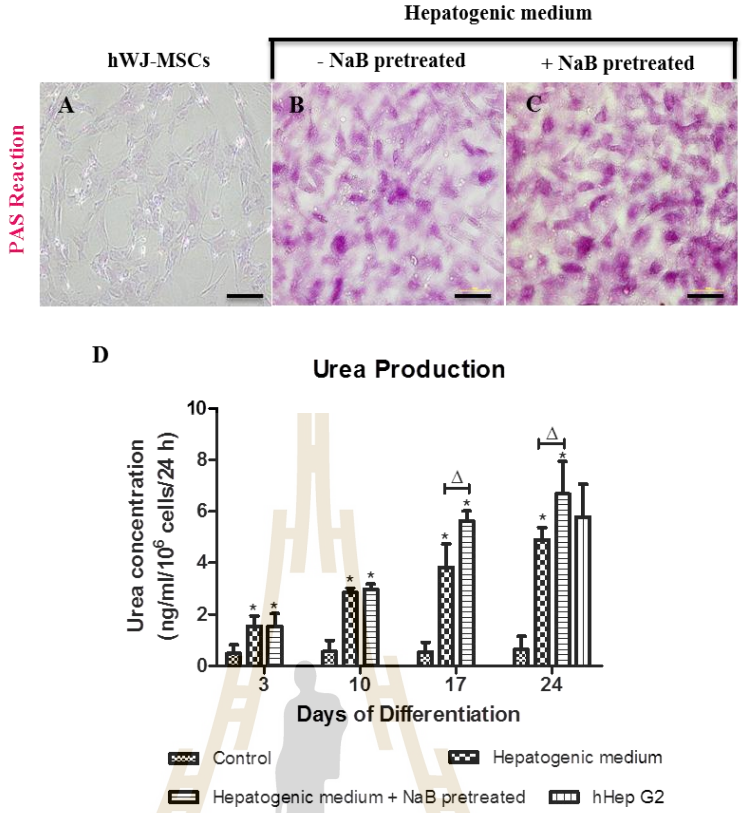

Figure 4.9 Functional evaluations of the differentiated hWJ-MSCs with and without NaB pre-treatment in EGF and bFGF supplementation. **(A-C)** Glycogen storage of the differentiated hWJ-MSCs with and without NaB pre-treatment in EGF and bFGF supplementation at day 24, respectively, were characterized by PAS staining following the differentiation period. Scale bar: 100 µm. **(D)** The capacity of the differentiated hWJ-MSCs with and without NaB pre-treatment in EGF and bFGF supplementation to produce urea after differentiation for 24 days was determined by colorimetric following the differentiation period. hHepG2 was used as positive control. Data were shown as mean ± SD, Δ,$*p < 0.05$.

CHAPTER V

DISCUSSION AND CONCLUSION

Nowadays, transplantation of undifferentiated umbilical cord-MSCs (UC-MSCs) has been applied in the degenerative diseases patients including liver diseases. Although, the transplantation can improve the liver function of the model of end-stage liver disease (MELD) symptoms, the survival rate is still low (Zhang and Wang, 2013). Other cell types may be needed if one wants to practice cell-based therapy. Based on their advantages, hWJ-MSCs seem to possess beneficial effect on liver disease treatment. The hWJ-MSCs-derived hepatocyte-like cells might be viewed as an alternative promising resource.

In this study, the early population of MSCs was obtained from human umbilical cord WJ tissue-by-tissue explants according to isolation procedure (Petsa et al., 2009) after removing umbilical blood vessels. Our data showed that hWJ-MSCs positively expressed MSC markers; CD73, CD90 and CD105, but did not expressed CD34-hematopoietic marker. They can also be differentiated into osteoblasts, chondrocytes and adipocytes of tri-mesodermal lineage differentiation potential *in vitro*. Therefore, these data indicated that the hWJ-MSCs used in this study could possess the hMSC characteristics of the International Society for Cellular Therapy (ISCT) criteria (Dominici et al., 2006).

This study has improved the method for *in vitro* hWJ-MSCs differentiation

into hepatocyte-like cells by using the pre-treatment of NaB combined with EGF and bFGF in the pre-treatment step of hepatogenic differentiation. Talèns-Visconti and colleagues (2006) used EGF and bFGF pre-treatment for 48 h of MSCs differentiation toward hepatocyte-like cells in order to arrest cell proliferation. Besides, the specification of foregut-midgut organ-specific lineages of endoderm derived from ESC differentiation includes hepatocytes, was successfully obtained by bFGF at low concentration (Ameri et al., 2010). To help the endodermal lineage specification, we changed the pre-treatment from 48 to 72 h for hepatogenic differentiation. Previously, another HDACi VPA pre-treatment prior to the hepatogenic differentiation of mBM-MSCs (Chen et al., 2009) and hBM-MSCs (Dong et al., 2013) successfully obtained hepatocyte-like cells. Here, we the optimized NaB concentration (0-5 mM) in the pre-treatment step to drive endodermal lineage specification. Normally, CXCR4 is used as endoderm marker specification in ESCs differentiation into endodermal lineage (King et al., 2008). Besides, SOX17 and GATA6 are also widely used as endoderm markers (An et al., 2014; Takayama et al., 2013). The SOX17 is a high-mobility-group box domain (HGM domain) transcription factor, essential for definitive endoderm formation (Wang et al., 2015). The transcription factor GATA6, zinc finger type that is the one type of GATA factors, important in mesendoderm specification; heart (mesoderm) and liver (endoderm) formation (reviewed in Gordillo, Evans and Gouon-Evans, 2015). Surprisingly a greater effect than that of the conventional method was found. Our result showed that in the pre-treatment medium (PTM) + 1 mM NaB group, endodermal-related gene expressions, *CXCR4, SOX17* and *GATA6* were up-regulated higher than in other groups. The SOX17-positive cells were also detected in the PTM + 1 mM NaB group greater than other groups.

The balance of HDACs and histone acetyltransferase (HATs) regulate histone acetylation of epigenetic modification. HDAC1 usually expressed to suppress gene expression (Liu et al., 2014). HDAC1 is known to be involved in endoderm organogenesis, including development of zebra fish (Noël et al., 2008). It is possible that the NaB may control the cellular histone acetylation in chromatin remodeling during MSC differentiation into the specific cell types, similar to the osteoblasts and smooth muscle cell types (Liu et al., 2014; Chen et al., 2007; Lee et al., 2009). Previous studies showed that class I HDACs, such as *hdac1* and *hdac3* mutants in *in vivo* study of zebrafish, were inhibited by tricostatin A (TSA) (Noël et al., 2008) and valproic acid (VPA) (Farooq et al., 2008) which defected embryonic endoderm specification, including hepatogenesis. But, *in vitro* non-mutant study of VPA pre-treatment in hepatogenic differentiation of hUC-MSCs obtained endodermal and hepatocyte-like cells (An et al., 2014). As inhibitor of HDACs, NaB, similar VPA group of HDACi, should enhance MSC differentiation to hepatocyte-like cells by inhibiting the enzymatic capacity of HDACs. Our data indicated that the PTM + 1 mM NaB group inhibited partially HDAC1 activity in gene and protein expressions, we proposed that the low concentration of NaB (1 mM) combined with EGF and bFGF in pre-treatment could drive endodermal-related gene up-regulation greater than the higher concentration (2.5 and 5 mM) and without NaB pre-treatment (0 mM) groups. Therefore, the partial inhibition of HDAC1 activity by NaB pre-treatement with EGF and bFGF maybe synergistically promote hWJ-MSCs differentiation toward endodermal lineage specification. However, the relationship between NaB and EGF/bFGF enhancement hWJ-MSCs differentiate into endodermal lineage via HDAC1 inhibition should be further investigated.

NaB is an HDAC inhibitor that has been proven to inhibit the proliferation of MSCs (Liu et al., 2014; Lee et al., 2009). The study of Liu and colleagues (2014) showed that 1 mM NaB treatment, arrest proliferation of hWJ-MSCs, however, in this study 1 mM NaB only slightly suppress MSC proliferation but not significantly different from the control, whereas other concentrations (2.5 and 5 mM) showed significantly suppression when compare to the control and the 1 mM NaB treatment groups. Therefore, the 1 mM NaB treatment was chosen for further study. To emphasize the previous evidences, NaB has been shown to promote hepatogenic differentiation from ESCs in pre-treatment (Hay et al., 2008; Cao et al., 2010) and differentiation (Rambhatla et al., 2003; Sharma et al., 2006; Zhou et al., 2007; Mizumoto et al., 2008; Ren et al., 2010; Yan et al., 2011) steps of the differentiation. Mizumoto and colleagues (2008) found that NaB alone cannot promote hepatogenic differentiation from ESCs in pre-treatment step similar to the previous study of MAPCs differentiation into hepatocyte-like cells (Schwartz et al., 2002). Nevertheless, the combination of NaB and activin A can promote hepatogenic differentiation greater than NaB or activin A alone (Mizumoto et al., 2008). This evidence was confirmed from the previous report of Cao and colleagues (2010) that NaB combined with bFGF and BMP4 consistently performed greater than growth factors, bFGF and BMP4 alone or bFGF and BMP4 combination.

To induce MSCs toward hepatocyte-like cells, bFGF, HGF and nicotinamide play a key role in the early step of hepatic induction. The FGFs has an essential role in ventral foregut endoderm specification. HGF also plays a critical role in the development and regeneration of liver cells (Zhang, Lie and Wei, 2009) and nicotinamide are necessary for small hepatocyte colonies proliferation, its

differentiation from rat primary hepatocytes in *in vitro* (Chen and Zheng, 2011). At the differentiation and maturation stages, OSM, dexamethasone and ITS have been used on hepatogenic differentiation of MSCs (Zhao et al., 2009; Prasajak and Leeanansaksiri, 2013; Talèns-Visconti et al., 2006; Ameri et al., 2010; Chen et al., 2009). OSM, a member of interleukin-6 (IL-6) family, is involved in the main maturation fate of fetal hepatogenesis (Sidhu, Liu and Omiecinski, 2004). Dexamethasone and ITS are required for maintaining the expression of liver-enriched transcription factors (LETFs) and cell survival, respectively, which are essential for stimulating liver-specific genes transcription (Prasajak and Leeanasaksiri, 2013; Sidhu, Liu and Omiecinski, 2004). With the primitive origin of hWJ-MSCs, they are more immature MSCs than other adult MSCs sources (Zhao et al., 2009). Therefore, the lager amount of exogenous HGF (40-50 ng/ml) was used in order to trigger hepatogenic signal transduction system (Zhang, Lie and Wei, 2009; Zhao et al., 2009). Here, our result confirmed that after hWJ-MSCs treated with 20 ng/ml HGF in the first step of hepatogenic differentiation protocol for 21 days with pre-treatment step for 48 h, they cannot transdifferentiate into mature hepatocytes (data not shown). This data probably confirmed the previous study of Campard and colleagues (2008) that hWJ-MSCs can be induced to hepatocyte-like cells with 20 ng/ml HGF but with much longer time. Therefore, we changed the HGF concentration from 20 to 40 ng/ml, and showed that hWJ-MSCs were driven into hepatocyte-like cells greater than previous study.

AFP, an early hepatic differentiation marker of fetal hepatocytes, has been investigated in hWJ-MSCs-derived hepatocyte-like cells (Zhang, Lie and Wei, 2009; Zhao et al., 2009, Prasajak and Leeanansaksiri, 2013). HNF3β or FOXA2 is a LETF

that has a crucial role in liver development and specified pre-hepatic fate through liver-specific gene during hepatogenic differentiation (Chen et al., 2009). Our data demonstrated that the differentiated hWJ-MSCs cells expressed *HNF3β* and *AFP* mRNA at an early stage of development. In the differentiated hWJ-MSCs cells with NaB pre-treatment their mRNA levels were much higher. However, for protein expression of AFP and HNF3β markers were detected strongest on day 24 of the differentiated hWJ-MSCs with NaB pre-treatment group. The hWJ-MSCs derived from more primitive origin seem to reserve the immature phenotype even though they have driven toward specific cell types (Prasajak and Leeanansaksiri, 2013). Compare to other HDACi chemicals, VPA pre-treatment on hepatogenic differentiation of BM-MSCs, AFP and HNF3β gene and protein expression patterns similar to previous reports (Chen et al., 2009; Dong et al., 2013), but *HNF3β* gene expression were up-regulated overtime. Therefore, this result indicated that the differentiated hWJ-MSCs with NaB pre-treatment were driven toward hepatoblast-like cells greater than the differentiated hWJ-MSCs cells without NaB pre-treatment.

In the mid to late stage of liver development, the differentiated hWJ-MSCs with and without NaB pre-treatment achieved characteristic of gene expressions and immunophenotypes of hepatic features by expressed CK18 and ALB. Because CK18 and ALB have been reported to be mid-late and late stages of differentiation markers of liver organogenesis (Zhang, Lie and Wei, 2009). Compare to other HDACi group, VPA pre-treatment on hepatogenic differentiation of BM-MSCs (Chen et al., 2009; Dong et al., 2013) and UC-MSCs (An et al., 2014), ALB gene and protein expression patterns were similar to our data. Besides, the strongest PAS positive signals were detected in the differentiated hWJ-MSCs with NaB pre-treatment on day 24 of the late

stage of differentiation. However, the differentiated hWJ-MSCs with NaB pre-treatment were not difference detected urea production at all stage of differentiation, they were significantly detected urea production difference from the control group on day 10, 17 and 24. Therefore, this result corresponds to previous study that they can be transdifferentiated into functional matured hepatocyte-like cells by storage glycogen (Campard et al., 2008; Zhang, Lie and Wei, 2009; Zhao et al., 2009; Prasajak and Leeanansaksiri, 2013) and urea production (Campard et al., 2008; Zhao et al., 2009; Prasajak and Leeanansaksiri, 2013) at the late stage of differentiation. According to previous study, glycogen storage from VPA pre-treatment (An et al., 2014; Chen et al., 2009; Dong et al., 2013) could give stronger PAS signal than VPA non-pre-treatment. However, previous study found that VPA pre-treatment (Dong et al., 2013; An et al., 2014) in hepatogenic differentiation of MSCs could gradually increase urea production, especially, at the late stage of differentiation. Significantly difference between groups, our study found that between groups of the differentiated hWJ-MSCs with and without NaB pre-treatment were not different at the late stage of differentiation. Therefore, the increase time of culture might increase the urea production better.

Taken together, hWJ-MSCs can be differentiated into endodermal, hepatoblast-like and hepatocyte-like cells through HDAC1 inhibition by pre-treatment with NaB and combined with EGF and bFGF in the culture condition. Thus, hWJ-MSCs have benefits over other adult MSCs and an attractive source for drug screening and cell-based therapy in liver diseases in the future.

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APPENDICES

APPENDIX A

Supporting Information

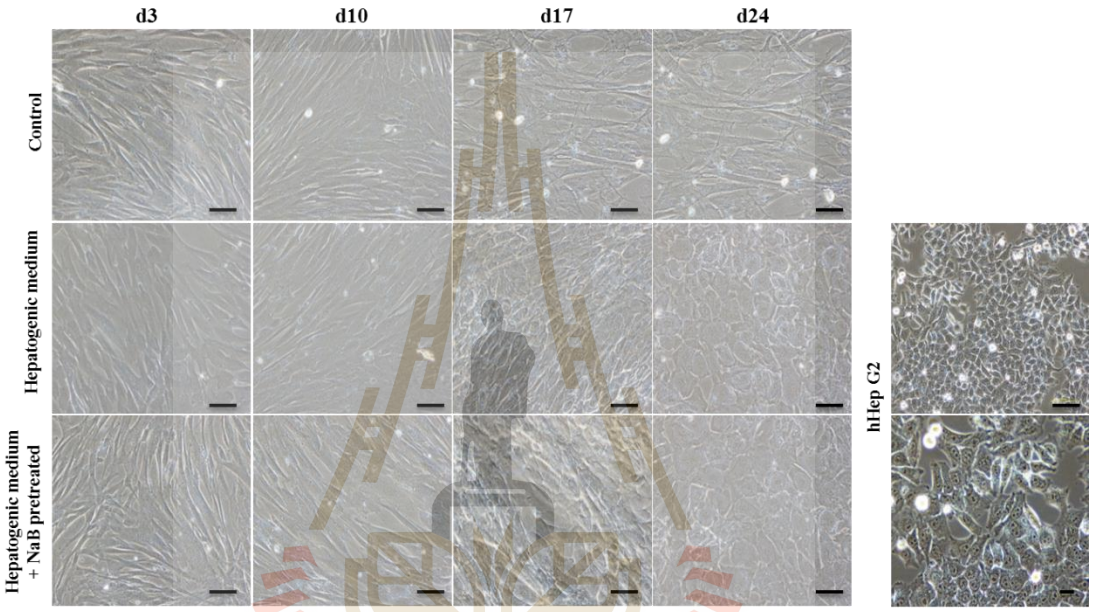


Figure S1 Hepatic-like cell features of the differentiated hWJ-MSCs. Morphology of the differentiated hepatocyte-like cells from hWJ-MSCs with and without NaB pre-treatment in EGF and bFGF supplementation at day 3, 10, 17 and 24 and hHep G2 cell lines were observed under a phrase contrast microscope following the differentiation period. Scale bar: 10 (hHep G2, right-handed of bottom panel) and 50 μm.

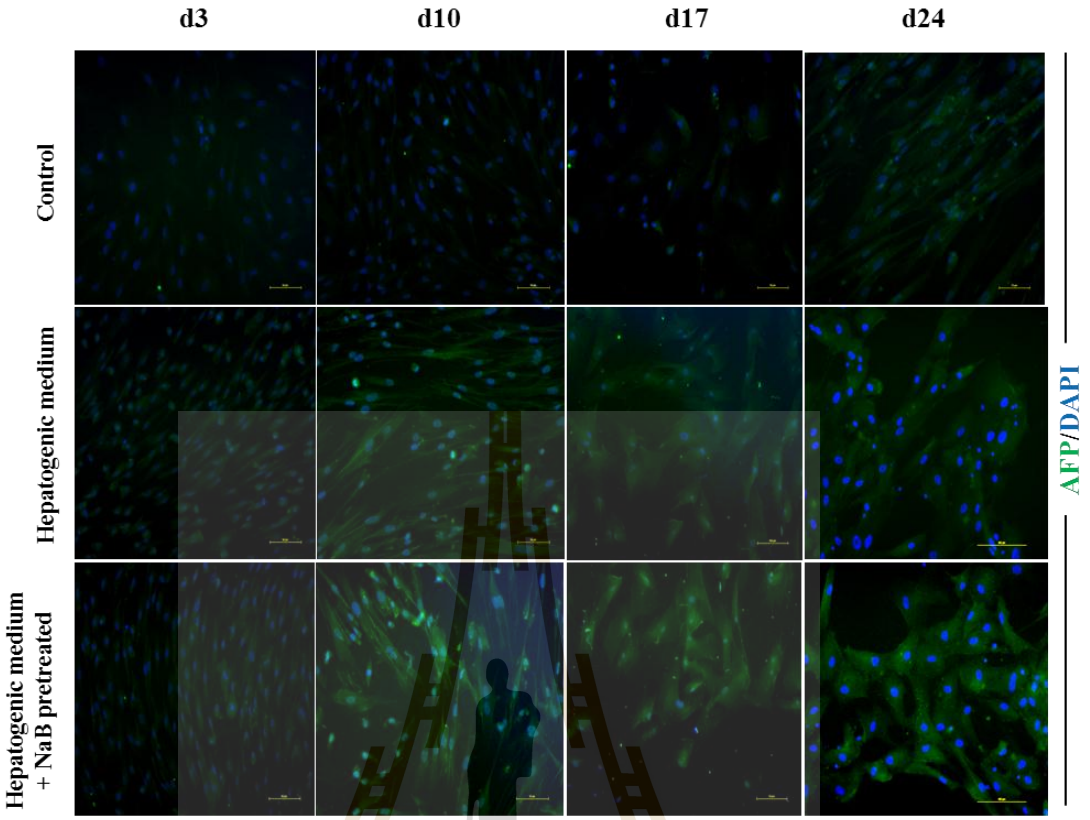


Figure S2 Hepatic-specific protein expression of the differentiated hWJ-MSCs with and without NaB pre-treatment in EGF and bFGF supplementation by immunofluorescent analysis at day 3, 10, 17 and 24 of differentiation. The differentiated hWJ-MSCs with and without NaB pre-treatment in EGF and bFGF supplementation were stained with a specific antibody for AFP. Scale bar: 50 μm.

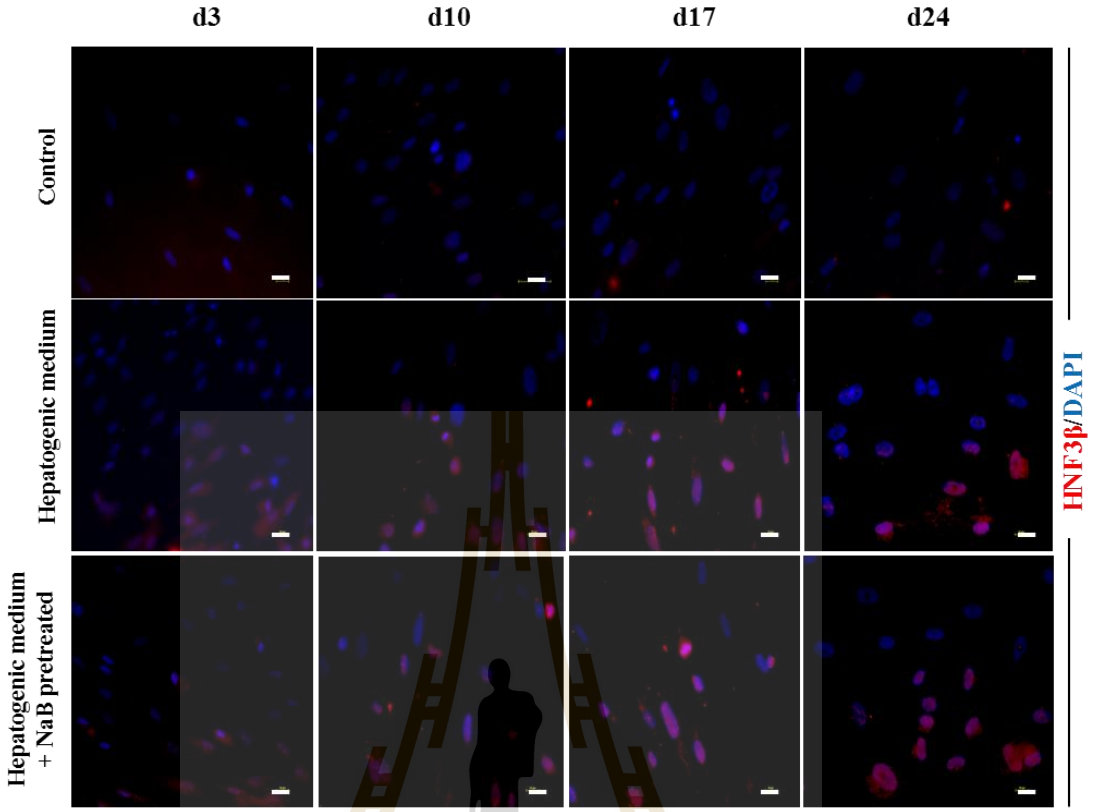


Figure S3 Hepatic-specific protein expression of the differentiated hWJ-MSCs with and without NaB pre-treatment in EGF and bFGF supplementation by immunofluorescent analysis at day 3, 10, 17 and 24 of differentiation. The differentiated hWJ-MSCs with and without NaB pre-treatment in EGF and bFGF supplementation were stained with a specific antibody for HNF3β. Scale bar: 10 μm.

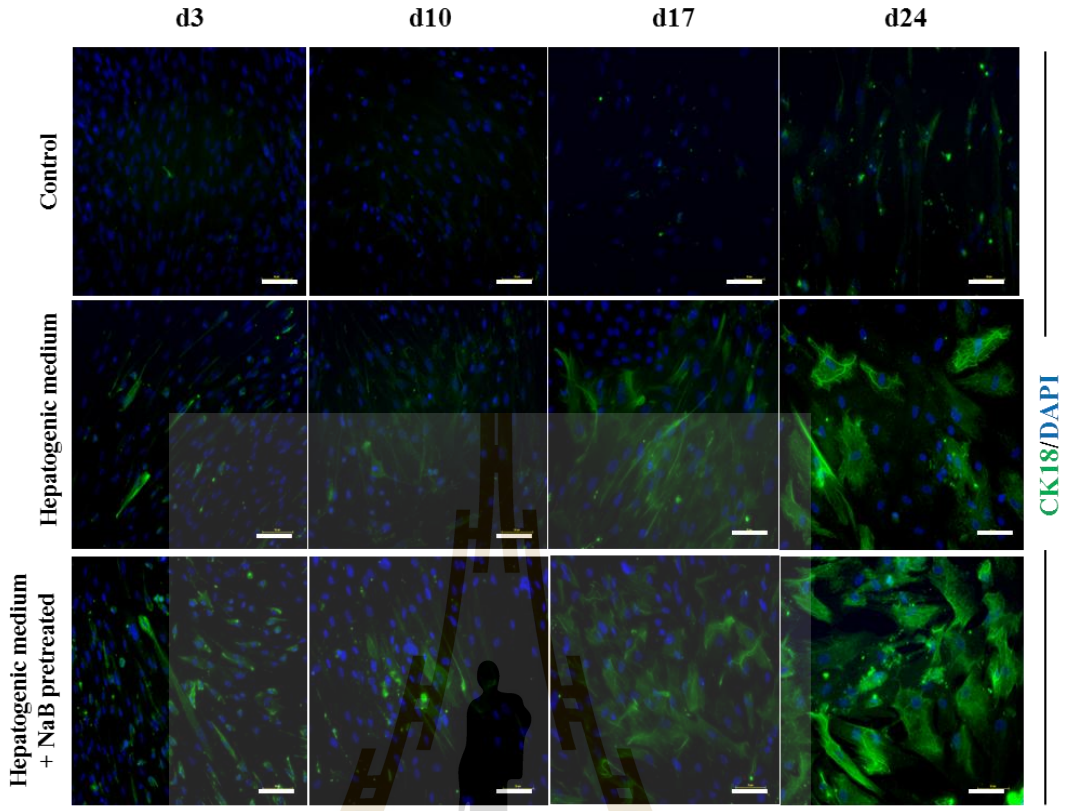


Figure S4 Hepatic-specific protein expression of the differentiated hWJ-MSCs with and without NaB pre-treatment in EGF and bFGF supplementation by immunofluorescent analysis at day 3, 10, 17 and 24 of differentiation. The differentiated hWJ-MSCs with and without NaB pre-treatment in EGF and bFGF supplementation were stained with a specific antibody for CK18. Scale bar: 50 μm.

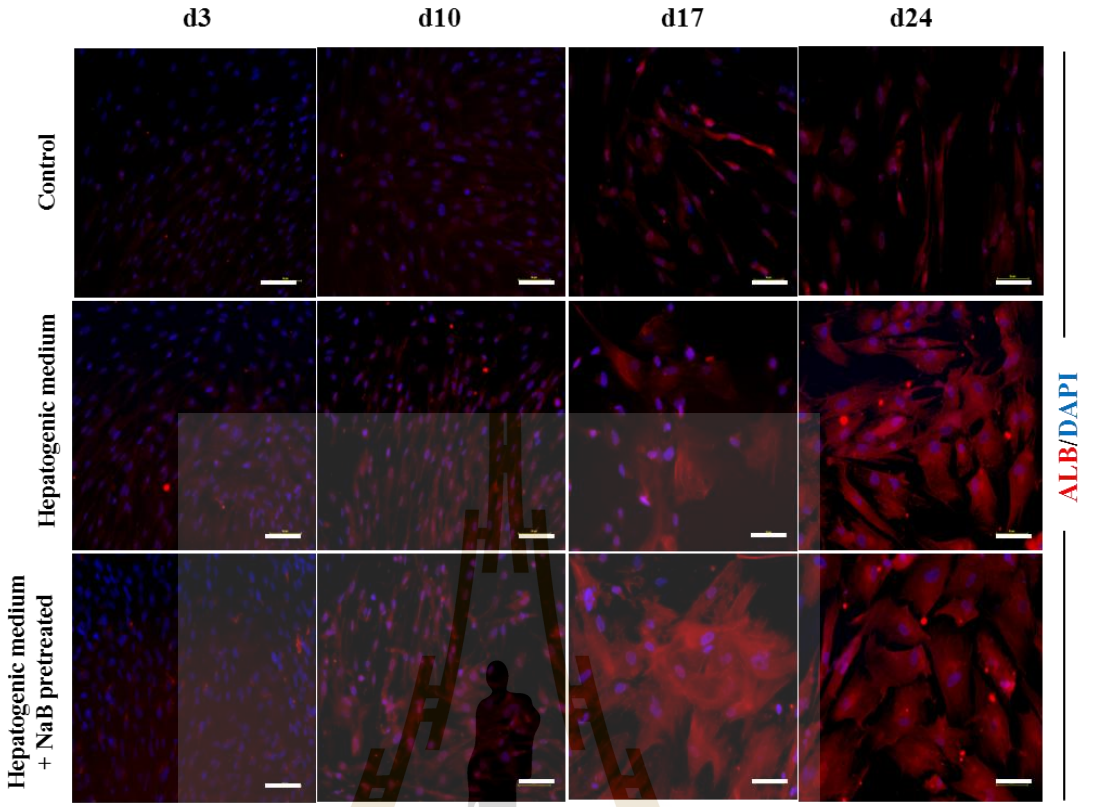


Figure S5 Hepatic-specific protein expression of the differentiated hWJ-MSCs with and without NaB pre-treatment in EGF and bFGF supplementation by immunofluorescent analysis at day 3, 10, 17 and 24 of differentiation. The differentiated hWJ-MSCs with and without NaB pre-treatment in EGF and bFGF supplementation were stained with a specific antibody for ALB. Scale bar: 50 μm.

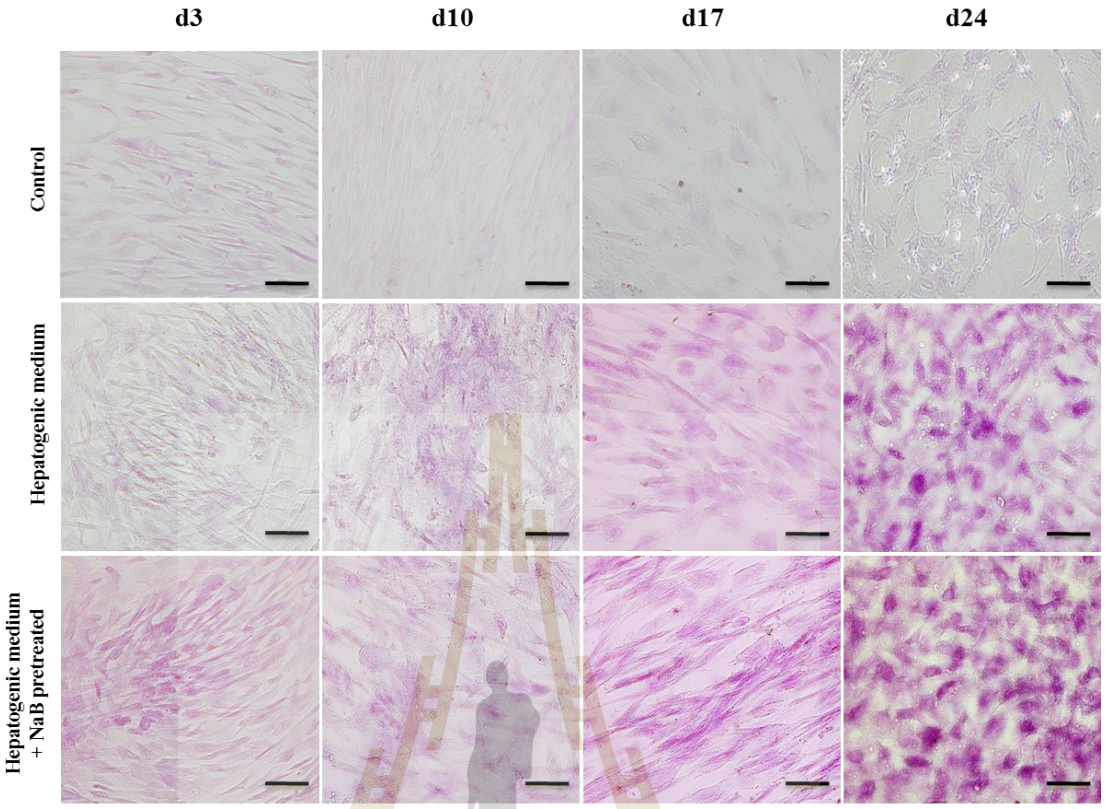


Figure S6 Functional evaluation of the differentiated hWJ-MSCs with and without NaB pre-treatment in EGF and bFGF supplementation. Glycogen storage of the differentiated hWJ-MSCs with and without NaB pre-treatment in EGF and bFGF supplementation at day 3, 10, 17 and 24, respectively, were characterized by PAS staining following the differentiation period. Scale bar: 100 μm.

APPENDIX B

Table 1 Relative fold expression of endodermal-related genes compared to control (hWJ-MSCs) at day 3 of differentiation (N=3).

Genes / **Conditions**	***CXCR4***	***SOX17***	***GATA6***	***HDAC1***
hWJ-MSCs (Control)	1±0.23	1±0.26	1±0.25	1±0.66
Pre-treatment medium (PTM)	1.68±0.66	0.46±0.06	0.65±0.16	2.80±0.72
PTM + 1 mM NaB	12.79±0.72	19.60±1.57	4.87±0.40	1.70±0.67
PTM + 2.5 mM NaB	1.14±0.15	1.12±0.20	2.70±0.69	0.74±0.11
PTM + 5 mM NaB	0.82±0.11	0.40±0.07	0.74±0.24	0.59±0.28
NIH3T3 cells	-	-	-	9.73±0.44

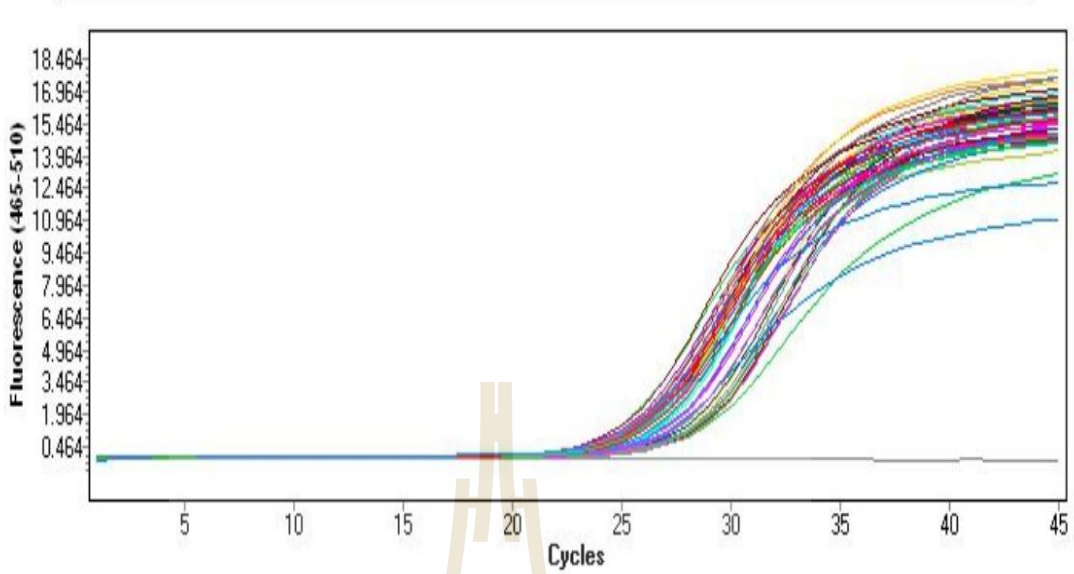
Table 2 Relative fold expression of hepatic genes compared to control at day 3, 10, 17 and 24 of differentiation (N=3).

Genes / Conditions	*AFP*				*HNF3β*			
	Day 3	**Day 10**	**Day 17**	**Day 24**	**Day 3**	**Day 10**	**Day 17**	**Day 24**
hWJ-MSCs (negative control)	1±0.16	1±0.43	1±0.48	1±0.36	1±0.26	1±0.49	1±0.90	1±0.41
Hepatogenic medium	2.56±0.85	15.66±3.18	7.26±1.26	0.50±0.31	1.21±0.46	5.50±0.32	10.06±1.08	3.27±0.05
Hepatogenic medium + NaB pre-treated	6.50±1.25	28.77±5.29	11.69±1.87	3.92±0.70	7.33±0.54	13.34±0.25	2.87±0.90	1.15±0.04
hHep G2 cells (positive control)	-	-	-	193.21±10.16	-	-	-	61.99±19.28

Table 2 Relative fold expression of hepatic genes compared to control at day 3, 10, 17 and 24 of differentiation (N=3) (**Continued).**

Genes / Conditions	*CK18*				*ALB*			
	Day 3	**Day 10**	**Day 17**	**Day 24**	**Day 3**	**Day 10**	**Day 17**	**Day 24**
hWJ-MSCs (negative control)	1±0.28	1±0.38	1±0.55	1±0.28	1±0.25	1±0.19	1±0.14	1±1.56
Hepatogenic medium	1.81±0.08	1.77±0.50	1.68±0.34	2.89±0.59	1.38±0.15	2.70±0.51	2.32±0.21	3.62±0.98
Hepatogenic medium + NaB pre-treated	1.99±0.32	3.37±1.04	6.82±0.43	9.46±1.32	3.65±0.44	3.94±1.32	4.94±1.41	7.25±1.39
hHep G2 cells (positive control)	-	-	-	141.20±22.94	-	-	-	119.99±12.89

A



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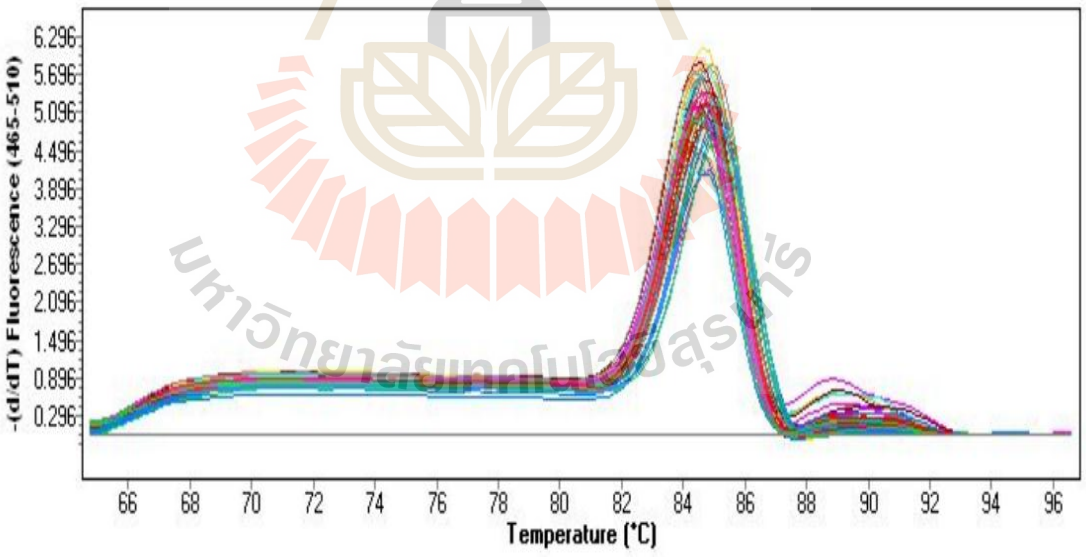
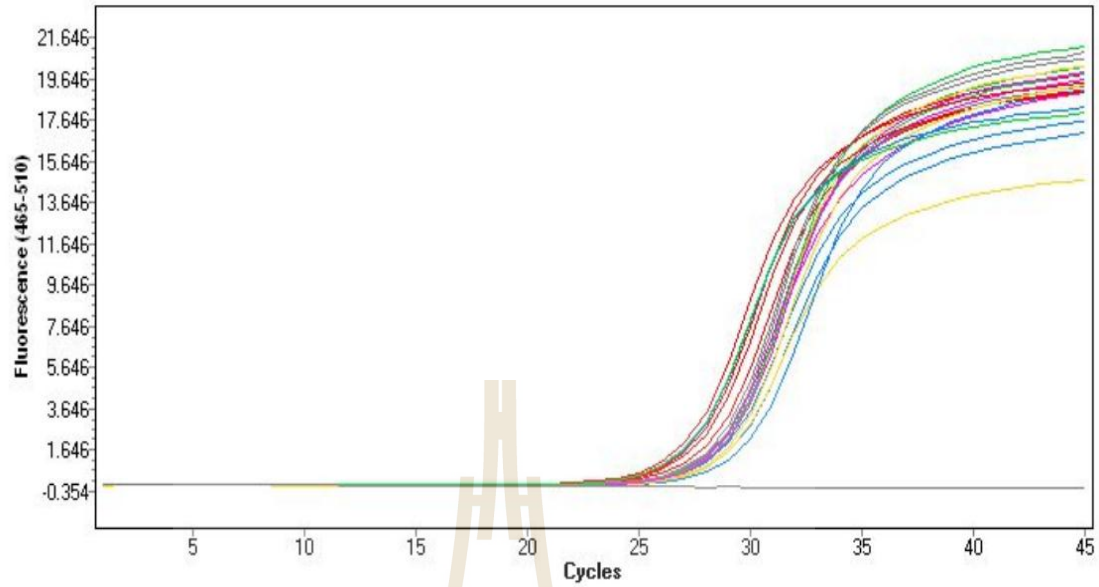


Figure 1 A representative validation curve of real time PCR for *GAPDH* gene.

(A) The amplification curve for the *GAPDH* gene.

(B) The dissociation curve of PCR products of *GAPDH* gene.

A



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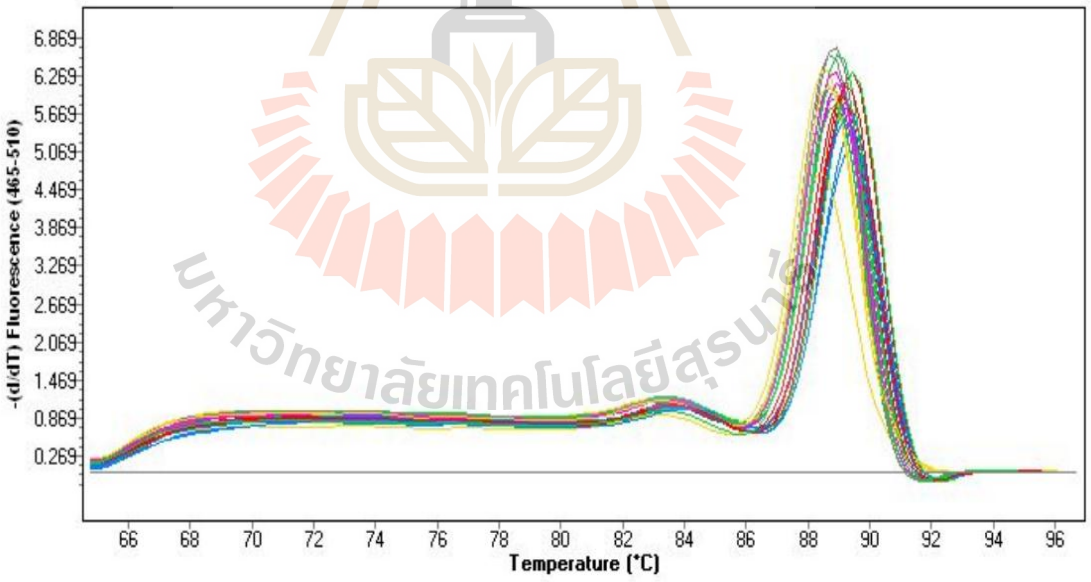
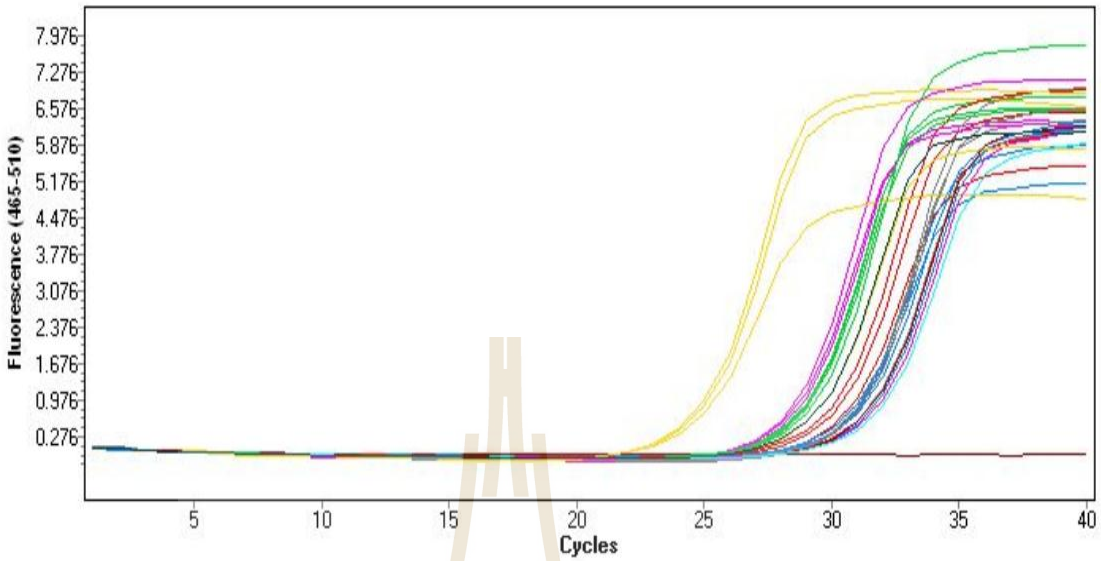


Figure 2 A representative validation curve of real time PCR for *GATA6* gene.

(A) The amplification curve for the *GATA6* gene.

(B) The dissociation curve of PCR products of *GATA6* gene.

A



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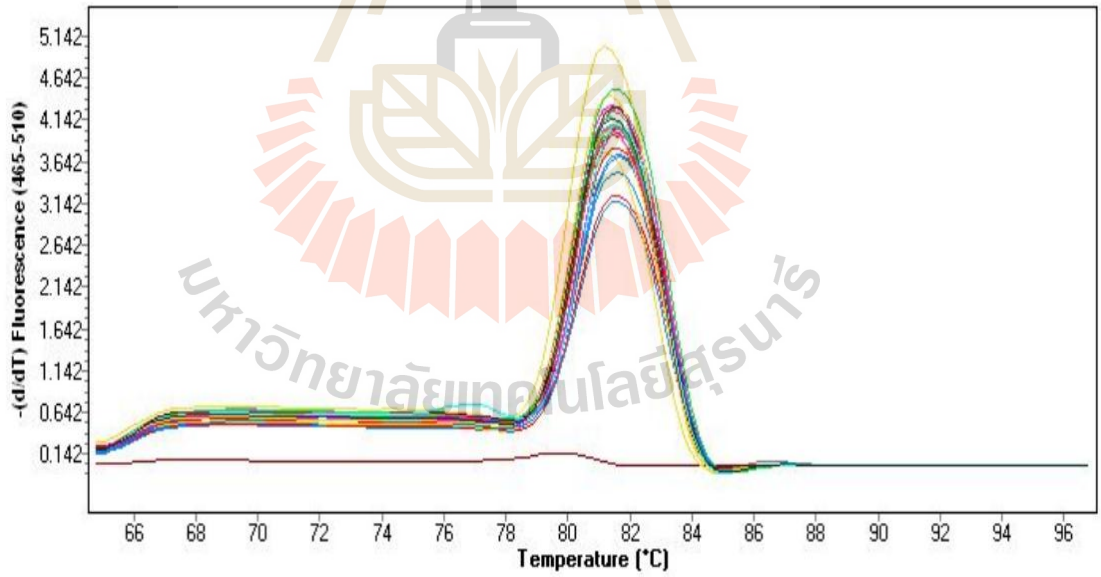
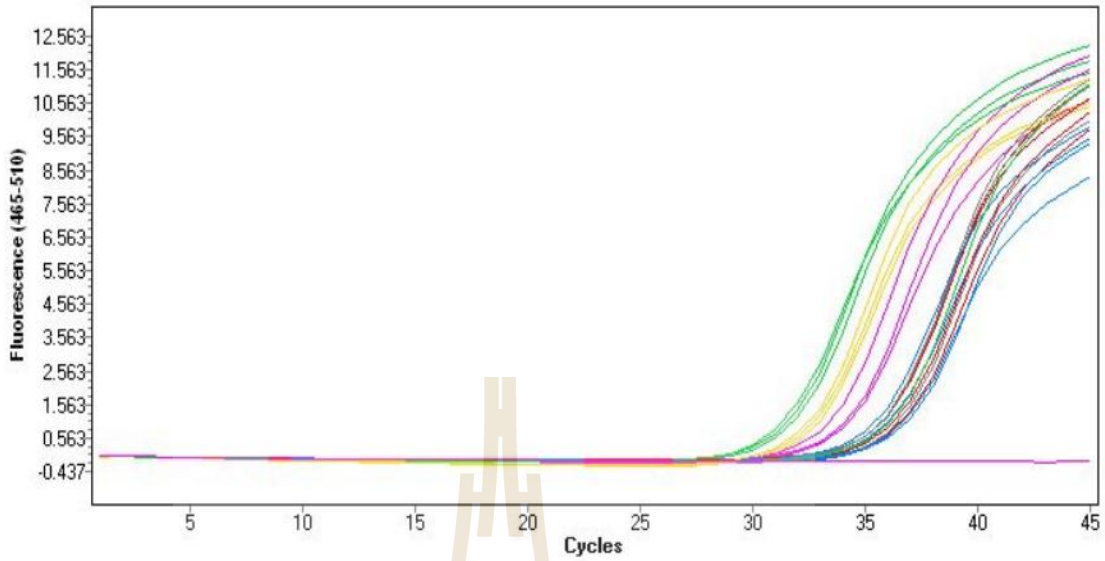


Figure 3 A representative validation curve of real time PCR for *CXCR4* gene.

(A) The amplification curve for the *CXCR4* gene.

(B) The dissociation curve of PCR products of *CXCR4* gene.

A



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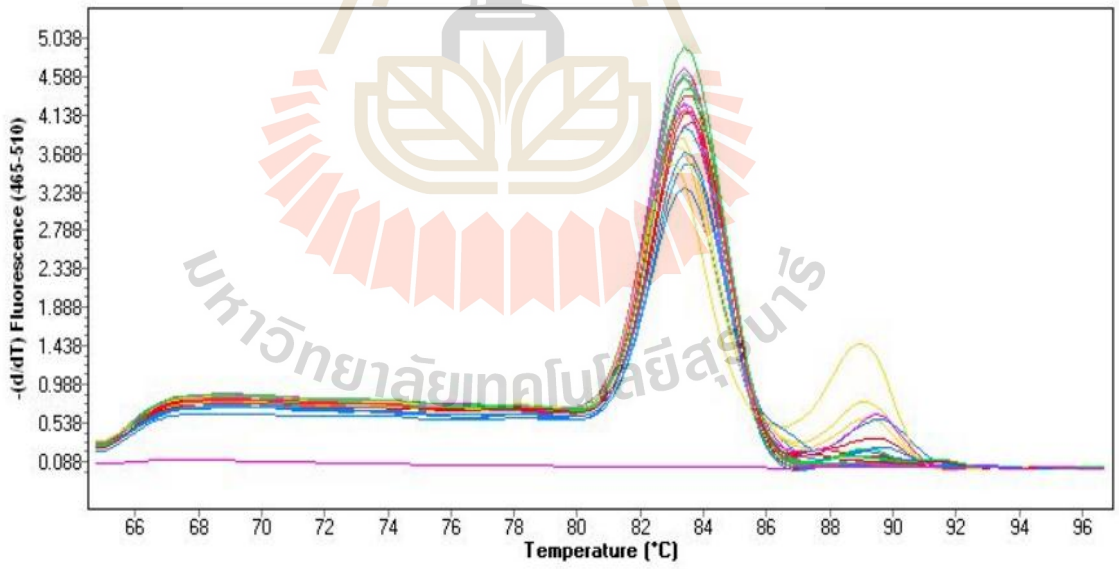
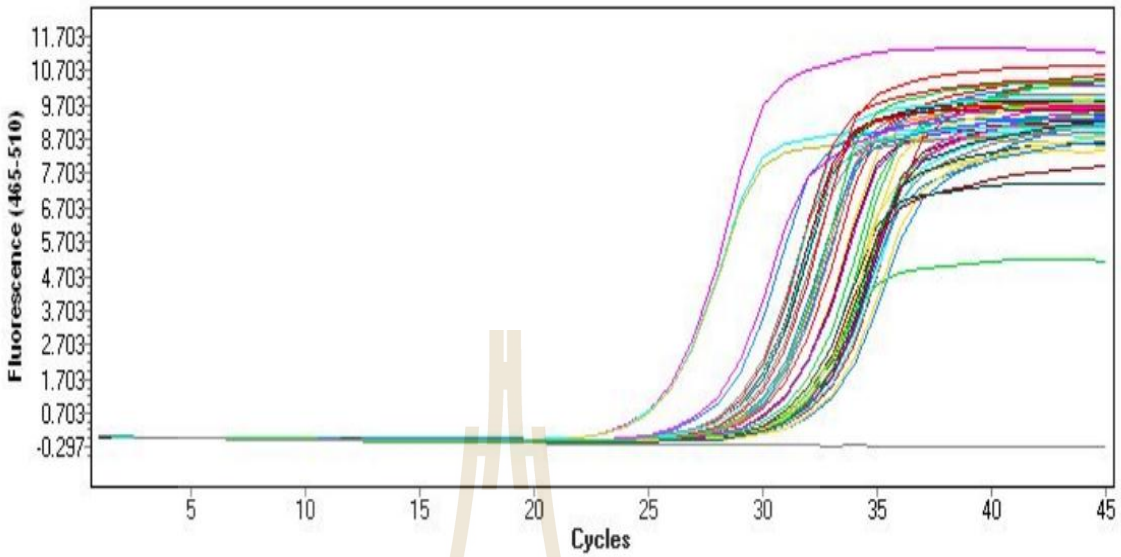


Figure 4 A representative validation curve of real time PCR for *SOX17* gene.

(A) The amplification curve for the *SOX17* gene.

(B) The dissociation curve of PCR products of *SOX17* gene.

A



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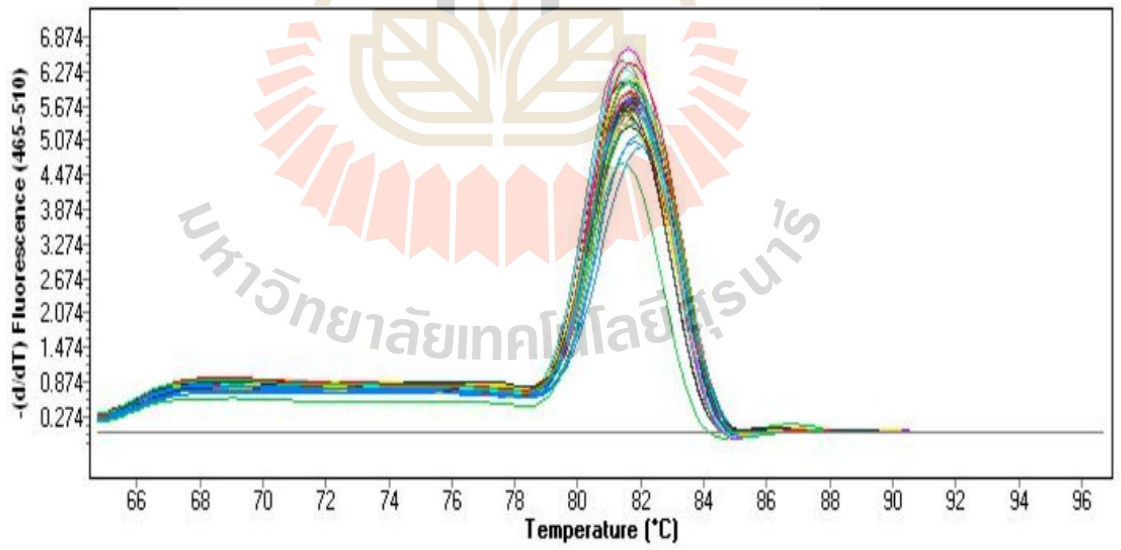
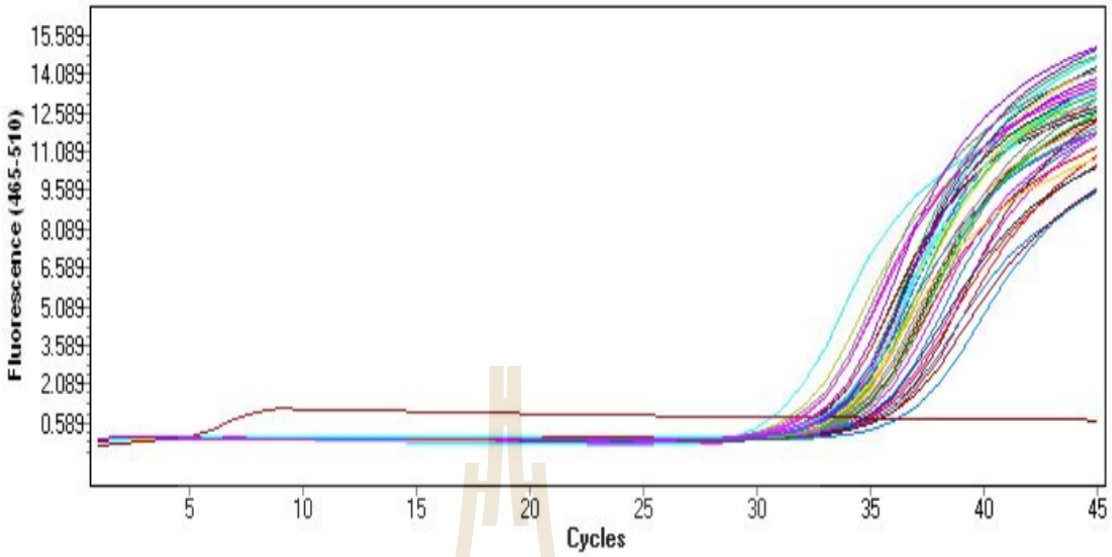


Figure 5 A representative validation curve of real time PCR for *AFP* gene.

(A) The amplification curve for the *AFP* gene.

(B) The dissociation curve of PCR products of *AFP* gene.

A



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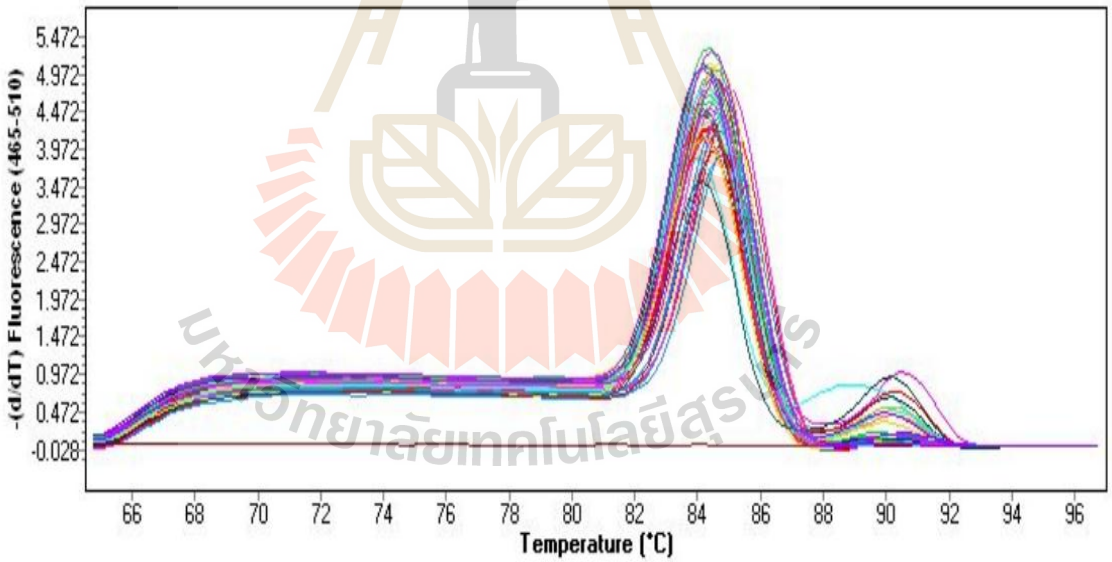
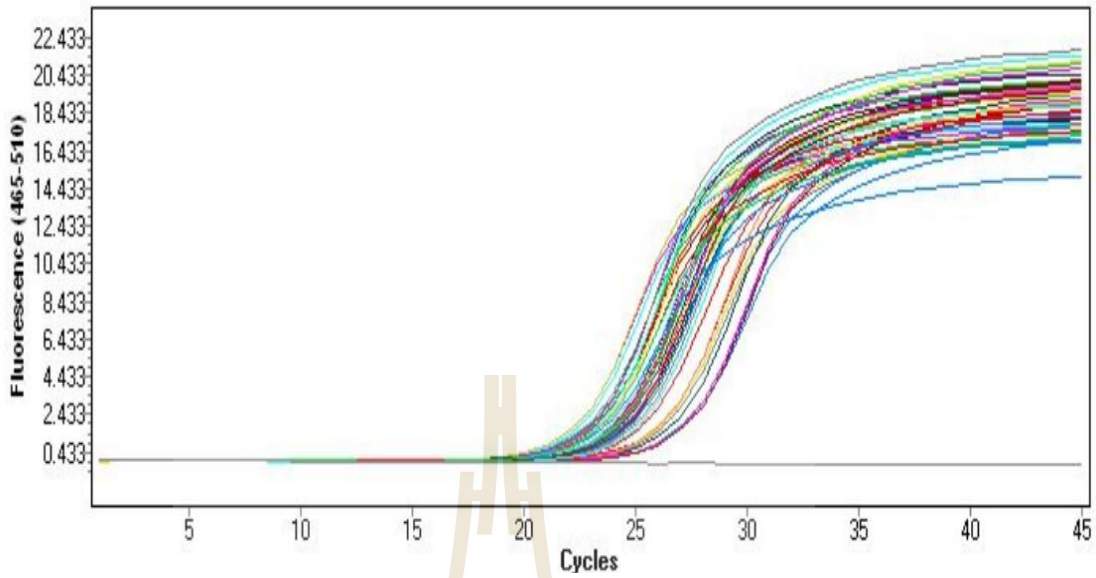


Figure 6 A representative validation curve of real time PCR for *HNF3β* gene.

(A) The amplification curve for the *HNF3β* gene.

(B) The dissociation curve of PCR products of *HNF3β* gene.

A



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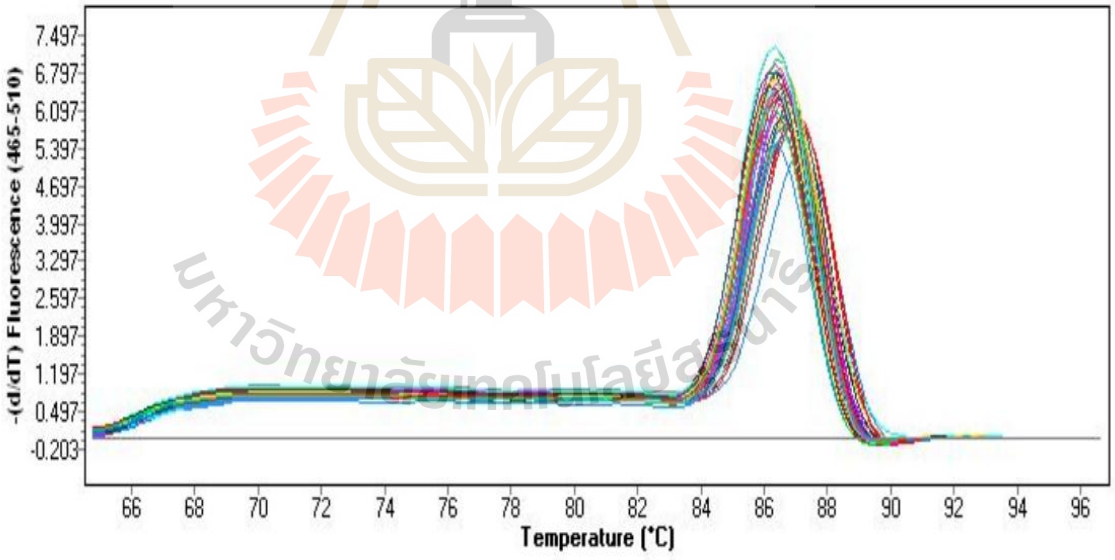
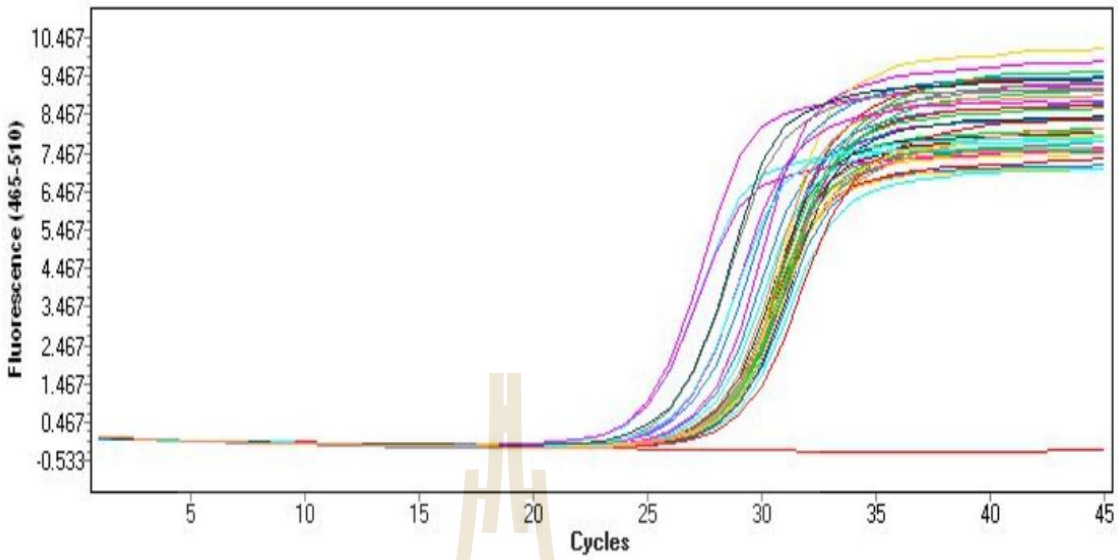


Figure 7 A representative validation curve of real time PCR for *CK18* gene.

(A) The amplification curve for the *CK18* gene.

(B) The dissociation curve of PCR products of *CK18* gene.

A



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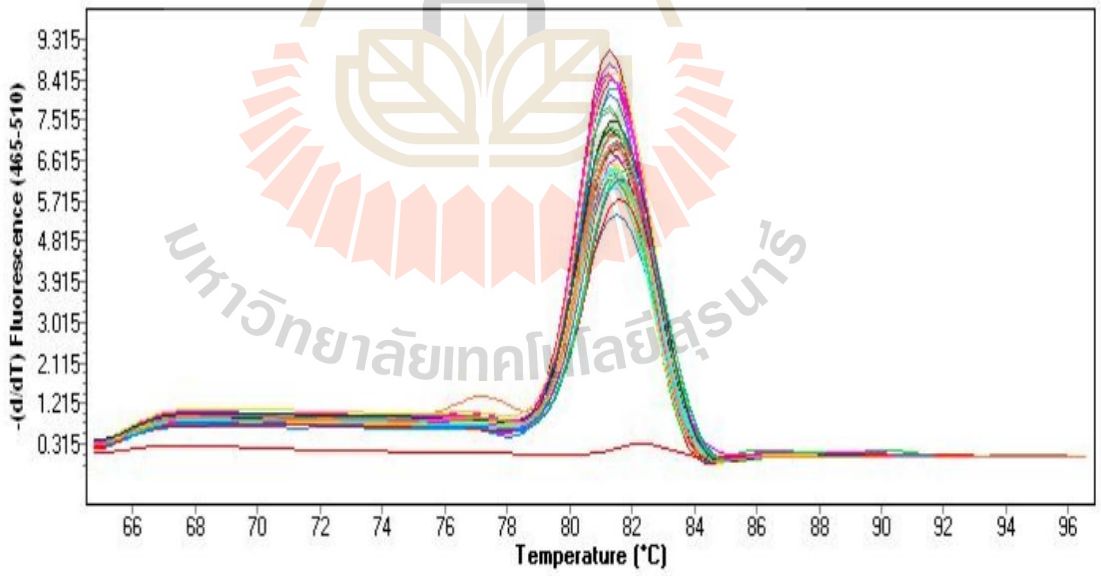
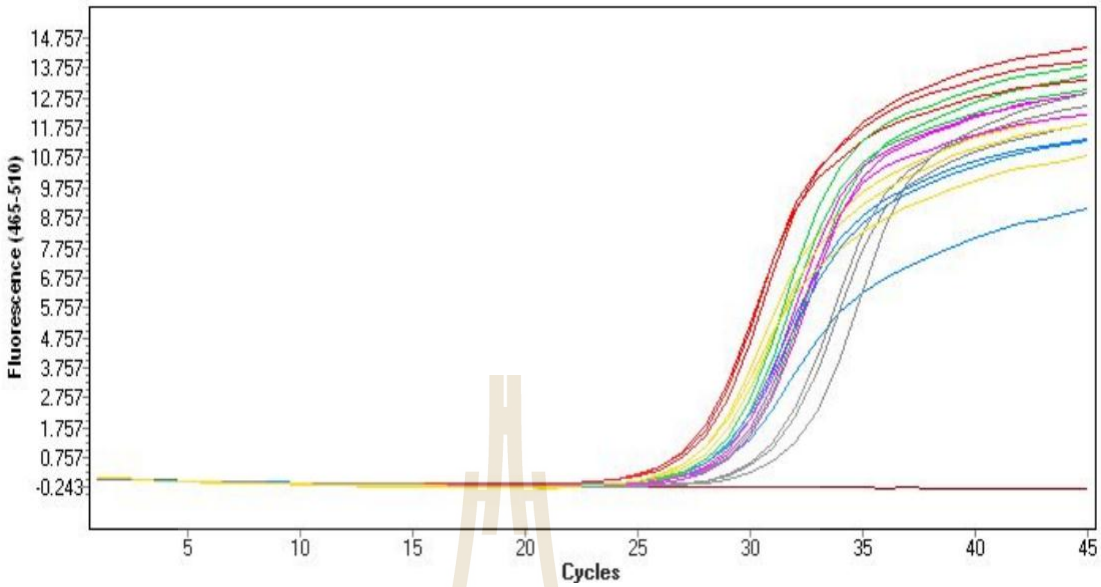


Figure 8 A representative validation curve of real time PCR for *ALB* gene.

(A) The amplification curve for the *ALB* gene.

(B) The dissociation curve of PCR products of *ALB* gene.

A



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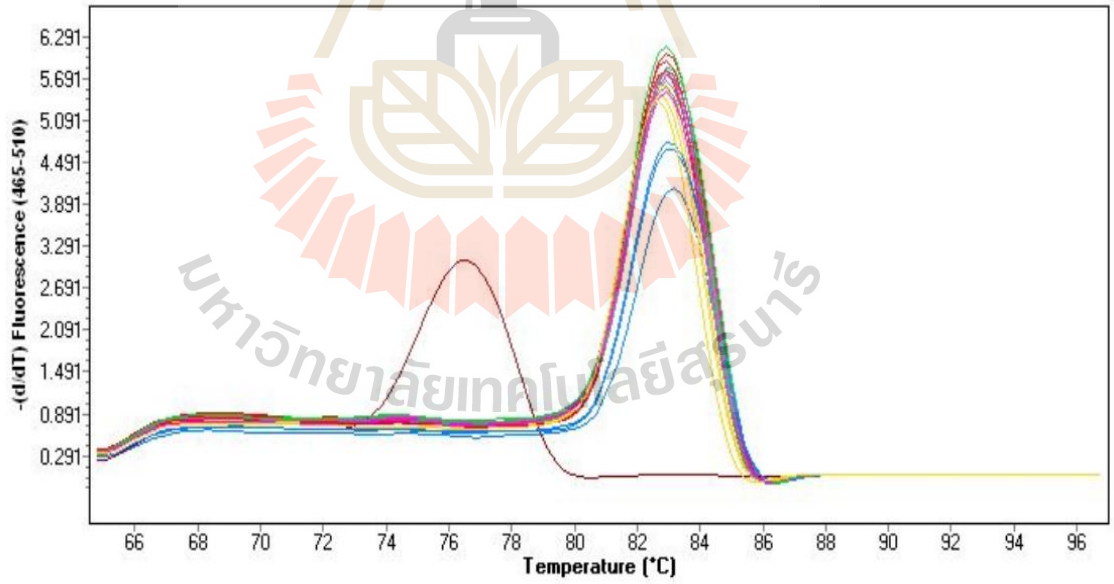

Figure 9 A representative validation curve of real time PCR for *HDAC1* gene.

(A) The amplification curve for the *HDAC1* gene.

(B) The dissociation curve of PCR products of *HDAC1* gene.

APPENDIX C

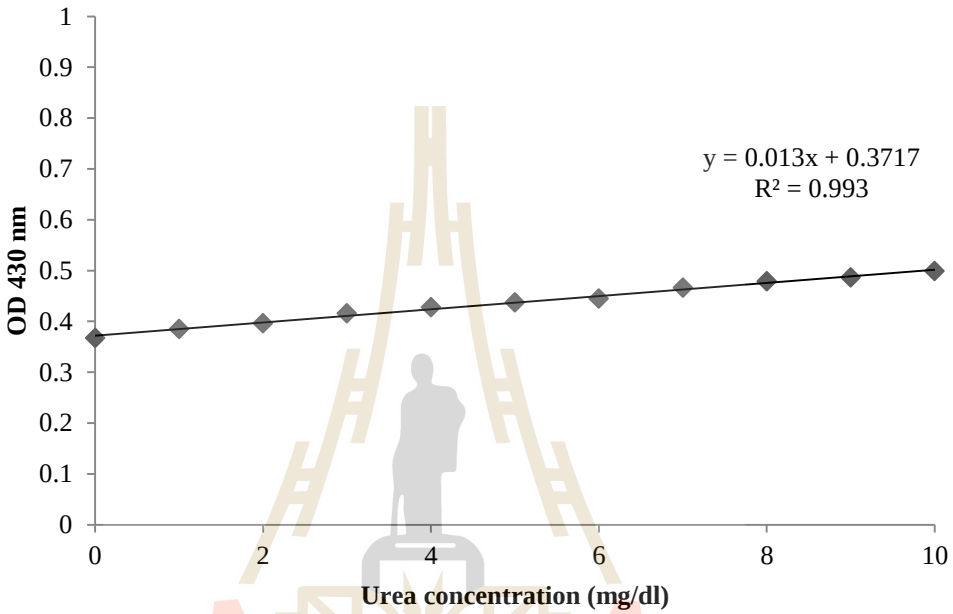

Figure 10 Urea standard curve at 430 nm by colorimetric assay.

Table 3 Urea production of the differentiated hWJ-MSCs with and without NaB pre-treatment groups compared to the control (hWJ-MSCs) group at day 3, 10, 17 and 24 of differentiation (N=3).

Day / Conditions	Urea production (ng/ml/10^6 cell/24 h)			
	3	**10**	**17**	**24**
hWJ-MSCs (Negative control)	0.48±0.33	0.55±0.43	0.54±0.38	0.64±0.51
Hepatogenic medium	1.54±0.41	2.85±0.16	3.82±0.92	4.91±0.46
Hepatogenic medium + NaB pre-treated	1.54±0.49	2.98±0.20	5.64±0.37	6.68±1.26
hHep G2 cells	-	-	-	5.79±1.27

APPENDIX D

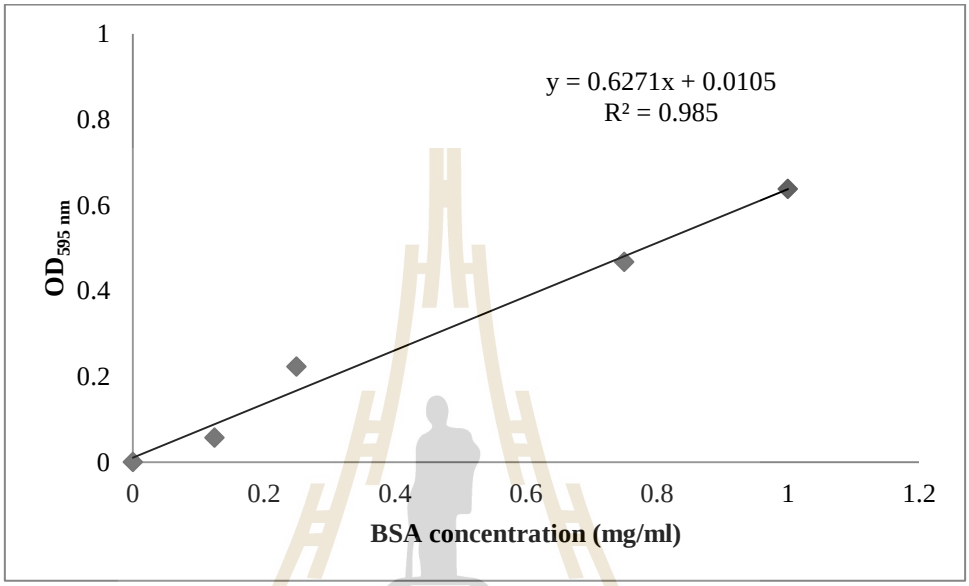

Figure 11 BSA standard curves at 595 nm by Bradford assay.

BIOGRAPHY

Wachira Panta was born in Prachinburi, Thailand on December 6th, 1990. He finished his high school from Prachinkallayanee School in Pracinburi. In 2012, he received a Bachelor Degree (BS) in Medical Science from Burapha University, Chonburi, Thailand. Then, he became interested in stem cell research and its application on medical science. Later on, he decided to obtain Master Degree (MS) in Biotechnology at Suranaree University of Technology, Nakhon Ratchaseema, Thailand. His MS study was supported by Bangkok Stem Cells, Co., Ltd. grant under superadvisor, Assoc. Prof. Dr. Rangsun Parnpai. His MS thesis was the effect of sodium butyrate on hepatogenic transdifferentiation of human Wharton's Jelly-derived mesenchymal stem cells. Parts of this work have been presented at the 7th SUT Stem Cell Conference 2016 on September 10th-11th, 2016 at Suranaree University of Technology, Nakhon Ratchaseema, Thailand.