THE EFFECTS OF HIGH GLUCOSE ON ADIPOGENIC AND OSTEOGENIC DIFFERENTIATION OF MSCS DERIVED FROM BONE MARROW, CHORION, PLACENTA AND UMBILICAL CORD

BY

MISS WEERAWAN HANKAMOLSIRI

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE PROGRAM IN MEDICAL SCIENCES
FACULTY OF MEDICINE
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ABSTRACT

Type 2 diabetes is a disease in which the level of blood glucose is abnormal high due to a decreased responsiveness of patient's own cells to insulin. Previous studies demonstrated that accumulation of visceral fat in the abdominal area increases insulin resistant and increases risk of developing diabetes. Moreover, diabetic patients also have reduced bone mineral density (BMD) leading to an increased risk of osteoporotic fractures. Although, it is known that significant percentages of osteocytes and adipocytes in our body are derived from mesenchymal stem cells (MSCs), the effects of high glucose on the adipogenic and osteogenic differentiation of MSCs is still poorly characterized. Therefore, the present study aims to investigate the effects of high glucose on proliferation as well as adipogenic and osteogenic differentiation of bone marrow and gestational tissue-derived MSCs. MSCs derived from bone marrow (BM-MSCs), chorion (CH-MSCs), placenta (PL-MSCs) and umbilical cord (UC-MSCs) established in this study exhibited typical MSC characteristics and were

able to differentiate to adipocytes and osteocytes under appropriated culture conditions. The results showed that high glucose reduced the proliferation but enhanced adipogenic differentiation of all MSCs examined. The numbers of adipocytes and the extents of Oil Red O staining of MSCs cultured in high glucose condition were greater than those of their normal glucose counterparts. The expression levels of adipogenic genes *PPARɣ*, *ADIPOQ* and *LPL* were also up-regulated when MSCs were cultured in high glucose. In contrast, high glucose did not affect the expression levels of osteogenic gene and the osteogenic differentiation levels of all MSCs examined. In conclusion, our study demonstrated that hyperglycemic condition associated with diabetes upregulated the expression levels of adipogenic gene *PPARɣ*, *ADIPOQ* and *LPL* which enhanced the differentiation of MSCs toward adipocyte-lineages. The increased number of adipocyte like-cells might increase the level of insulin resistance in diabetic patients, and therefore compromise the outcome of their treatment. The knowledge gained from this study will increase our understanding on the mechanism underlying the effects of high glucose on proliferation, adipogenic and osteogenic differentiation of MSCs and might lead to an improvement in the treatment of diabetes and related diseases in the future.

Keywords: glucose, MSCs, proliferation, adipocyte, osteocyte, differentiation

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Weerawan Hankamolsiri

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LIST OF ABBREVIATIONS

Symbols/Abbreviations	Term
%	Percent
/	Per
<	Less than
ADIPOQ	Adiponectin
ALP	Alkaline phosphatase
BM	Bone marrow
BM-MSCs	Bone marrow mesenchymal stem cells
CH	Chorion
CH-MSCs	Chorion mesenchymal stem cells
°C	Degree of Celsius
CD	Cluster of differentiation
cm	Centimeter
CO_2	Carbondioxide
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
et al.	Et alii, and colleagues
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
g	Gram
GLUT4	Glucose transporter type 4
h	Hour
LPL	Lipoprotein lipase
M	Molar or mole per liter (concentration)
mg	Milligram
mg/L	Milligram per liter
mg/mL	Milligram per milliliter
min	Minute
mL	Milliliter

mM	Millimolar
OD	Optical density
OCN	Osteocalcin
OSX	Osterix
PPARγ	Peroxisome proliferator activated receptor gamma
PBS	Phosphate buffer saline
pH	Log concentration of (H^+)-1
PL	Placenta
PL-MSCs	Placenta mesenchymal stem cells
qRT-PCR	Quantitative real time polymerase chain reaction
rpm	Revolution per minute
Runx2	Runt-related transcription factor 2
SEM	Standard error of mean
SREBP-1c	Sterol regulatory element-binding proteins 1c
UC	Umbilical cord
UC-MSCs	Umbilical cord mesenchymal stem cells
µg	Microgram
µL	Microliter
µg/mL	Microgram per milliliter
α	Alpha
β	Beta
γ	Gamma

CHAPTER 1
INTRODUCTION

1.1 Introduction

Diabetes mellitus (DM) is a group of metabolic diseases in which there are high blood sugar levels. Diabetes is caused by either the pancreas cannot produce enough insulin or the patient's cells develop insulin resistant. Diabetic patients are at risk of developing several disabling and life-threatening health problems including, cardiovascular diseases, cerebrovascular diseases, and diabetic retinopathy.

One of the important contributing factor for diabetic development is obesity which is defined as abnormal or excessive fat accumulation in the body. Accumulation of body fat, especially around the waist (abdominal adiposity) causes fat cells in that area to release pro-inflammatory chemicals. The released chemicals were reported to reduce the sensitivity of several cells to insulin leading to the development of type 2 diabetes (1, 2). In addition to type 2 diabetes, obesity also contributed to the development of other debilitating diseases, such as coronary artery disease, high blood pressure, metabolic syndrome and cancer among others. It has been previously reported that adipocytes which are the major cellular parts of fat tissues are derived, at least in part, from somatic stem/progenitor cells called mesenchymal stem cells(3).

Mesenchymal stem cells or mesenchymal stromal cells (MSCs) are multipotent stem/progenitor cells which were isolated for the first time from human bone marrow in 1976 by Friedenstein and colleague(4). MSCs have potential to differentiate to various mesodermal lineages, including bone, cartilage, fat and other marrow stromal connective tissues which play important roles in normal hemopoiesis (5). In previous study reported that 0.001% to 0.01% of mononuclear cells isolated on density gradient gave rise to plastic adherent fibroblast like colonies (6).Apart from the bone marrow, MSCs are also located in the other tissue of the human body such as umbilical cord tissue (7), chorion tissue and placenta tissue (8, 9).MSCs that isolated

from other tissue of human body were a popular choice for preclinical research and clinical trials of variety of diseases (10).

Several recent studies demonstrated that high glucose has negative effect on the proliferative capacity of MSCs derived from bone marrow (BM-MSCs) of rat(11, 12). Moreover, high glucose also increase adipogenic differentiation and lipid accumulation of mouse BM-MSCs (13), osteoblastic MG-63 cell (14, 15) and muscle-derived stem cells (16), which was accompanied by the upregulation of several adipogenic marker genes, including *PPARγ, aP2, resistin, adipsin, SREBP-1C, lipoprotein lipase, adiponectin* and *GLUT4*(13, 14, 16). In contrast to adipogenic differentiation, the osteogenic differentiation and the expressions of osteogenic marker genes, such as *Runx2*, *collagen type I*, *osteonectin* and *osteocalcin* in osteoblastic MG63 cell lines were suppressed by high glucose (12, 14).

Although the effects of high glucose on the proliferation and differentiation of bone marrow-derived MSCs have been previously reported, the effects of high glucose on the biological properties of MSCs derived from other sources, such as chorion, placenta and umbilical cord, as well as the mechanisms underlying those effects are currently unknown. To address those scientific questions, the present study aims to investigate the effects of high glucose on the proliferation, adipogenic differentiation and osteogenic differentiation of MSCs derived from several gestational tissues, including chorion, placenta and umbilical cord, as well as the mechanisms underlying those effects. The knowledge gained from this study may increase our understanding of the pathophysiological mechanism of both diabetes and obesity and might be used to develop novel therapeutic interventions for these debilitating diseases in the near future.

1.2 Objectives

1.2.1 Overall objective

To study the effects of high glucose on adipogenic and osteogenic differentiation of bone marrow and gestational tissue-derived MSCs

1.2.2 Specific objectives

1. To isolate and characterize MSCs from human bone marrow, chorion, placenta and umbilical cord.

2. To study the proliferative capacity of MSCs derived from human bone marrow, chorion, placenta and umbilical cord which are cultured in high glucose condition in comparison to those cultured in normal glucose condition.

3. To study the adipogenic differentiaion capacity of MSCs derived from human bone marrow, chorion, placenta and umbilical cord which are cultured in high glucose condition in comparison to those cultured in normal glucose condition.

4. To study the osteogenic differentiaion capacity of MSCs derived from human bone marrow, chorion, placenta and umbilical cord which are cultured in high glucose condition in comparison to those cultured in normal glucose condition.

5. To study the expression profiles of adipogenic genes in MSCs derived from bone marrow, chorion, placenta and umbilical cord which are cultured in high glucose condition in comparison to those cultured in normal glucose condition.

6. To study the expression profile of osteogenic genes in MSCs derived from bone marrow, chorion, placenta and umbilical cord which are cultured in high glucose condition in comparison to those cultured in normal glucose condition.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Diabetes

Diabetes mellitus is a chronic disease in which levels of blood glucose are elevated due to an insufficient insulin production or an ineffectiveness of the body to use insulin. Insulin deficiency which disturbs carbohydrate, lipid and protein metabolism leads to chronic hyperglycemia. In 2013, approximately 382 million people worldwide were estimated to have diabetes. If the current trends continues, the number of diabetic patients might increase to 592 million by the end of 2035 (17).

2.1.1 Types of Diabetes mellitus

Diabetes mellitus can be categorized into 3 types:

2.1.1.1 Type 1 diabetes

Type 1 diabetes, also called Insulin-dependent diabetes, juvenile diabetes or Immune-mediated diabetes was caused by a cellular-mediated autoimmune destruction of the insulin-producing beta cells of the pancreas and, therefore reduce the patient's ability to produce insulin. The disease, which accounts for 5-10% of all diabetes, mostly affect children or young adults (17, 18).

Although the causes of type I diabetes are still poorly characterized, several autoimmune immune disorders, such as Grave's disease, Hashimoto's thyroiditis and Addison's disease were reported to be associated with type 1 diabetes (18).

2.1.1.2 Type 2 diabetes

Type 2 diabetes, also called non-insulin dependent diabetes or adult-onset diabetes is the most common type of diabetes which accounts for 90-95% of all diabetes. In type 2 diabetes, the body is able to produce insulin but its cells are not responsive to insulin's effects (in another word those cells develop "insulin resistance") leading to high blood glucose.

Most type 2 diabetic patients are obese who have an increased percentage of body fat which is accumulated in the abdominal region. Apart from obesity, there are other important risk factors which are associated with type 2 diabetes, including

- poor diet
- physical inactivity
- advancing age
- family history of diabetes
- ethnicity
- high blood glucose during pregnancy

Most type 2 diabetic patients remain ignorant of their illness for several years due to the slow progression of the disease during which the damages inpatient's organs are slowly built up.

2.1.1.3 Gestational diabetes

Gestational diabetes refers to the pregnant women who develop insulin resistance and high blood glucose during pregnancy. Although gestational diabetes usually disappears after birth, women who have developed gestational diabetes during her pregnancy are at risk of developing type 2 diabetes later in life. Moreover, children born to mothers with gestational diabetes also have a higher chance of developing obesity and type 2 diabetes.

2.1.2 Diabetes complications

Patients with diabetes have an increased risk of developing serious long-term complications affecting several important organs, including:

2.1.2.1 Cardiovascular disease (CVD), which affects heart and vessels, is the most common cause of death and disability among diabetic patients. It is currently well established that diabetes is one of the major risk factors for cardiovascular disease together with hypertension, dyslipidemia and smoking.

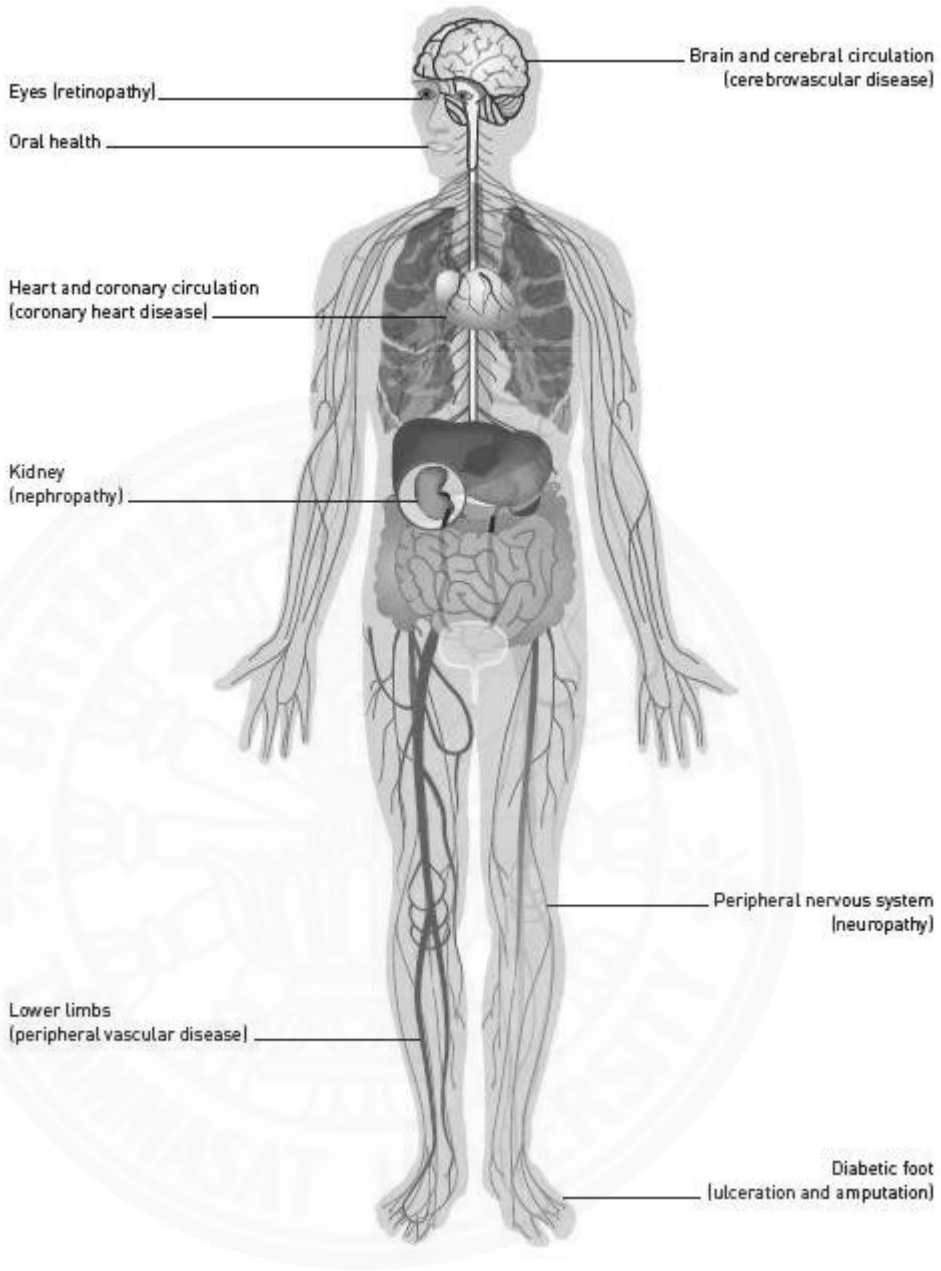

Figure 2.1 The major diabetes complications[17]

2.1.2.2 Retinopathy, which is a leading cause of blindness in diabetic patients, is caused by the persistent injury of blood vessels that nourish retina. The fluid that leaked from those injured vessel cause retinal tissue to swell and in several cases leading to clouding of vision and blindness. High glucose, hypertension and high cholesterol are reported to be the main contributing factors of retinopathy.

2.1.2.3 Nephropathy, which is the degeneration of kidney, is caused by the damage of small blood vessels that innervate kidney which compromise

renal blood supply. Several previous reports indicate that the risk of developing nephropathy in diabetic patients is significantly higher that of general population.

2.1.2.4 Neuropathy, which is the degeneration of nerve tissue, is caused by the damage of small blood vessels that supply nerves. The ischemic/injured nerve cells cannot effectively carry massages between brain and the other parts of body leading to pain, numbness and loss of feeling in several body parts, especially the extremities.

2.1.2.5 Diabetic foot, which is the chronic ulceration affecting feet, is the result of damage to nerves and blood vessels that supply feet. The lack of blood and nerve supply leads to infection and chronic ulceration of feet, which increased risk of amputation.

2.2 Overweight and Obesity

Overweight and obesity, which is the accumulation of excess fat in several bodyparts, play a major role in the pathogenesis of several metabolic and cardiovascular disorders including type 2 diabetes. Currently, obesity has reached epidemic proportions globally, with more than 1 billion adults being either overweight or obese in 2003. In 2013 the number increased to more than 2 billion (19).

For adults, overweight and obesity ranges could be determined and categorized by the body mass index (BMI). Body mass index (BMI) is defined as a person's weight in kilograms divided by the square of height in meter (kg/m^2) (Table 2.1).

Table 2.1 Overweight and obesity ranges

Body mass index (BMI)	Weight category
< 18.5	Underweight
18.5 - 24.9	Ideal weight
25 - 29.9	Overweight
30 - 39.9	Obese
>40	Very obese

However, BMI alone is not the reliable marker for predicting the level of intra-abdominal fat accumulation which increases the risk of metabolic syndrome. The results of computed tomography (CT) scans of two men, whose BMI and amount of total body fat are similar, shows the marked difference in the visceral fat area (VFA) (Figure 2.2). It appears that VFA of one of the subject (top panel) is approximately 50% higher than the other subject (bottom panel), despite the fact that both subjects have similar BMI values (Despres and Lemieux 2006).

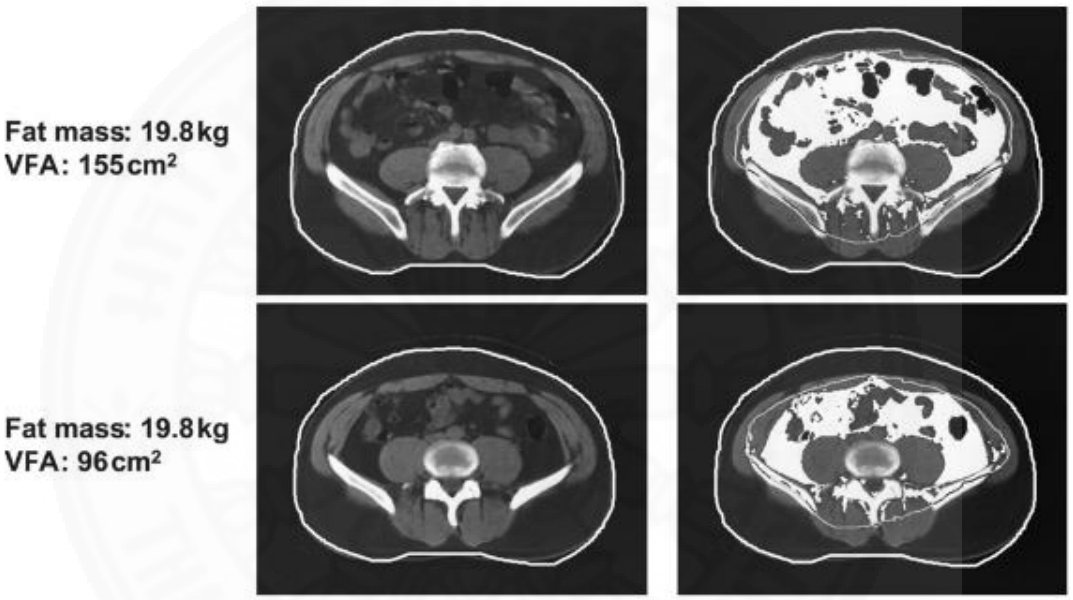

Figure 2.2 Visceral fat area (VFA) of two subjects with comparable BMI [20]

Apart from BMI, waist circumference (WC) has also been used as an indicator for measuring the accumulation of body fat around the waist (abdominal adiposity). The previous study reported that WC values are closely correlated with total abdominal fat mass measured by computer tomography (21). Several previous reports suggest that abdominal fat may present a greater risk of developing hypertension, cardiovascular diseases and diabetes than fat deposited elsewhere in the body. The visceral fat which accumulate in the abdominal area release an increased amounts of fatty acids, glycerol, hormones, pro-inflammatory cytokines and other

factors that involved in the development of insulin resistance leading to the development of type 2 diabetes (22).

2.2.1 Overweight and Obesity-Related Health Problems

Overweight and obesity increase risks of developing several health problems, such as

2.2.1.1 Coronary artery disease (CAD), which is a condition in which a waxy substance called plaque builds up inside the coronary arteries leading to the blockage of coronary arteries and reduces blood flow to the heart muscle causing a heart attack.

2.2.1.2 Persistent high blood pressure which damages endothelial lining of blood vessel and causing atherosclerosis

2.2.1.3 Type 2 diabetes which is caused, at least in part, by the insulin resistant associated with obesity

2.2.1.4 Metabolic syndrome which is a name for group of physical conditions that include diabetes, hypertension and dyslipidemia.

2.3 Osteoporosis

Osteoporosis is an important bone disorder characterized by low bone mass, increased fragility, decreased bone quality, and increased risk of fracture. According to the diagnostic criteria provided by World Health Organization (WHO), osteoporosis is defined by the decreasing of Bone mineral density (BMD) at the hip or lumbar spine to the level less -2.5 SD, as determined by dual energy X-ray absorptionmetry (DXA). The most common fractures are those of the vertebrae (spine), proximal femur (hip) and distal forearm (wrist) (23).

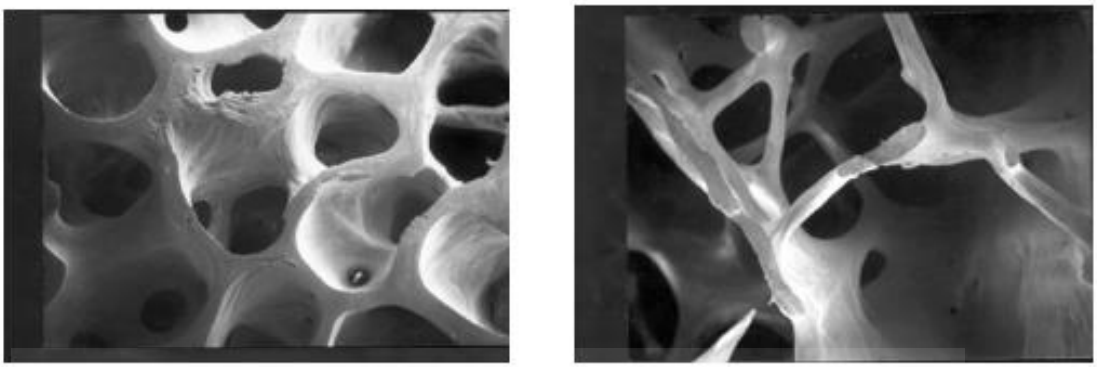

Figure 2.3 Micrographs of normal and osteoporotic bone [24]

Osteoporosis is usually asymptomatic until there is a fracture of the affected bone. The incidence of fractures increases with age, and associated with an increased mortality rate and overall functional decline. Diabetes mellitus has been found to be associated with metabolic bone diseases, osteoporosis and low-impact fracture. In type 1 diabetes, both male and female patients were found to have lower bone mass at hip, femoral neck and spine, which may lead to an increased incidence of bone fractures (25-28). In contrast to type 1 diabetes, the bone disorders associated with type 2 diabetes appear to be controversial. Yamaguchi and colleagues (29) demonstrated that, 187 males with type 2 diabetes, have an average increase in BMD at femoral neck and lower prevalence of vertebral fracture. Similarly, Petit and colleagues (30) reported a higher BMD in elderly patients with type 2 diabetes when compared to age-matched non-diabetic volunteers. On the other hand, Yaturu and colleagues (31) found a significantly lower BMD of hip in type 2 diabetic patients when compared to age-matched non-diabetic volunteers. This result is in agreement with the previous study demonstrated that high glucose concentrations markedly suppressed cell growth, mineralization, and expression of several osteoblast-related markers, including *runt-related transcription factor-2* (*Runx2*), *type I collagen*, o*steocalcin* and *osteonectin* in osteoblast-like MG63 cells (14).

2.4 Mesenchymal stem cells

Mesenchymal stem cells or mesenchymal stromal cells (MSCs) were first discovered by Friedenstien and colleagues in 1970, discovering that single cell suspension of bone marrow is able to generate colonies of adherent fibroblast-like cells when cultured. MSCs can differentiate into bone, cartilage and adipose tissue both *in vitro* and *in vivo.* MSCs are primarily enriched from the bone marrow, which is normally harvested from the upper part of the hip and the iliac crest. However, other populations of MSCs have been found from various tissue including peripheral blood (32), adipose tissue (33), skin tissue (34), trabecular bone (35), umbilical cord blood (36), umbilical cord tissue, amniotic membrane and fluid (7), chorion tissue and placenta tissue (8, 9).

The International Society for Cellular Therapy proposed criteria for defining multi-potent human MSCs: 1) MSCs are adherence to plastic in standard culture condition. 2) MSCs are expressed a specific set of cell surface markers including CD73, CD90 and CD105 together with lack of expression of the hematopoietic markers CD34, CD45, CD14 and human leukocyte antigen (HLA)-DR. 3) MSCs are differentiated into multiple mesenchymal lineages including osteocytes, adipocytes and chondrocytes (37, 38).

2.4.1 Morphology of MSCs

Previous studies explained that MSCs isolated from bone marrow comprise a single phenotypic population forming symmetric, spindle-shape colonies (homology up to 98%). Most recent studies, the single cell derived colonies are morphologically heterogeneous, containing at least two different cell types: small spindle shape cells and large cuboidal or flattened cells. Additionally, the cells which enriched for the small cells had a greater potential for multipotential differentiation than samples enriched for the large cells (39).

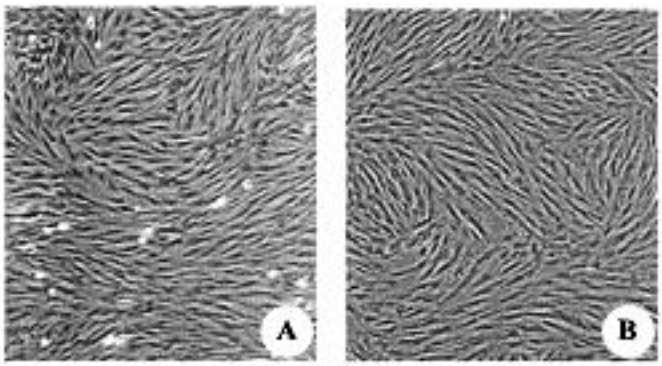

Figure 2.4 Morphology of human mesenchymal stem cells (MSCs) derived from bone marrow A) MSCs at passage 0 B) MSCs at passage 1 (X100) [40]

2.4.2 Differentiation potential of MSCs

In earlier studies, it was shown that MSCs could differentiate to several mesodermal-derived tissues, including osteoblasts, chondrocytes, adipocytes, fibroblasts, and myoblasts (41). In vitro osteogenic differentiation could be achieved by culturing MSCs in medium containing β-glycerophosphate, ascorbic acid and dexamethasone for approximately 2-3 weeks (42). The level of osteogenic differentiation could be assessed by measuring alkaline phosphatase activity and the amount of calcium accumulation (6) as well as the expression levels of osteogenic genes, such as *Runx2*, *Osterix* and *Osteocalcin*. In vitro adipogenic differentiation could be archived by treating MSCs with a hormonal cocktail containing dexamethasone, isobutyl methyl xanthine (IBMX) and indomethacin (43, 44). The level of adipogenic differentiation could be assessed by Oil Red O staining as well as the expression level of adipogenic genes, such as *Peroxisome proliferator activated receptors* ɣ (*PPARɣ*), *lipoproteins lipase* (*LPL*), *adiponectin* (*ADIPOQ*), *glucose transporter 4* (*GLUT4*) and *SREBP-1C* (44).

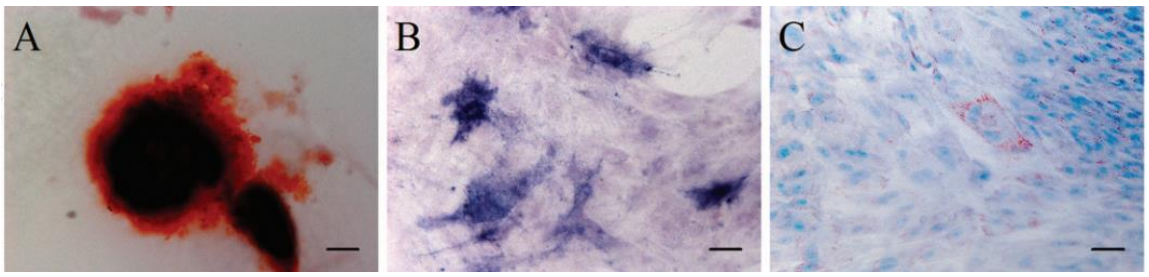

Figure 2.5 Multilineage differentiation potential of bone marrow derived MSCs at passage 3 A) osteogenic differentiation was detected by Alizarin red staining and B) alkaline phosphatase activity C) adipogenesis was indicated by the accumulation of lipid vacuoles using Oil Red O staining
(Scale bars = 100 µm.) [45]

2.5 The effect of high glucose condition on proliferation

Several previous studies suggested that hyperglycemic condition associated with diabetes has negative effects on the proliferation of rat BM-MSCs (11, 12), osteoblastic MG-63 cell (14, 15). Moreover, high glucose has also been reported to induce cellular senescence and increase apoptosis while the reduction of glucose level enhanced proliferation, decreased apoptosis and increased number colonies of forming units of cultured MSCs (46, 47).

Liu and colleague (48) founded that high glucose reduced basal level of VEGF production in rMAPCs by inhibiting JAK/STAT signaling pathway. Similarly, Kuki and colleague (49) demonstrated that hyperglycemia induced EPC senescence by altering the activity of p38 MAPK and JAK/STAT signaling pathways which regulate various cell functions, such as growth factor production, proliferation and apoptosis. In contrast to that study, Brent R. Weil and other (47) reported that the activity of JAK/STAT and p38 MAPK were unaffected by short term incubation with high glucose.

2.6 The effect of high glucose on adipogenic and osteogenic differentiation of MSCs

Adipogenesis involves two major stages: the recruitment and proliferation of pre-adipocytes and the differentiation of pre-adipocytes to adipocytes (50). The previous studies demonstrated that high glucose increased the level of adipogenic differentiation and lipid accumulation in mouse BM-MSCs (13), osteoblastic MG-63 cells (14) and muscle-derived stem cells (16), high glucose can increased adipogenic differentiation. High glucose also up-regulated the expression levels of several adipogenic genes, including *PPARγ, aP2, resistin, adipsin, SREBP-1C*, l*ipoprotein lipase*, *adiponectin* and *GLUT4* (13, 14, 16). On the other hand, high glucose also inhibited osteogenic differentiaton and suppressed the expression of several ostogenic genes, including *Runx2, collagen I, osteonectin* and *ostocalcin*(12, 14, 15) in osteoblastic MG-63 cells and mouse BM-MSCs. The effect of high glucose on the differentiation of MG-63 was shown to be mediated by the activation of cAMP/PKA/ERK pathways (14).

2.7 Adipogenic differentiation

Adipose tissue is consisted of several cell types including adipocytes, pre-adipocytes, fibroblasts, endothelial cells as well as adipose-derived MSCs which can differentiate to adipocyte (3).

Adipogenesis is usually divided into two phases: the determination phase involving the conversion of stem cells to pre-adipocytes and the terminal differentiation phase involving the maturation of pre-adipocytes to mature adipocytes. During the terminal differentiation phase, several genes which play important roles in the process of lipid transport, lipid synthesis, insulin signaling and production of adipocyte-specific protein were upregulated (Figure. 2.6) (3). Information regarding those adipogenic genes and their roles during the process of adipogenesis are reviewed as follows:

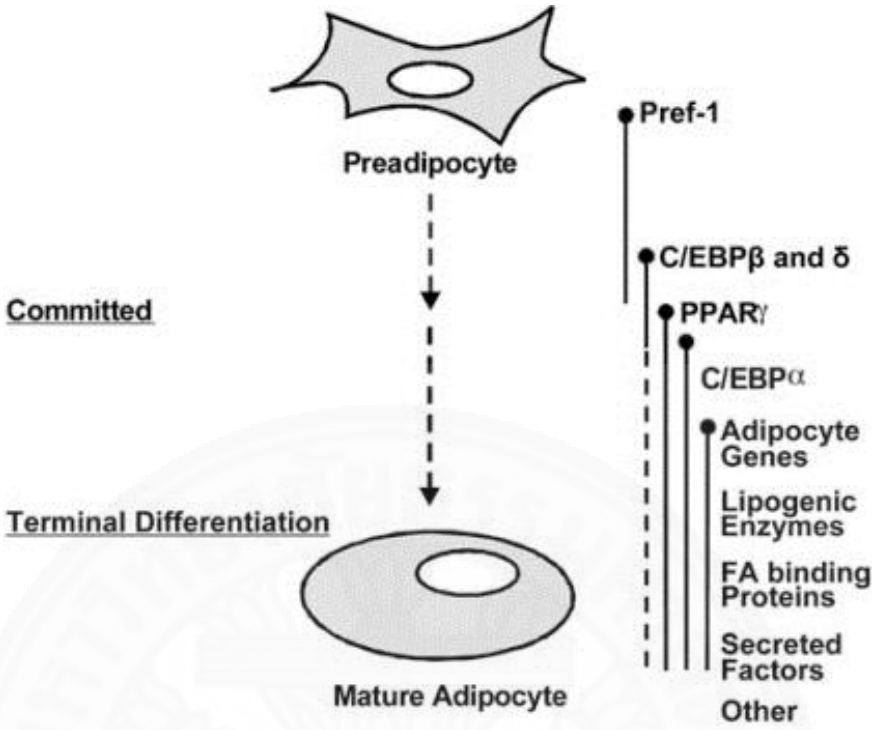

Figure 2.6 Development of mature adipocytes from preadipocytes[51]

2.7.1 Role of PPARγ on adipogenic differentiation

Peroxisome proliferator activated receptors *(PPARs*) are members of the steroid/thyroid hormone receptor gene superfamily (52). *PPARs* interacting with binding sites on targeted gene by forming heterodimers with retinoid X receptors (RXRs) and these heterodimers regulate transcription of various genes (Fig 2.6). *PPARs* have three subtypes including *PPARα, PPARδ,* and *PPARγ*. While *PPARα* and *PPARδ* were expressed during adipogenesis, *PPARγ* was more-specific to adipocytes. The expression of *PPARγ* gene was rapidly up-regulated during an early phase of adipogenesis (53, 54) and its expression is required to maintain the phenotype of mature adipocytes (55). *PPARγ*, which expressed predominantly in adipose tissue and macrophages, played important roles in the regulation of lipid and glucose metabolisms, and was associated with obesity and other related disease (56, 57).*PPARγ* was principally regarded as the master regulator of adipogenesis, for no other factor can rescue adipocyte formation in the event of *PPARγ* knockout, and generally all proadipogenic cell signaling pathway converge to control the expression of *PPARγ*(58). Selective knockout of *PPARγ* in adipose tissue also caused a

significant reduction in adipocyte function and lead to progressive lipodystrophy, insulin resistance and many other metabolic disorders (59). In contrast, the overexpression of *PPARɣ* could directly up-regulate the expression of several adipogenic genes, including *fatty acid synthase, GLUT4, acetyl CoA carboxylase*, adipocyte selective fatty acid binding protein and insulin receptor.

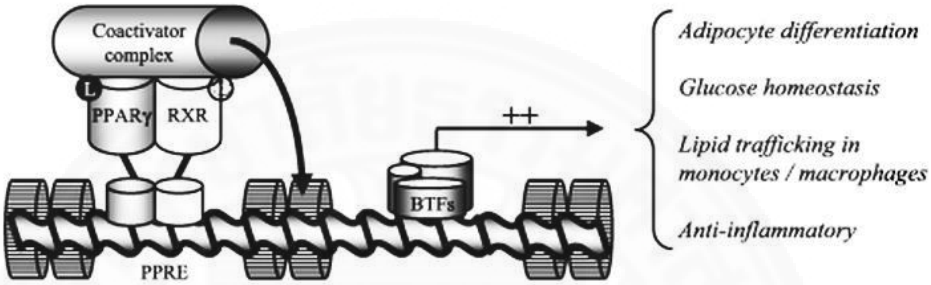

Figure 2.7*PPARγ-RXR* heterodimer promoters [60]

2.7.2 Role of *adiponectin* on adipogenic differentiation

Adiponectin or *ADIPOQ* gene encodes secreted protein which expressed exclusively in adipocytes. *Adiponectin* has been shown to play important roles in the regulation of glucose and lipid metabolism. When secreted, *adiponectin* forms multimeric protein complex either among themselves (Figure 2.8) or with other transcription factor, such as RXR (Figure 2.7) (61), through the formation of di-sulfide bonds (62).

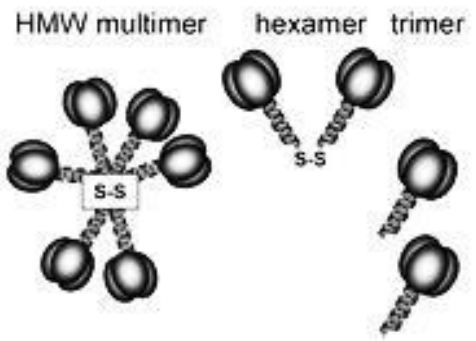

Figure 2.8 Structure of adiponectin [62]

Concentration of *adiponectin* was shown to be reduced in both mice and patients with type 2 diabetes (63-66). Administration of insulin sensitizing, anti-diabetic drug could increase the level of *adiponectin* in type 2 diabetic patients (67). Furthermore, high level of *adiponectin* was also reported to lower the risk of developing type 2 diabetes (68). In agreement with this result, the decrease *adiponectin* levels were reported to increase risk of developing coronary artery disease (CAD) (69).

Fu and colleague (70) demonstrated that 3T3-L1 fibroblasts which overexpressed *adiponectin* gene accumulated lipid earlier during adipogenesis and contained greater amounts of lipid droplets when compared with controls. In addition, *adiponectin* treatment increased AMPK phosphorylation and fatty acid oxidation in the skeletal muscle of mice (71, 72).

2.7.3 Role of *GLUT4* on adipogenic differentiation

Glucose transporter type 4 (*GLUT4*) is the insulin-sensitive glucose transporter which play important roles in the insulin-stimulated glucose uptake by adipose tissue, skeletal muscle and cardiac muscle which specifically express this protein. Insulin regulates glucose uptake of these cells by recruiting membrane vesicles containing *GLUT4* to their surface, where they facilitate glucose uptake by facultative diffusion. Once in the cytoplasm, the glucose was phosphorylated and thereby trapped inside cells. The effect of insulin on *GLUT4* distribution was reversible. Therefore, the level of glucose uptake by muscle and adipocytes could be regulated by modulating the number of *GLUT4* glucose transporters on their surface. Carvalho and colleagues (73) also reported that the insulin-sensitivity of transgenic mice lacking *GLUT4* was decreased while the insulin-sensitivity of transgenic mice overexpressing *GLUT4* was increased. In addition, there is also a report demonstrated that the reduction of GLUT4 expression in obese subjects was directly correlated with the development of insulin resistance (74).

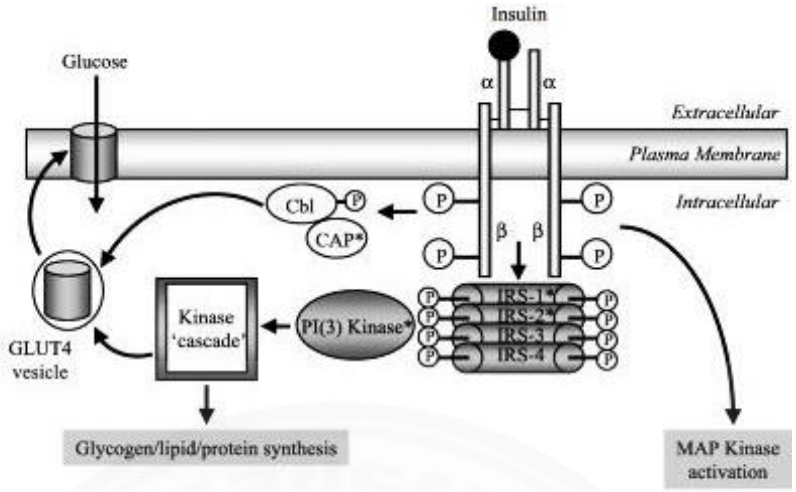

Figure 2.9 Insulin stimulated glucose uptake is mediated by GLUT4 [60]

2.7.4 Role of *SREBP-1C* on adipogenic differentiation

Sterol regulatory element-binding transcription factor 1c (*SREBP*-1c) is basic helix-loop-helix-leucine zipper transcription factors which induces lipid biosynthesis by upregulating the expression of lipogenic genes involving in fatty acid synthesis and acetyl-CoA carboxylase in response to insulin signaling (75). Although *SREBP-1C* is expressed in most tissues of mice and human, it expression level is especially high in the liver, white adipose tissue, adrenal gland, and brain (76). During adipocyte differentiation, the expression of *SREBP-1C* induces *PPARγ* expression (Fig. 2.10) (77). *SREBP-1C* is often associated with *adipocyte determination and differentiation factor 1* (*ADD1*) (76) and their roles in adipogenesis were suggested by the observation that their expression was upregulated during the maturation of pre-adipocyte cell lines (77).

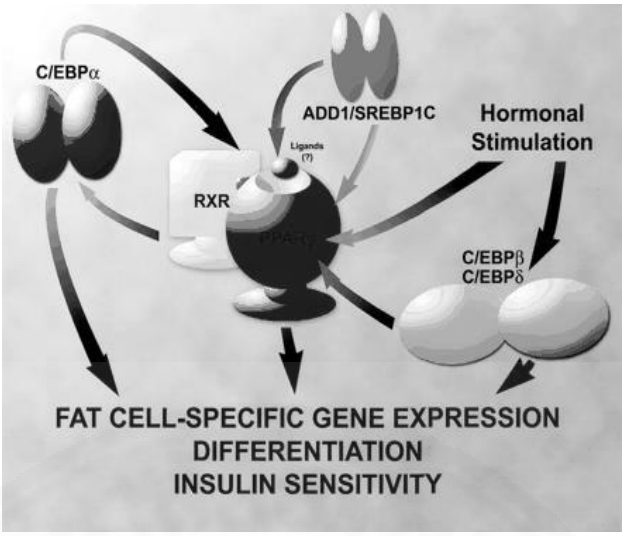

Figure 2.10 The transcriptional control of adipogenesis involves the activation of several families of transcription factors [78]

2.7.5 Role of *Lipoprotein lipase* on adipogenic differentiation

Lipoprotein lipase (*LPL*) is a member of the lipase gene family, which includes pancreatic lipase, hepatic lipase and endothelial lipase. *LPL* is a key enzyme in the metabolism of triglyceride-rich lipoprotein and has been implicated in the pathogenesis of dyslipidemia in conditions associated with insulin resistance. *LPL* gene encodes lipoprotein lipase, which expressed in endothelial cells, cardiomyocytes, muscle, and adipose tissue. *LPL* hydrolyse triglyceride in chylomicrons and very low density lipoproteins (VLDLs) to free fatty acids, which can then be re-esterified and stored in the adipocytes (Fig. 2.10) (79, 80). In addition, *LPL* mRNA expression was a direct correlated with intracellular lipid storage (81). Previous studies in mammalian cell lines and primary cell cultures have demonstrated that *LPL* was an early marker of adipocyte differentiation, since the expression of *LPL* gene was detected in the early stage of adipocyte differentiation and its expression was maintained throughout the entire differentiation process (82, 83). The expression of *LPL* gene in rat adipocytes was regulated by insulin at both transcriptional and post-transcriptional levels (84, 85). Moreover, the previous studies on 3T3-L1 adipocytes also demonstrated that insulin could increase the expression level of *LPL* gene and enhance its activity without affecting transcription of the gene (85, 86).

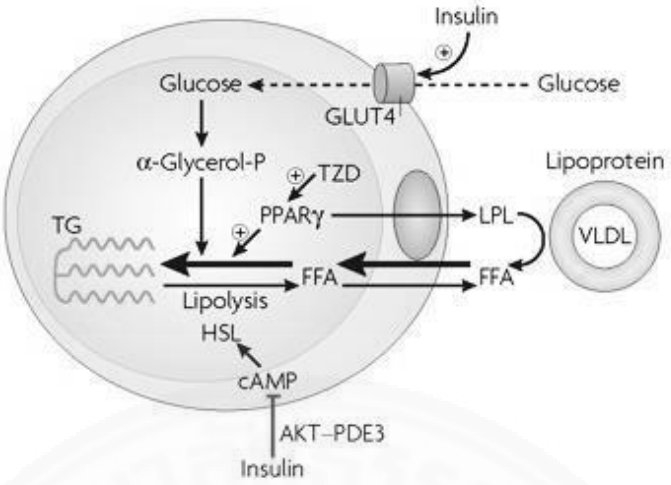

Figure 2.11*LPL* hydrolyses TG from circulating very low density lipoprotein (VLDL), releasing FFA to be re-esterified and stored in the adipocytes [87]

2.8 Osteogenic differentiation

Bone is a composite tissue that consists of organic components, such as type I collagen and non-collageneous proteins, as well as inorganic components which are mostly comprised of hydroxyapatite crystal (88).

Huang and colleague (88) proposed that the osteogenic differentiation of MSCs could be divided into three stages consisted of cell proliferation stage, early differentiation stage characterized by the expression of alkaline phosphatase (ALP) and collagen type I (89) and maturation stage characterized by the up-regulation of osteocalcin and osteopontin, followed by calcium and phosphate deposition (88, 90).

Information regarding the osteogenic genes which play important roles during the process of osteogenesis was reviewed as follows.

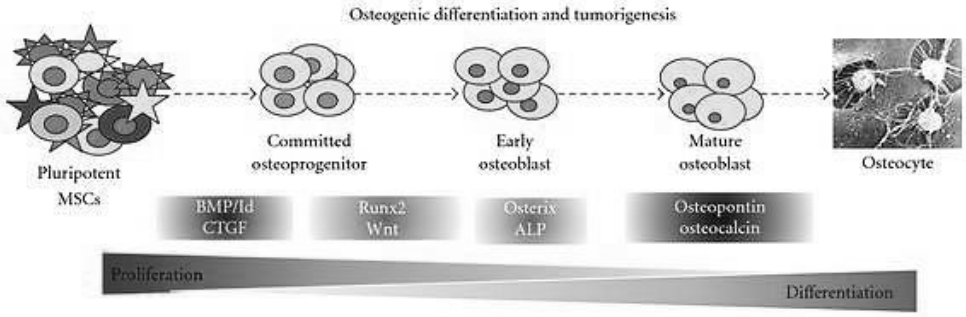

Figure 2.12 Mesenchymal stem cells (MSCs) progress down the osteogenic differentiation cascade [91]

2.8.1 Role of *Runx2* on osteogenic differentiation

The *Runt-related transcription factor (Runx*) family consists of three distinct proteins which are *Runx1*, *Runx2* and *Runx3*. All of them are comprised of a varying α subunit with the same β subunit (92). Members of the *Runx* family have various roles in determining stem cell commitment: *Runx1* determines hematopoietic stem cell differentiation (93), *Runx2* determines osteoblastic and chondrogenic cell differentiation (94). In addition, *Runx3* has roles in epithelial differentiation, neurogenesis, and chondrocyte differentiation (95, 96). To regulate osteogenic differentiation, *Runx2* activates and regulates the targeted genes of many signaling pathways, such as transforming growth factor-beta 1 (TGF-β1), BMP, Wingless type (Wnt), Hedgehog (HH), and (Nel)-like protein type 1 (NELL-1) (97-99). Otto and colleague (100) showed that mice with a homozygous mutation of Cbfa-1 gene failed to generate differentiated osteoblasts and die shortly after birth. The Runx2 null phenotypes cannot be rescued by the overexpression of other osteogenic factors, although the cleidocranial dysplasia-like phenotype of Runx2+/− mice can be partially rescued (101, 102). Although Runx2 is not a key regulator of adipocyte differentiation, its function in promoting osteogenesis may prevent the differentiation of MSCs toward adipocyte-lineage (103).

2.8.2 Role of *Osterix* on osteogenic differentiation

Osterix or *OSX* is a zinc-finger containing transcription factor essential for osteoblast differentiation and bone formation. *OSX* is thought to act in downstream of *Runx2* and regulate the expression of osteoblast including, *osteocalcin, osteonectin, osteopontin, bone sialoprotein* and *collagen type I* (104, 105). Previous studies also reported that the single nucleotide polymorphism (SNP) in *OSX* gene is associated with bone mineral density (BMD) of both children and adults, suggesting that *OSX* may continue to play an important role in postnatal osteogenesis (106, 107). Deletion of *OSX* gene in mouse models results is a complete lack of ossification. Moreover, MSCs of those *OSX* null mice also failed to generate osteoblasts (104, 108). Interestingly, the expression level of *Runx2* gene in the *OSX* null mice remain unaffected indicating that *OSX* may act downstream or independently of *Runx2*(104, 109, 110). Moreover, the deletion of *Runx2* (but with BMP-2 treatment) also has no effect on the expression level of *OSX* gene (111).

2.8.3 Role of Osteocalcin on osteogenic differentiation

Osteocalcin (*OCN)* or bone ɣ-carboxyglutamic acid protein (BGLP) is secreted by cell of osteoblast lineage. It is one of the few osteoblast specific genes and plays important role in the differentiation of osteoblast progenitor cell (112). Previous studies explained that high serum osteocalcin levels are correlated with increase in bone mineral density (BMD) and used as a biochemical marker for bone turnover (113, 114). Clemens and Karsenty (115) demonstrated that insulin signaling in osteoblasts was a positive regulator of both postnatal osteogenesis and bone resorption. It has also been demonstrated that insulin signaling in osteoblasts induced the expression of osteocalcin by increasing osteoclast activity.

CHAPTER 3
METHODOLOGY

3.1 Collection of human specimen

The samples of chorion, placenta and umbilical cord were obtained from pregnant women after normal deliveries at Thammasat Chalermprakiat Hospital.

Bone marrow samples were obtained from sternum or iliac crest of normal healthy volunteers. Subjects which have any clinical history of malignancy, metabolic disorder, or infectious disease, were excluded.

This study was approved by Ethical Committees of the Faculty of Medicine, Thammasat University (No. 141/2555).

3.2 Isolation and culture of bone marrow-derived MSCs

Ten ml of bone marrow aspirated from sternum or iliac crest of normal healthy volunteer was placed in 50 ml sterile tube (Costa, corning, USA) containing 500 µl of heparin (LEO 5,000 i.u./u.i./ml). The aspirate of bone marrow was diluted with equal volume of phosphate-buffer saline (PBS) and was carefully layered over Ficoll-Hypaque solution (GE Healthcare, Bio-science AB, Sweden). After density gradient centrifugation at 100xg (Hettich, Universal 320K, USA) for 30 min at 20°C (break 3), mononuclear cells (MNCs) was removed from the interphase layer, washed twice with 15 ml washing buffer [PBS containing 100 U/ml penicillin and 100 µg/ml streptomycin (GibcoBRL, USA)] and centrifuged at 380xg for 5 min at room temperature. Cell numbers was enumerated using hematocytometer. Bone marrow-derived MNCs were cultured in complete medium [Dulbecco's Modified Eagle's Medium (DMEM; GibcoBRL, USA) containing 10% fetal bovine serum (FBS; GibcoBRL, USA), 2 mM L-glutamine (GibcoBRL, USA), 100 U/ml penicillin and 100 µg/ml streptomycin] at density of $1x10^6$cells/cm^2 in 25-cm^2 tissue culture flasks (Costa, corning, USA). The cells were cultured at 37°C in a humidified tissue culture incubator with 5% carbon dioxide. After 72 h, non-adherent cells were removed and

fresh medium were added to the flasks. The cultures were maintained in this condition with complete change of culture medium every 3-4 days. Culture flasks were observed continuously to get hold of developing colonies of fibroblast like cells. After culture for 7-12 days, the plastic-adherent cells (about 80-90% confluence) were sub-cultured using 0.25% trypsin-EDTA (GibcoBRL, USA) and replated at density of $1x10^4$cell/cm^2 for further expansion. Some batches of continuously sub-culture cells were cryopreserved in freezing medium (90% FBS and 10% DMSO) and stored in liquid nitrogen for future use.

3.3 Isolation and culture of gestational tissue-derived MSCs

Chorion tissue (diameter ~5 cm), placenta tissue (size ~3x3x1 cm) and umbilical cord (length ~2-4 cm)obtained from pregnant women after cesarean section were washed with washing buffer and minced into small pieces of approximately 1-2 mm^2 in size. The tissues were then digested with 1.6 mg/ml collagenase XI (Sigma-Aldrich, USA) and 200 mg/ml deoxyribonuclease I (Sigma-Aldrich, USA) for 4 h at 37°C with shaking. Subsequently, the cells were washed twice with washing buffer and cultured with complete medium in 25cm^2 tissue culture flasks (Costa, corning, USA). The cells were cultured at 37°C in a humidified tissue culture incubator with 5% carbon dioxide. The cultures were maintained at 37°C in a humidified tissue culture incubator with 5% carbon dioxide. The culture medium was changed every 3-4 days. Culture flasks were observed continuously to get hold of developing colonies of fibroblast–like cell. The plastic-adherent cells (about 80-90% confluence) were sub-cultured using 0.25% trypsin-EDTA (GibcoBRL, USA) and replated at density of $1x10^4$cell/cm^2 for further expansion. Some batches of continuously sub-culture cells were cryopreserved in freezing medium (90% FBS and 10% DMSO) and stored in liquid nitrogen for future use.

3.4 Immunophenotypical characterization of MSCs

Primary cultures from bone marrow, chorion, placenta and umbilical cord at passage 2 to 5 were detached with 0.25% trypsin-EDTA and washed twice with PBS. In each sample, $4x10^5$ cells were resuspended in 50 µl of PBS and incubate with 5 µl of fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated antibody against CD34 (BioLegend, USA), CD45 (BioLegend, USA), CD73 (BioLegend, USA), CD90 (BioLegend, USA) or CD105 (BD Bioscience, USA) for 30 min at 4°C in the dark. After washing with PBS, the cells were fixed with 1% paraformaldehyde in PBS. The positive cells were identified by comparison with isotype-match controls [FITC-conjugated mouse immune-globulin G1 (IgG1) and PE-conjugated mouse immunoglobulin G2a (IgG2a)]. At least twenty thousand labeled cells were acquired and analyzed using flow cytometry (FACScalibur™, Becton Dickinson, USA) and CellQuest® software (Becton Dickinson, USA).

3.5 Adipogenic differentiation of MSCs

The adipogenic differentiation potential of chorion, placenta, umbilical cord and bone marrow-derived MSCs was examined using passage 3 to 5 of cultured cells, according to the manufacturer's protocol. Briefly, $7.5x10^4$ cells of MSCs were cultured with MSC growth medium in 35-mm^2 dishes (Costa, Corning, USA). The cells were allowed to adhere overnight. Subsequently, the cells were washed with PBS and 2 ml of adipogenic differentiation medium (in house) as described in appendix C was added. The cells were cultured at 37°C in a humidified tissue culture incubator with 5% carbon dioxide. The medium was changed every 3 days. After of culture, 2 to 4 weeks the cells were washed with PBS and fixed with 40% formalin vapor for 10 min at room temperature. Subsequently, the cells were washed twice with distilled water and stained with 0.5% Oil Red O (Sigma-Aldrich, USA) in 60% isopropanol for 20 min with shaking at room temperature. Subsequently, the cells were washed twice with distilled water and observed under inverted microscope (Nikon TS100, Japan). For control group, the cells were maintained in MSCs growth medium, carried out in pararell to the experiments and stained in the same manner.

3.6 Osteogenic differentiation of MSCs

The osteogenic differentiation potential of chorion, placenta, umbilical cord and bone marrow-derived MSCs was examined using passage 3 to 5 of the cultured cells, according to the manufacturer's protocol. Briefly, $3x10^4$ cells of MSCs were cultured with MSC growth medium in 35-mm^2 dishes. The cells were allowed to adhere overnight. Subsequently, the cells were washed with PBS and 2 ml of osteogenic differentiation medium (in house) as described in appendix C was added. The cells were cultured at 37°C in a humidified tissue culture incubator with 5% carbon dioxide. The medium was changed every 3 days. After 2 to 4 weeks of culture, the cells were washed with PBS and fixed with 4% paraformaldehyde at 4°C for 20 min. The cells were washed with PBS and stained for alkaline phosphatase activity using BCIP®/NBT Liquid substrate (Sigma-Aldrich, USA) for 30 min at room temperature. Subsequently, the cells were washed twice with distilled water and observed under inverted microscope (Nikon TS100, Japan). For control group, the cells were maintained in MSCs growth medium, carried out in pararell to the experiments and stained in the same manner.

3.7 Proliferation assay of cultured MSCs

MSCs (passage3) derived from bone marrow, chorion, placenta and umbilical cord were seeded at $1x10^3$ cells in 24 well plate containing 500 µl of complete medium with or without 25mM D-glucose in triplicates. The cultures were maintained at 37°C in a humidified tissue culture incubator with 5% carbon dioxide for several intervals (0, 2, 4, 6, 8, 10, 12, and 14 days). Subsequently, these MSCs were treated with 0.25% trypsin-EDTA and cell numbers were determined using hematocytometer. The mean numbers of cells were calculated and plotted against culture time to generate a growth curve.

3.8 Determination of adipogenic differentiation of MSCs in high glucose condition

3.8.1 Oil Red O staining assay

MSCs derived from bone marrow, chorion, placenta and umbilical cord (7.5×10^4cells) were cultured in 6-well plates (Costa, Corning, USA) with complete medium and allowed to adhere to the plate overnight. Subsequently, the cells were washed with PBS and 2 ml of adipogenic differentiation medium (in house) was added with or without 25mM D-glucose. The cultures of MSCs in DMEM medium were used control. The number of adipocyte-like cells were quantified on day 14 (BM-MSCs) and day 28 (CH-MSCs, PL-MSCs and UC-MSCs) using Oil Red O staining method. Briefly, the cells were washed with PBS and fixed with 40% formalin vapor for 10 min at room temperature. Subsequently, the cells were washed twice with distilled water and stained with 0.5% Oil Red O (Sigma-Aldrich, USA) in 60% isopropanol for 20 min with shaking at room temperature and then washed twice with distilled water. The number of adipocyte-like cells was counted under inverted microscope (Nikon TS100, Japan). To measure the amount of Oil Red O staining in each sample, the Oil Red O presented in each stained culture plates were extracted by incubation with 1ml isopropanol for 5 minute at room temperature with shaking. The optical density (OD) of each extracted Oil Red O sample was measured by microplate reader at the wavelength of 500 nm. The amount of extracted Oil Red O in each sample were calculated by comparing the measure OD values against a standard curve generated from 0 µg/ml to 500 µg/ml of Oil Red O (Appendix A).

3.9 Determination of osteogenic differentiation of MSCs in high glucose condition

3.9.1 Alkaline phosphatase activity assay

MSCs derived from bone marrow, chorion, placenta and umbilical cord (3×10^4cells) were cultured in 6-well plates (Costa, Corning, USA) with complete medium and allowed to adhere to the plate overnight. Subsequently, the cells were washed with PBS and 2 ml of osteogenic differentiation medium (in house) were

added with or without 25mM D-glucose. The cultures of MSCs in DMEM medium were used as control. After 7 days and 14 days of culture, the cells were washed with PBS and fixed with 4% paraformaldehyde at 4°C for 20 min. The cells were washed with PBS and stained for alkaline phosphatase activity using BCIP®/NBT Liquid substrate (Sigma-Aldrich, USA) for 30 min at room temperature. Subsequently, the cells were washed twice with distilled water and observed under inverted microscope (Nikon TS100, Japan). For control group, the cells were maintained in MSCs growth medium, carried out in parallel to the experiments and stained in the same manner.

3.10 Determination of the expression levels of adipogenic and osteogenic genes

3.10.1 RNA extraction

MSCs derived from bone marrow, chorion, placenta and umbilical cord (2×10^5cells) were cultured in 25cm^2 tissue culture flasks (Costa, Corning, USA) with complete medium and allowed to adhere to the plate overnight. The medium was removed and adipogenic or osteogenic differentiation medium was added. In high glucose condition, 25mM D-glucose was added to differentiation medium. The cultures of MSCs in DMEM medium were used as control.

In adipogenic differentiation, after 14 days in BM-MSCs and 28 days in CH-MSCs, PL-MSCs and UC-MSCs after incubation, total RNA were extracted from the cultured MSCs using PureLink™ RNA Mini Kit (Invitrogen, USA). In case of osteogenic differentiation, total RNA were extracted from BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs after incubated for 7 days and 14 days by PureLink™ RNA Mini Kit (Invitrogen, USA) following the manufacturer's instruction. Briefly, MSCs from each experiment were detached using 0.25% trypsin-EDTA and transferred to 1.5 ml RNase-free tube. To lyse the cells, 300 µl of lysis buffer containing 1% 2-mercaptoethanol were added and mixed with an RNase-free pipetted tip. Subsequently, one volume of 70% ethanol was added to each volume of cell homogenate and vortexed to mix thoroughly. The samples were then transferred to the spin cartridges (with the collection tubes) and centrifuged at 12,000xg for 15 s at room temperature. After discarding the flow-through, 700 µl of wash buffer I were added and then centrifuged at 12000xg for 15 s at room temperature. The spin

cartridges were placed into the new collection tube and added 500 µl of wash buffer II with ethanol to the spin cartridges. The spin cartridges centrifuged at 12,000xg for 15s at room temperature and the flow-through was discarded. The spin cartridges were reinserted into the same collection tubes and centrifuged at 12,000xg for 2 min to dry the membranes (with attached RNA). The spin cartridges were inserted into a recovery tube. To elude the RNA from the membrane, 50 µl of RNase-free water were added to the center of the spin cartridges. After incubating at room temperature for 1 min, the spin cartridges were centrifuged at 12,000xg for 2 min at room temperature. The purified RNA were collected and stored at -80°C for further processes.

3.10.2 First-strand cDNA synthesis

Messenger RNA was reverse transcribed to cDNA using SuperScript® III First Strand Synthesis Kit (Invitrogen, USA) following the manufacturer's instruction. Briefly, RNA/primer mixture containing 1 µg of total RNA, 1 µl of 50 µM oligo (dT), 1 µl of 10 mM dNTP mix and DEPC-treated water to 10 µl were prepared. The mixture was then be incubated on MyCycler Thermal cycler (Bio-Rad, USA) at 65°C for 5 min and placed on ice for at least 1 min to stop the reaction. Subsequently, cDNA Synthesis Mix containing 2 µl of 10X RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl of RNase OUT ™ (40 U/µl) and 1 µl of SuperScript ™ III RT (200U/µl) were added to RNA/primer mixture and incubated on Thermal cycler (Bio-Rad , USA) at 50°C for 50 min were be incubated at 4°C for 1 min. cDNA synthesis reactions were stored at -20°C or used for qRT-PCR immediately.

3.10.3 Quantitative real-time polymerase chain reaction (qRT-PCR)

The expression levels of adipogenic gene including *PPARɣ*, *SREBP-1c*, *ADIPOQ, GLUT4* and *LPL* or osteogenic genes including*Runx2*, *osterix*, and *osteocalcin* were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Primer sequences were shown in Table 3.1.

Quantitative real-time PCR samples were prepared using a SYBR® Green PCR Master Mix (Applied Biosystems, USA). The amplification was performed on a Step one plus™ Real-Time PCR system (Applied Biosystems; ABI USA) using 40 cycles of amplification (denaturation at 95°C for 10 s, annealing at 60°C for *10 s* and extension at 72°C for 20 s) after an initial activation at 95°C for 10 min. Melting curves were assessed to ensure that single products were quantified. Each sample was examined in duplicate and mean value was calculated. The quantitation was based on normalizing the gene of interest to the invariant control gene (*GAPDH*). The data were analyzed by comparative ΔΔCT method using StepOne ™ Software version 2.2 (Applied Biosystems; ABI, USA.) and presented as the relative mRNA level.

Table 3.1 The primers and the product size of adipogenic and osteogenic genes

Gene	Forward primer	Reverse primer	Product size
ADIPOQ	5'-CCTGGTGAGAAGGGTGAGAA-3'	5'-CAATCCCACACTGAATGCTG-3'	174
GLUT4	5'-CTTCGAGACAGCAGGGGTAG-3'	5'-ACAGTCATCAGGATGGCACA-3'	168
LPL	5'-TCAACTGGATGGAGGAGGAG-3'	5'-GGGGCTTCTGCATACTCAAA-3'	169
PPARγ	5'-GACCACTCCCACTCCTTTGA-3'	5'-AGGCTCCACTTTGATTGCAC-3'	168
SREBP1C	5'-TTCTCACCTCCCAGCTCTGT-3'	5'-GGAGGCTTCTTTGCTGTGAG-3'	173
Runx2	5'-GACAGCCCCAACTTCCTGT-3'	5'-CCGGAGCTCAGCAGAATAAT-3'	159
Osteocalcin	5'-CTCACACTCCTCGCCCTATT-3'	5'-TCAGCCAACTCGTCACAGTC-3'	245
Osterix	5'-TGCTTGAGGAGGAAGTTCAC-3'	5'-CTGCTTTGCCCAGAGTTGTT-3'	114
GAPDH	5'-CAATGACCCCTTCATTGACC-3'	5'-TTGATTTTGGAGGGATCTCG-3'	159

3.11 Statistical analysis

The data was presented as mean ± standard error of mean (SEM). Statistical comparisons were performed using the unpaired T-test. *P*-value of less than 0.05 was considered to be statistically significant.

CHAPTER 4
FINDING

4.1 Characteristics of MSCs derived from bone marrow and gestational tissues

4.1.1Morphology of MSCs derived from bone marrow and gestational tissues

To study the morphology of MSCs generated from bone marrow and gestational tissues, MSCs were isolated from bone marrow, chorion, placenta and umbilical cord. The MSCs were expanded in culture and their morphology was determined by light microscopy.

4.1.1.1 Morphology of bone marrow-derived MSCs (BM-MSCs)

Bone marrow mononuclear cells (BM-MNCs) were isolated from bone marrow as described in Section 3.2. Three days after initial seeding, some BM-MNCs attached to the plastic culture surface and formed small clusters of adherent spindle-shaped cells (Figure 4.1A). At culture day 7, these adherent spindle-shaped cells (Figure 4.1B) which exhibited typical MSC morphology rapidly proliferated and reached 80% confluence within 2 weeks. These BM-MSCs were successfully subcultured and expanded for several passages without losing their typical MSC morphology (Figure 4.1C and D)

4.1.1.2 Morphology of chorion-derived MSCs (CH-MSCs)

Pieces of chorion tissues were processed to isolate CH-MSCs as described in section 3.3. After cultured for 3 days, several clusters of adherent spindle shaped cells were observed (Figure 4.2A). At culture day 7, these adherent spindle-shaped cells (Figure 4.2B) which exhibited typical MSC morphology rapidly proliferated and reached 80% confluence within 3 weeks. These CH-MSCs were successfully subcultured and expanded for several passages without losing their typical MSC morphology (Figure 4.2C and D)

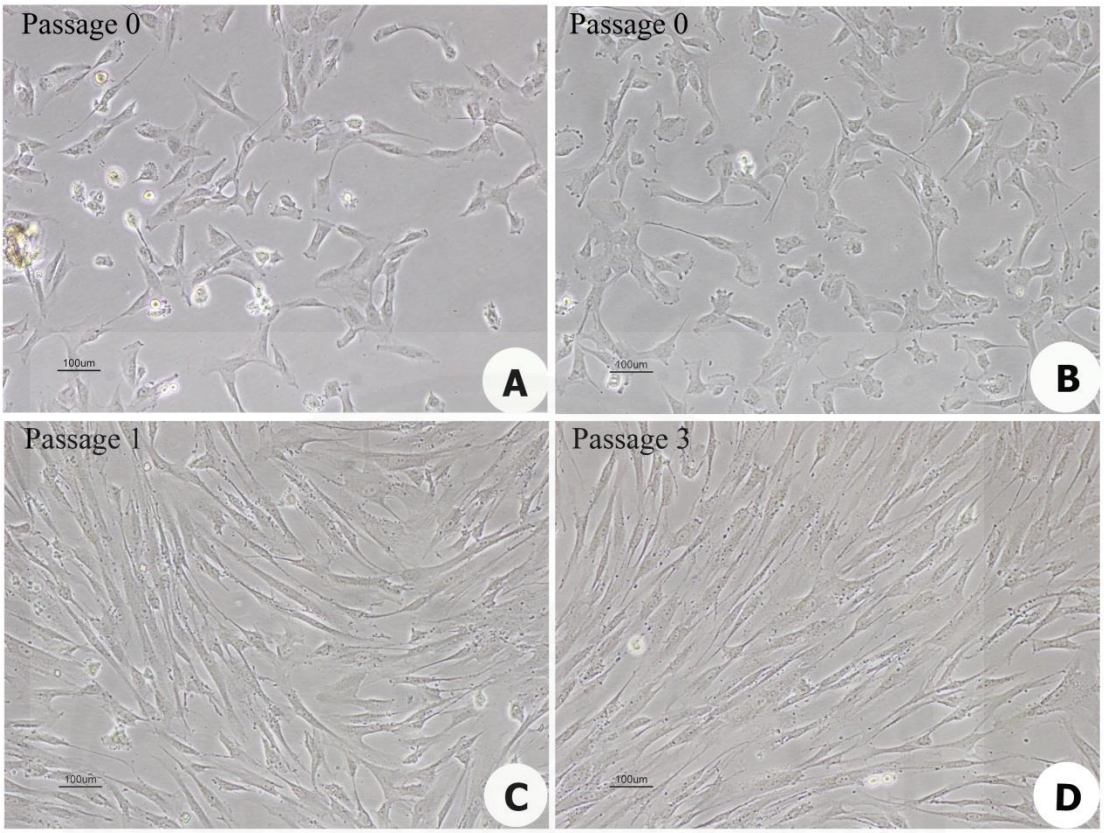

Figure 4.1 Morphology of bone marrow-derived MSCs (BM-MSCs)

Figure shows the morphology of adherent BM-MNCs after cultured for 3 (A) and 7 days (B) as well as the morphology of BM-MSCs after being subcultured for 1 (C) and 3 passages (D)

Scale bar = 100 µm.

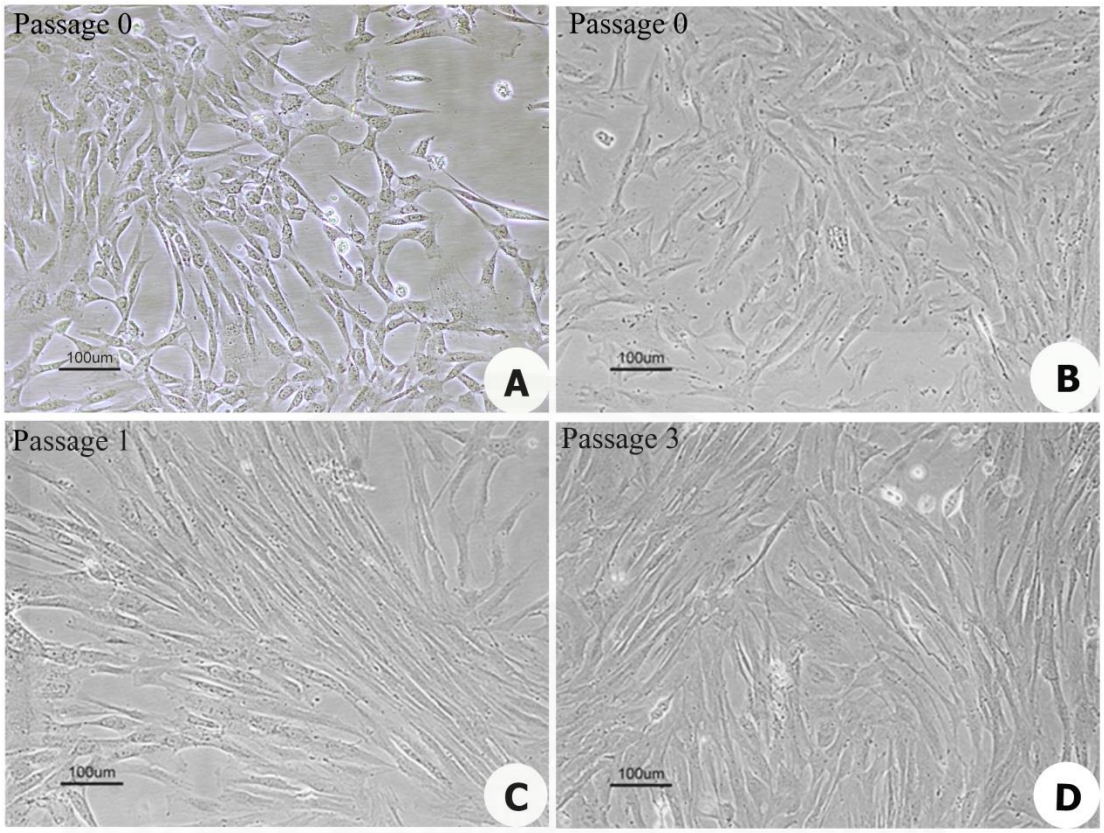

Figure 4.2 Morphology of chorion-derived MSCs (CH-MSCs)

Figure shows the morphology of adherent chorion cells after cultured for 3 (A) and 7 days (B) as well as the morphology of CH-MSCs after being subcultured for 1 (C) and 3 passages (D)

Scale bar = 100 µm.

4.1.1.3 Morphology of placenta-derived MSCs (PL-MSCs)

The placental tissues were collected and processed to isolate PL-MSCs as described in section 3.3. Three days after initial seeding, some placental cells attached to the plastic culture surface and exhibited short, spindle-shaped morphology (Figure 4.3A). At culture day 7, these adherent spindle-shaped cells (Figure 4.3B) which exhibited typical MSC morphology rapidly proliferated and reached 80% confluence within 2 weeks. These BM-MSCs were successfully subcultured and expanded for several passages without losing their typical MSC morphology (Figure 4.3C and D)

4.1.1.4 Morphology of umbilical cord-derived MSCs (UC-MSCs)

Short sections of umbilical cords were processed to isolate UC-MSCs as described in section 3.3. After cultured for 4 days, some umbilical cord cells adhered to culture surface and exhibited spindle-shaped morphology (Figure 4.4A). At culture day 7, the adherent spindle-shaped cells (Figure 4.4B) which exhibited typical MSC morphology rapidly proliferated and reached 80% confluence within 3 weeks. These UC-MSCs were successfully subcultured and expanded for several passages without losing their typical MSC morphology (Figure 4.4C and D)

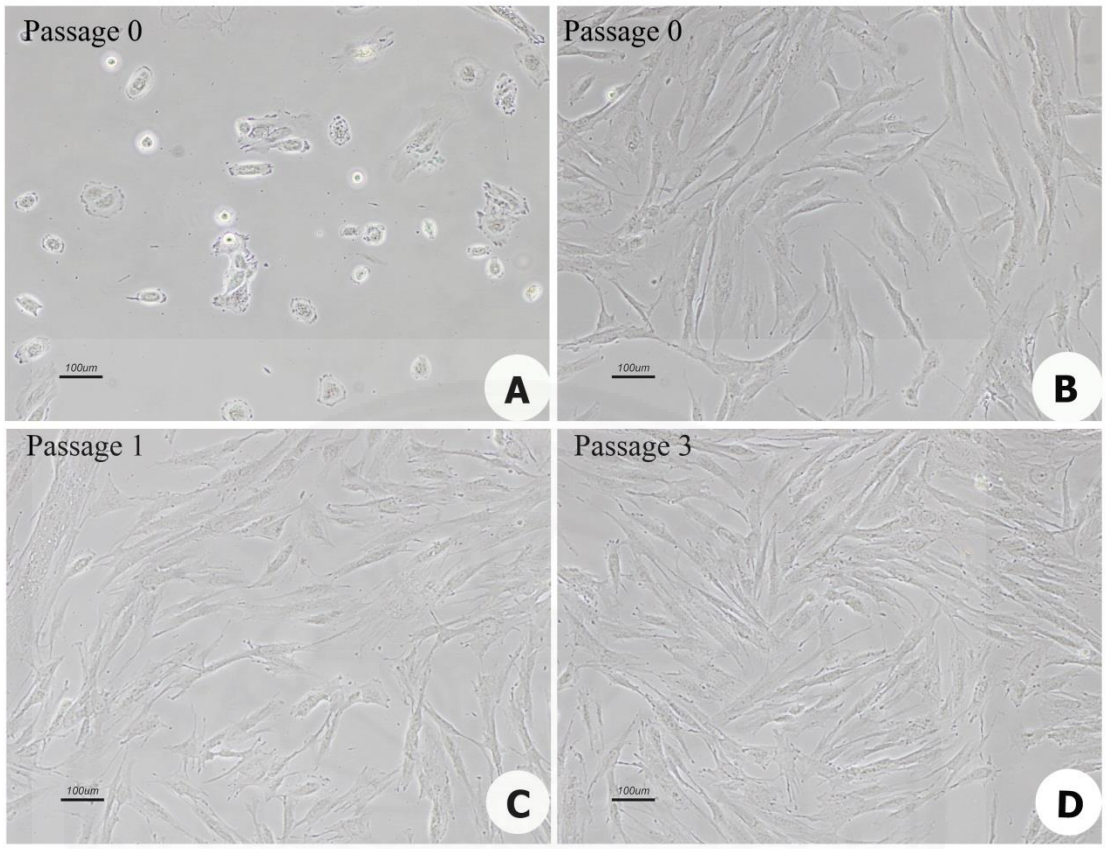

Figure 4.3 Morphology of placenta-derived MSCs (PL-MSCs)

Figure shows the morphology of adherent placental cells after cultured for 3 (A) and 7 days (B) as well as the morphology of PL-MSCs after being subcultured for 1 (C) and 3 passages (D)

Scale bar = 100 µm.

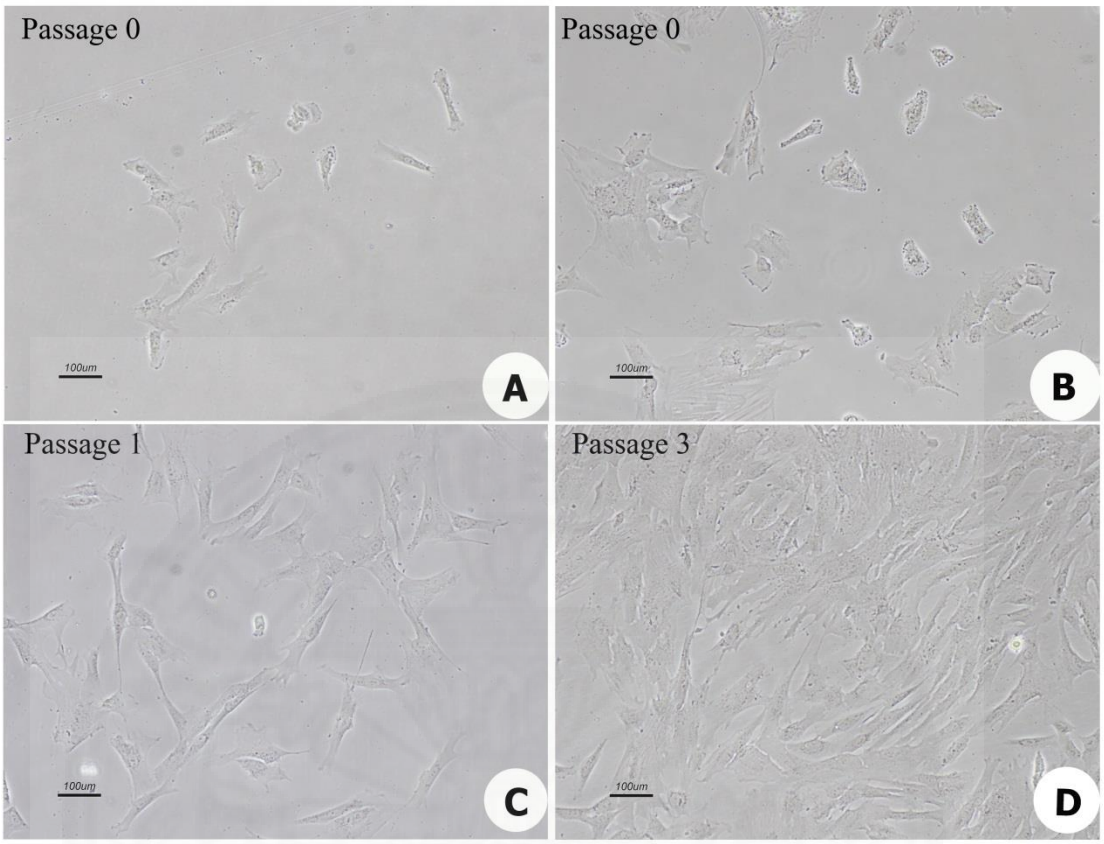

Figure 4.4 Morphology of umbilical cord-derived MSCs (UC-MSCs)

Figure shows the morphology of adherent umbilical cord cells after cultured for 3 (A) and 7 days (B) as well as the morphology of UC-MSCs after being subcultured for 1 (C) and 3 passages (D)

Scale bar = 100 µm.

4.1.2 Immunophenotypes of BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs

BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs were harvested by trypsinization as described in section 3.4 to analyze the expression profile of typical mesenchymal and hematopoietic markers including CD73, CD90, CD105, CD34, and CD45 by FACS calibur™ Flow cytometer using CellQuest™ software (Becton Dickinson, USA) as described in section 3.4. The results showed that BM-MSCs, CH-MSCs, UC-MSCs, and PL-MSCs expressed typical MSC markers CD73, CD90, CD105 but did not express hematopoietic markers CD34, CD45 (Table 4.1) (Figure 4.5).

Table 4.1 The surface marker expression profiles of MSCs from various sources

Percentages of the positive cells					
MSC sources	CD34	CD45	CD73	CD90	CD105
BM-MSCs	2.11±1.44	1.11±0.94	89.29±4.46	88.53±4.90	87.08±5.06
CH-MSCs	1.93±0.95	2.11±0.36	93.64±2.19	83.46±5.62	90.56±3.12
PL-MSCs	0.89±0.45	1.38±0.57	92.35±2.43	81.87±5.66	89.02±2.99
UC-MSCs	1.96±0.64	1.73±0.89	85.27±3.63	84.91±4.66	80.70±0.74

Data are presented at mean ± standard error of means (SEM) from 3 independent experiments

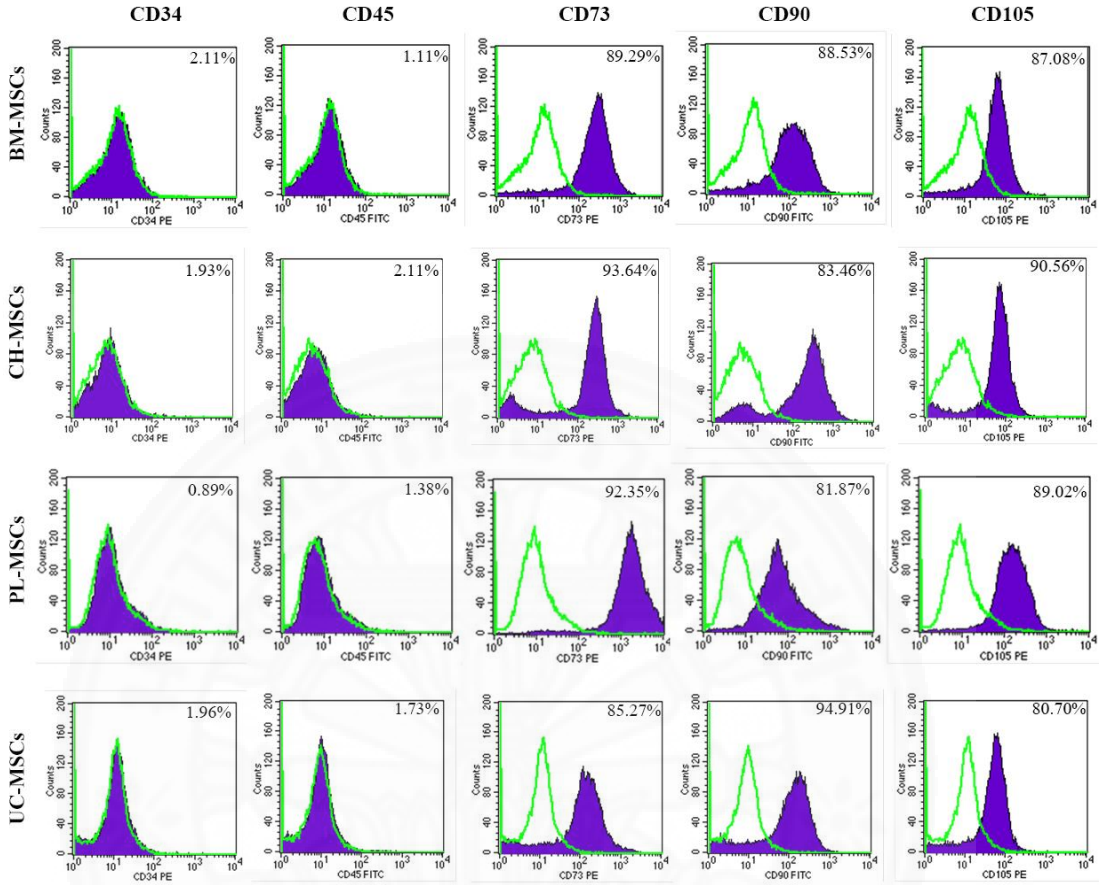

Figure 4.5 Immunophenotypes of BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs

Bone marrow and gestational tissue-derived MSCs between passage three to five were stained with mesenchymal and hematopoietic markers including CD73, CD90, CD105, CD34, and CD45. The green lines indicate isotype-matched mouse IgG antibody control staining. Bone marrow and gestational tissue-derived MSCs were positive for typical MSC markers CD73, CD90, CD105 and negative for hematopoietic markers CD34 and CD45.

4.1.3 Adipogenic differentiation potential of MSCs

To assess their adipogenic differentiation potentials, MSCs were harvested by trypsinization and were cultured in adipogenic differentiation medium as described in section 3.5. After cultured for 2 weeks, BM-MSCs changed their morphology from spindle-shaped cells to become adipocyte-like cells which are large round cells containing several lipid droplets in their cytoplasm which were positive for Oil Red O staining (Figure 4.6). Similar to BM-MSCs, CH-MSC, PL-MSCs, and UC-MSCs also showed the appearance of adipocyte-like cells which were positive for Oil Red O staining after cultured in adipogenic differentiation medium as show in figure 4.7-4.9. However, CH-MSC, PL-MSCs, and UC-MSCs took a longer period of time to differentiate toward adipocytes in comparison to BM-MSCs.

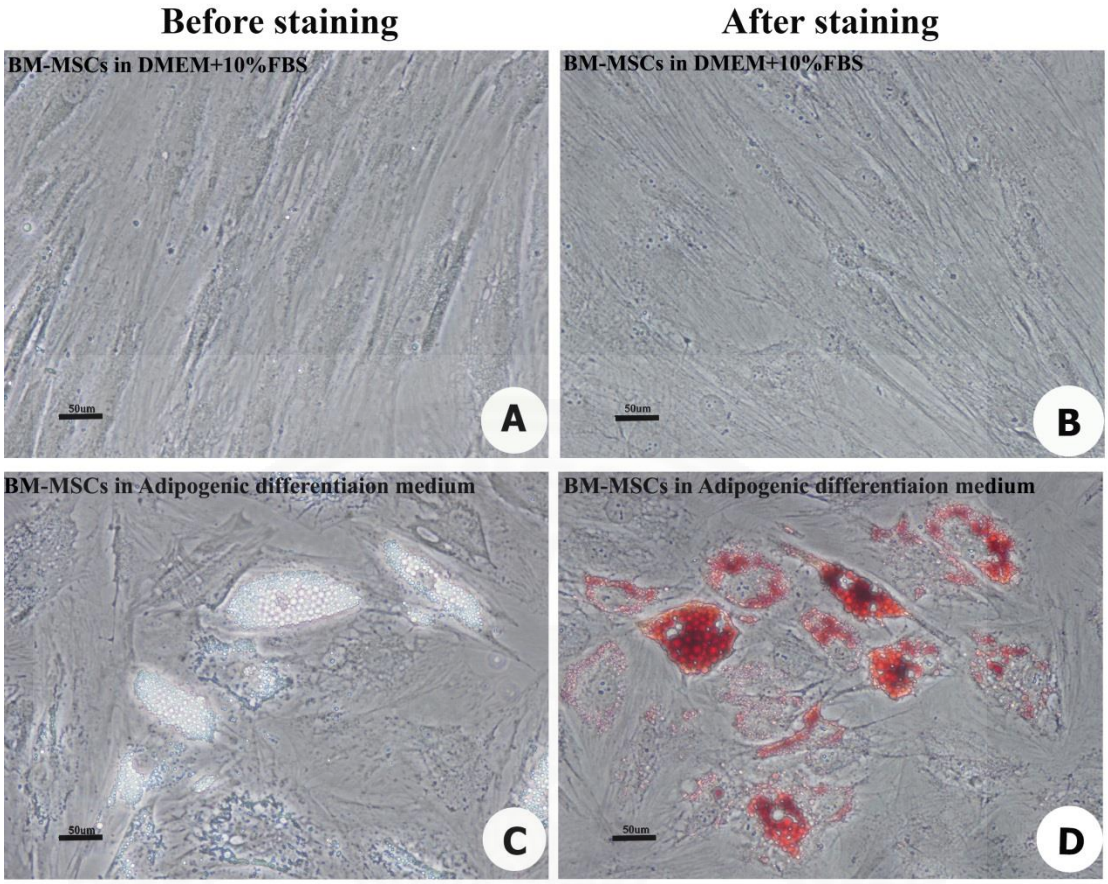

Figure 4.6 Adipogenic differentiation of BM-MSCs

BM-MSCs were cultured in adipogenic differentiation medium. After cultured for 2 weeks, the cells were stained with Oil Red O as described in section 3.5to determine the number of adipocytes.

A. Morphology of BM-MSCs after culture in DMEM supplemented with 10% FBS for 3 weeks before Oil Red O staining
B. Morphology of BM-MSCs cultured in DMEM supplemented with 10% FBS for 3 weeks after Oil Red O staining
C. Morphology of adipocyte-like cells derived from BM-MSCs which were cultured in adipogenic differentiation medium for 3 weeks before Oil Red O staining
D. Morphology of adipocyte-like cells derived from BM-MSCs which were cultured in adipogenic differentiation medium for 3 weeks after Oil Red O staining

   Scale bar: 50 µm

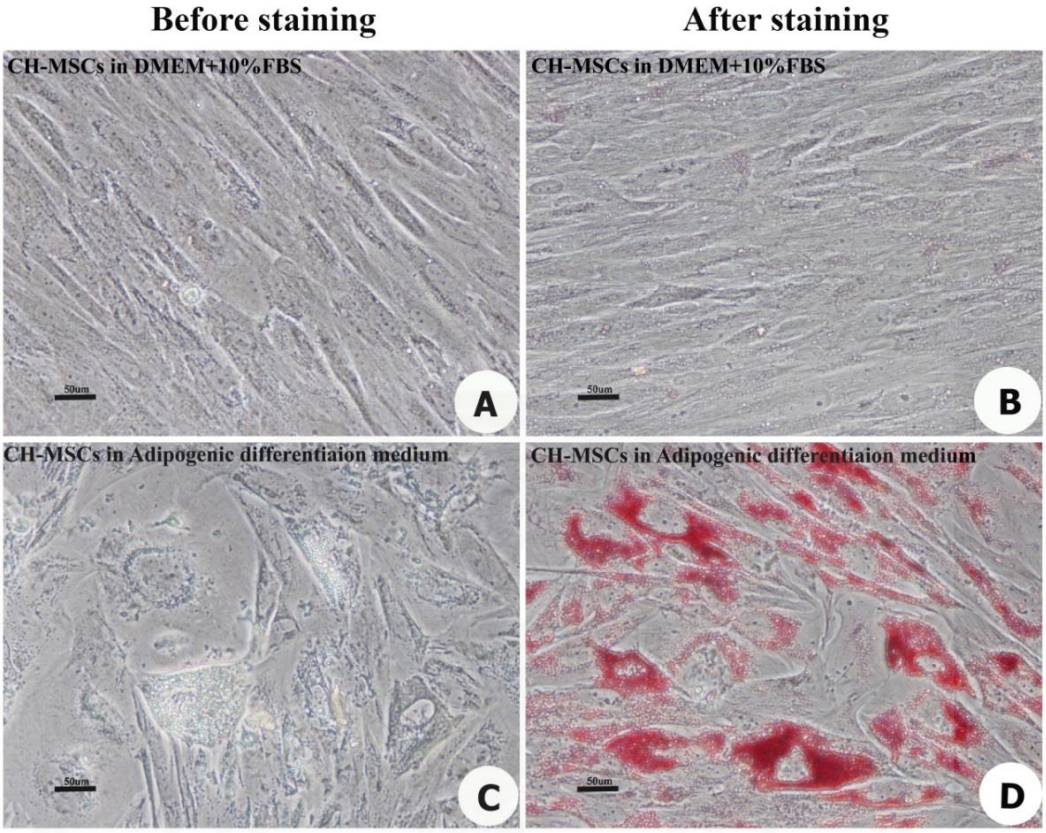

Figure 4.7 Adipogenic differentiation of CH-MSCs

CH-MSCs were cultured in adipogenic differentiation medium. After cultured for 3 weeks, the cells were stained with Oil Red O as described in section 3.5 to determine the number of adipocytes.

A. Morphology of CH-MSCs after culture in DMEM supplemented with 10% FBS for 3 weeks before Oil Red O staining
B. Morphology of CH-MSCs cultured in DMEM supplemented with 10% FBS for 3 weeks after Oil Red O staining
C. Morphology of adipocyte-like cells derived from CH-MSCs which were cultured in adipogenic differentiation medium for 3 weeks before Oil Red O staining
D. Morphology of adipocyte-like cells derived from CH-MSCs which were cultured in adipogenic differentiation medium for 3 weeks after Oil Red O staining Scale bar: 50 μm

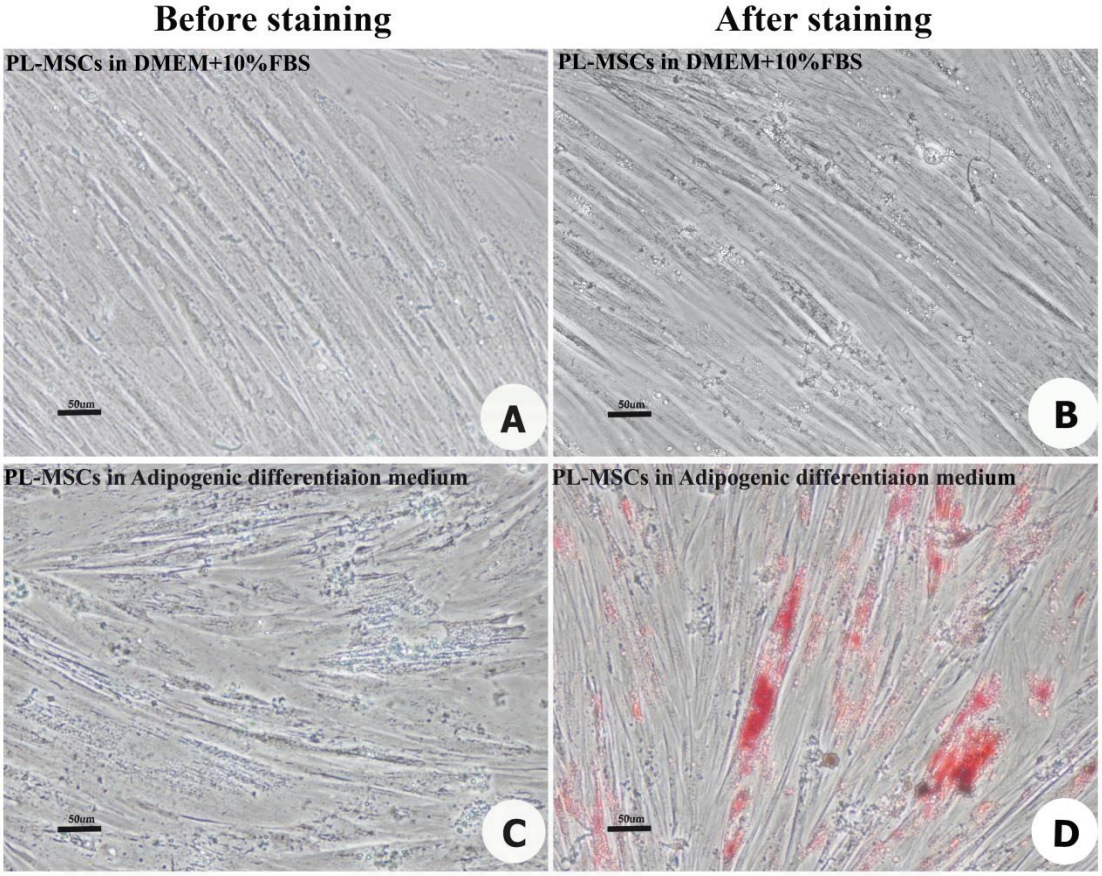

Figure 4.8 Adipogenic differentiation of PL-MSCs

PL-MSCs were cultured in adipogenic differentiation medium. After cultured for 3 weeks, the cells were stained with Oil Red O as described in section 3.5 to determine the number of adipocytes.

A. Morphology of PL-MSCs after culture in DMEM supplemented with 10% FBS for 3 weeks before Oil Red O staining
B. Morphology of PL-MSCs cultured in DMEM supplemented with 10% FBS for 3 weeks after Oil Red O staining
C. Morphology of adipocyte-like cells derived from PL-MSCs which were cultured in adipogenic differentiation medium for 3 weeks before Oil Red O staining
D. Morphology of adipocyte-like cells derived from PL-MSCs which were cultured in adipogenic differentiation medium for 3 weeks after Oil Red O staining Scale bar: 50 µm

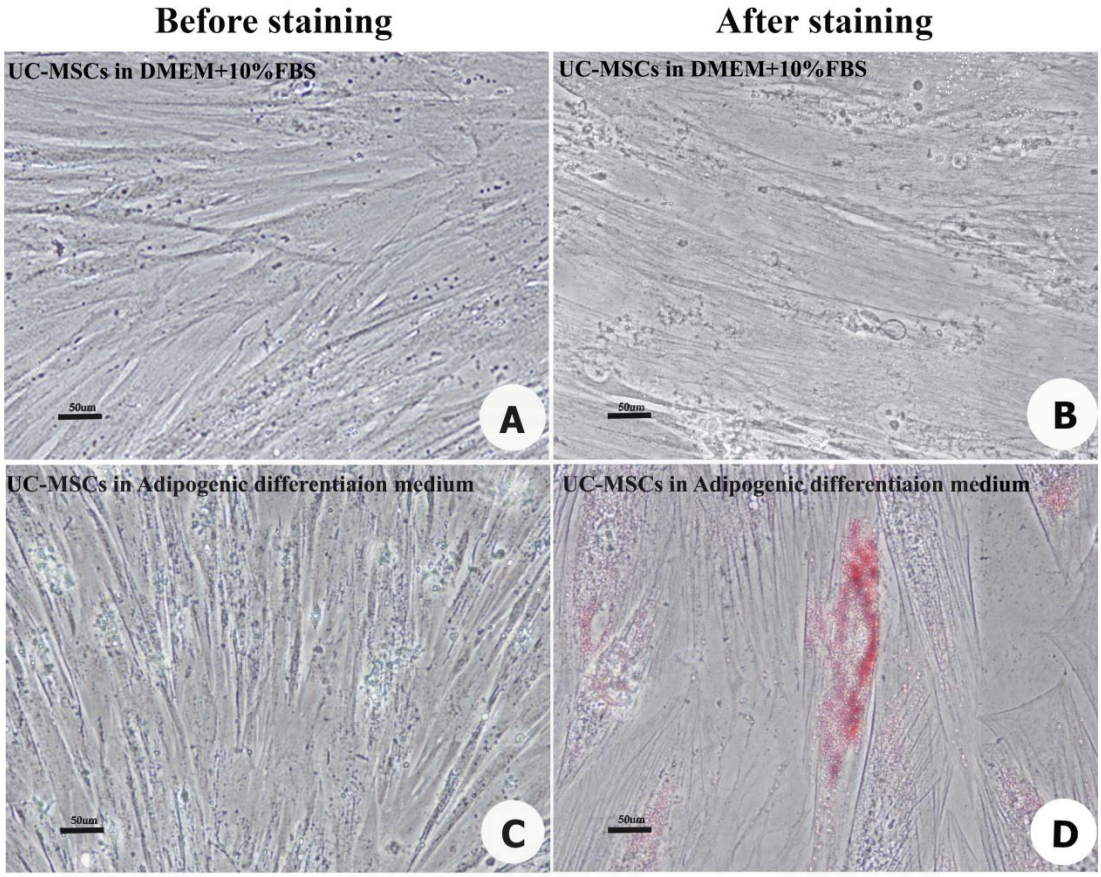

Figure 4.9 Adipogenic differentiation of UC-MSCs

UC-MSCs were cultured in adipogenic differentiation medium. After cultured for 3 weeks, the cells were stained with Oil Red O as described in section 3.5 to determine the number of adipocytes.

A. Morphology of UC-MSCs after culture in DMEM supplemented with 10% FBS for 3 weeks before Oil Red O staining
B. Morphology of UC-MSCs cultured in DMEM supplemented with 10% FBS for 3 weeks after Oil Red O staining
C. Morphology of adipocyte-like cells derived from UC-MSCs which were cultured in adipogenic differentiation medium for 3 weeks before Oil Red O staining
D. Morphology of adipocyte-like cells derived from UC-MSCs which were cultured in adipogenic differentiation medium for 3 weeks after Oil Red O staining Scale bar: 50 µm

4.1.4 Osteogenic differentiation potential of MSCs

To assess their osteogenic differentiation potentials, MSCs were harvested by trypsinization and were cultured in osteogenic differentiation medium as described in section 3.6. After cultured for 2 weeks, BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs differentiate to osteocyte-like cells which exhibited alkaline phosphatase activity while their non-treated counterparts which were cultured in complete medium did not exhibit alkaline phosphatase activity (Figure. 4.10- 4.13).

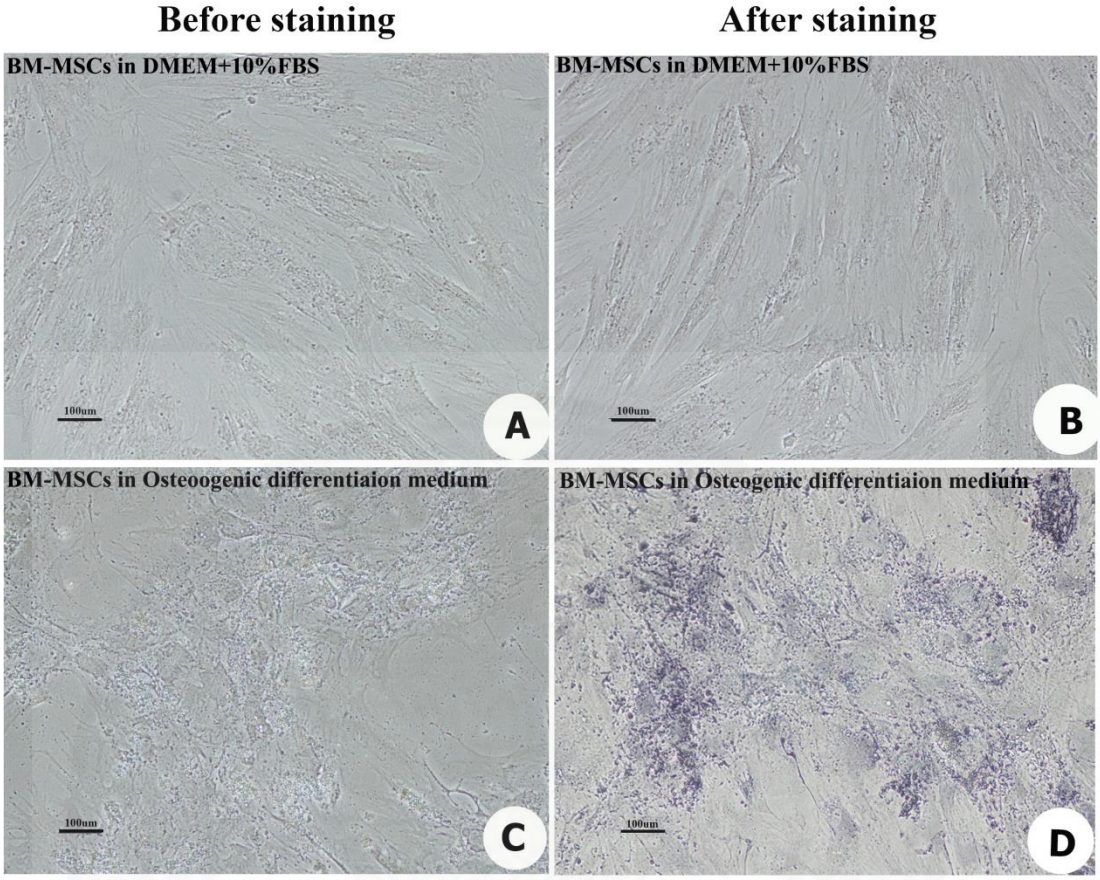

Figure 4.10 Osteogenic differentiation of BM-MSCs

BM-MSCs were cultured in osteogenic differentiation medium. After cultured for 2 weeks, the cells were subjected to alkaline phosphatase activity assay as described in section 3.6 to determine the level of osteogenic differentiation.

A. Morphology of BM-MSCs after culture in DMEM supplemented with 10% FBS for 2 weeks before subjected to alkaline phosphatase activity assay
B. Morphology of BM-MSCs cultured in DMEM supplemented with 10% FBS for 2 weeks after subjected to alkaline phosphatase activity assay
C. Morphology of osteocyte-like cells derived from BM-MSCs which were cultured in osteogenic differentiation medium for 2 weeks before subjected to alkaline phosphatase activity assay
D. Morphology of osteocyte-like cells derived from BM-MSCs which were cultured in osteogenic differentiation medium for 2 weeks after subjected to alkaline phosphatase activity assay.

   Scale bar: 100 µm

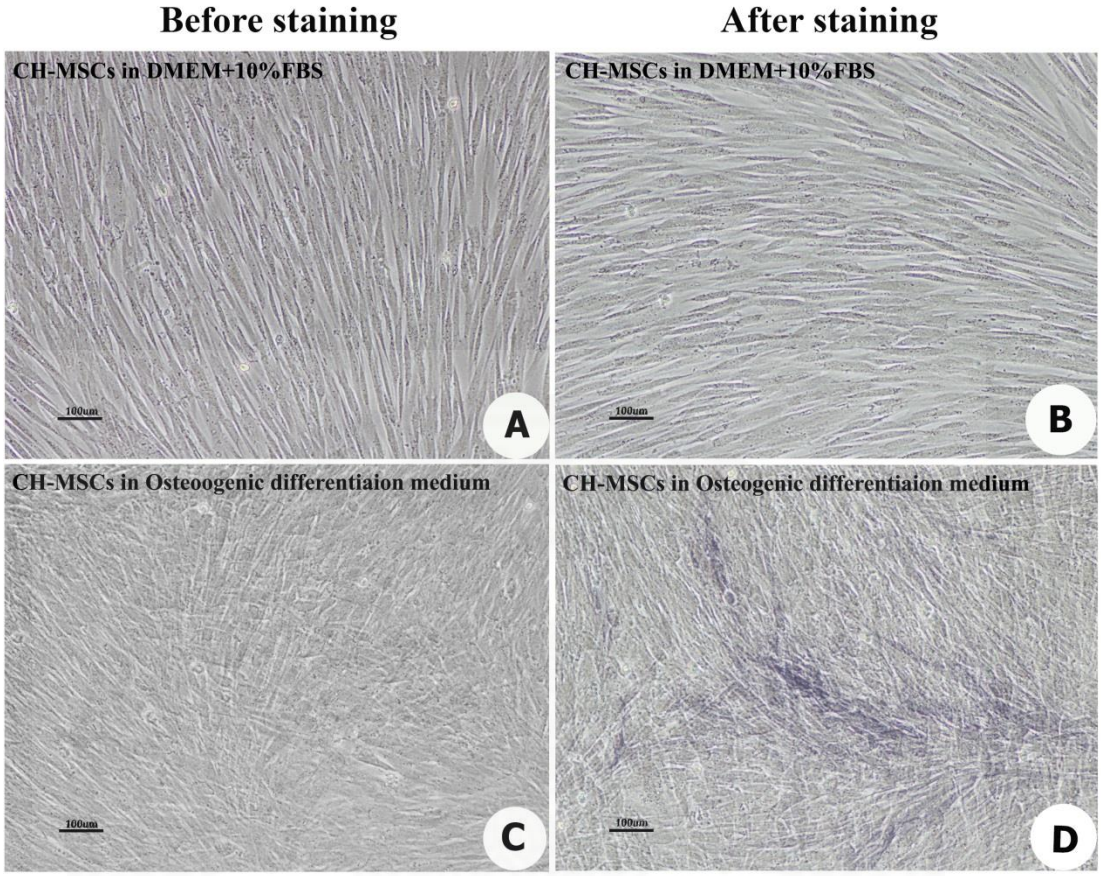

Figure 4.11 Osteogenic differentiation of CH-MSCs

CH-MSCs were cultured in osteogenic differentiation medium. After cultured for 2 weeks, the cells were subjected to alkaline phosphatase activity assay as described in section 3.6to determine the level of osteogenic differentiation.

A. Morphology of CH-MSCs after culture in DMEM supplemented with 10% FBS for 2 weeks before subjected to alkaline phosphatase activity assay
B. Morphology of CH-MSCs cultured in DMEM supplemented with 10% FBS for 2 weeks after subjected to alkaline phosphatase activity assay
C. Morphology of osteocyte-like cells derived from CH-MSCs which were cultured in osteogenic differentiation medium for 2 weeks before subjected to alkaline phosphatase activity assay
D. Morphology of osteocyte-like cells derived from CH-MSCs which were cultured in osteogenic differentiation medium for 2 weeks after subjected to alkaline phosphatase activity assay.

   Scale bar: 100 µm

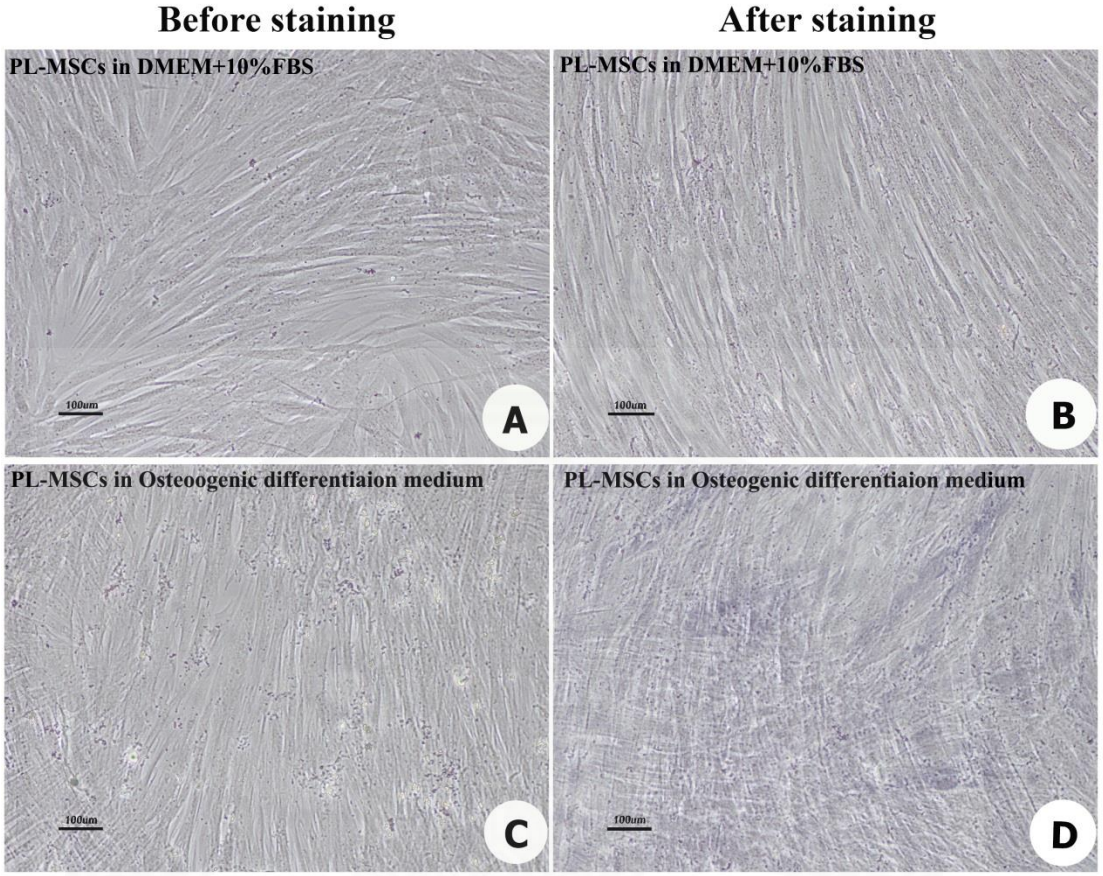

Figure 4.12 Osteogenic differentiation of PL-MSCs

PL-MSCs were cultured in osteogenic differentiation medium. After cultured for 2 weeks, the cells were subjected to alkaline phosphatase activity assay as described in section 3.6to determine the level of osteogenic differentiation.

A. Morphology of PL-MSCs after culture in DMEM supplemented with 10% FBS for 2 weeks before subjected to alkaline phosphatase activity assay
B. Morphology of PL-MSCs cultured in DMEM supplemented with 10% FBS for 2 weeks after subjected to alkaline phosphatase activity assay
C. Morphology of osteocyte-like cells derived from PL-MSCs which were cultured in osteogenic differentiation medium for 2 weeks before subjected to alkaline phosphatase activity assay
D. Morphology of osteocyte-like cells derived from PL-MSCs which were cultured in osteogenic differentiation medium for 2 weeks after subjected to alkaline phosphatase activity assay.

   Scale bar: 100 µm

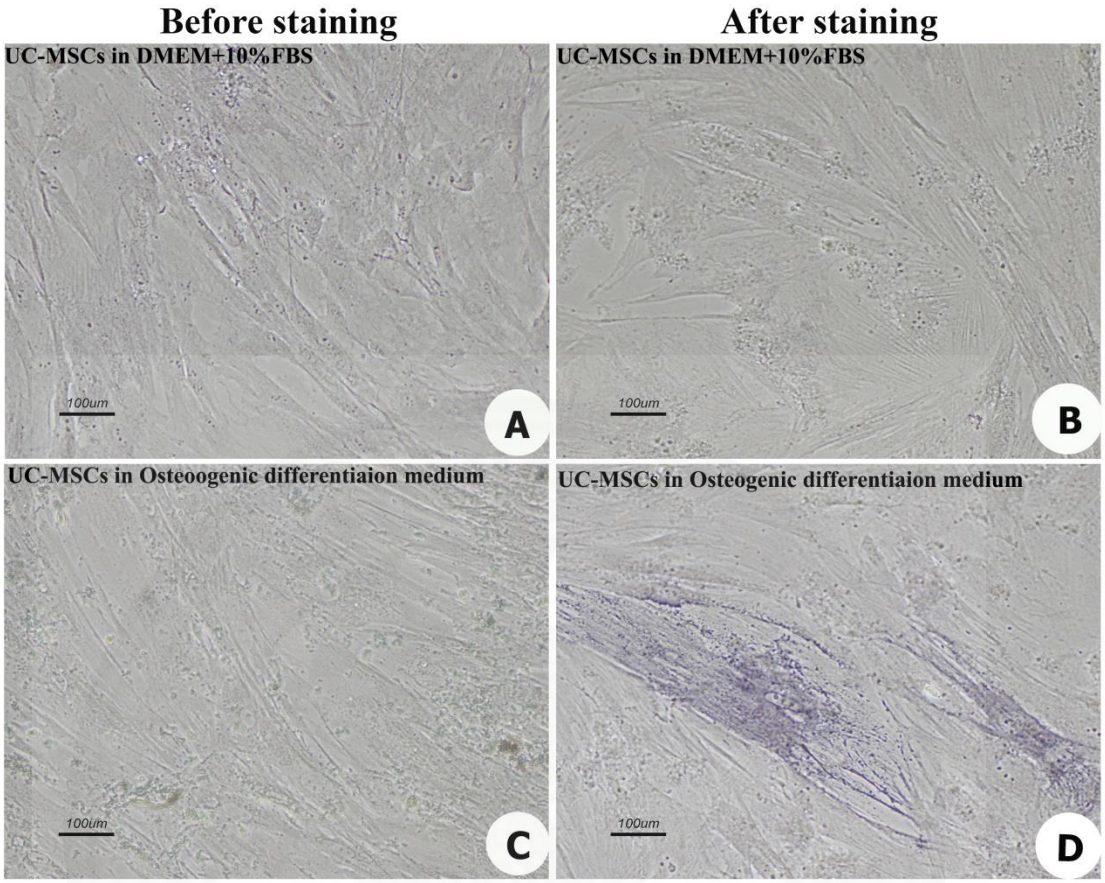

Figure 4.13 Osteogenic differentiation of UC-MSCs

UC-MSCs were cultured in osteogenic differentiation medium. After cultured for 2 weeks, the cells were subjected to alkaline phosphatase activity assay as described in section 3.6 to determine the level of osteogenic differentiation.

A. Morphology of UC-MSCs after culture in DMEM supplemented with 10% FBS for 2 weeks before subjected to alkaline phosphatase activity assay
B. Morphology of UC-MSCs cultured in DMEM supplemented with 10% FBS for 2 weeks after subjected to alkaline phosphatase activity assay
C. Morphology of osteocyte-like cells derived from UC-MSCs which were cultured in osteogenic differentiation medium for 2 weeks before subjected to alkaline phosphatase activity assay
D. Morphology of osteocyte-like cells derived from UC-MSCs which were cultured in osteogenic differentiation medium for 2 weeks after subjected to alkaline phosphatase activity assay.

   Scale bar: 100 µm

4.2 The effects of high glucose on MSCs proliferation

To study the effect of high glucose on MSC proliferation, $1x10^3$ 3rd passage BM-MSCs, CH-MSCs, UC-MSCs and PL-MSCs were seeded in an individual well of 24-well plate containing 500 µl complete medium supplemented with 25mM D-glucose as described in section 3.7. The cells were harvested at culture days 2, 4, 6, 8, 10, 12 and 14 to determine cell number. The results showed that from day 6 onward the growth of BM-MSCs cultured in complete medium supplemented with 25mM D-glucose was significantly decreased when compared with the growth of BM-MSCs cultured in normal glucose condition ($p<0.05$) (Table 4.2 and Figure 4.14). Similar to BM-MSCs, the growth rate of CH-MSCs, PL-MSCs and UC-MSCs cultured in high glucose condition was significantly lower than their normal glucose counterparts ($p<0.05$) (Table 4.3-4.5 and Figure 4.15-4.17).

Table 4.2 The number of BM-MSCs during a 14-day culture period in high glucose condition

Culture day	Number of BM-MSCs	
	DMEM+10%FBS	DMEM+10%FBS +25mM D-Glucose
0	$1.00x10^3 \pm 0.00$	$1.00x10^3 \pm 0.00$
2	$1.77x10^3 \pm 0.10$	$1.42x10^3 \pm 0.10$
4	$2.25x10^3 \pm 0.22$	$1.69x10^3 \pm 0.10$
6	$4.19x10^3 \pm 0.20$	$3.11x10^3 \pm 0.07$
8	$6.64x10^3 \pm 0.34$	$4.56x10^3 \pm 0.07$
10	$13.47x10^3 \pm 1.06$	$8.64x10^3 \pm 0.23$
12	$17.80x10^3 \pm 1.54$	$12.97x10^3 \pm 0.41$
14	$19.72x10^3 \pm 0.82$	$15.20x10^3 \pm 1.45$

Data were presented as mean ± standard error of means (SEM) from three independent experiments

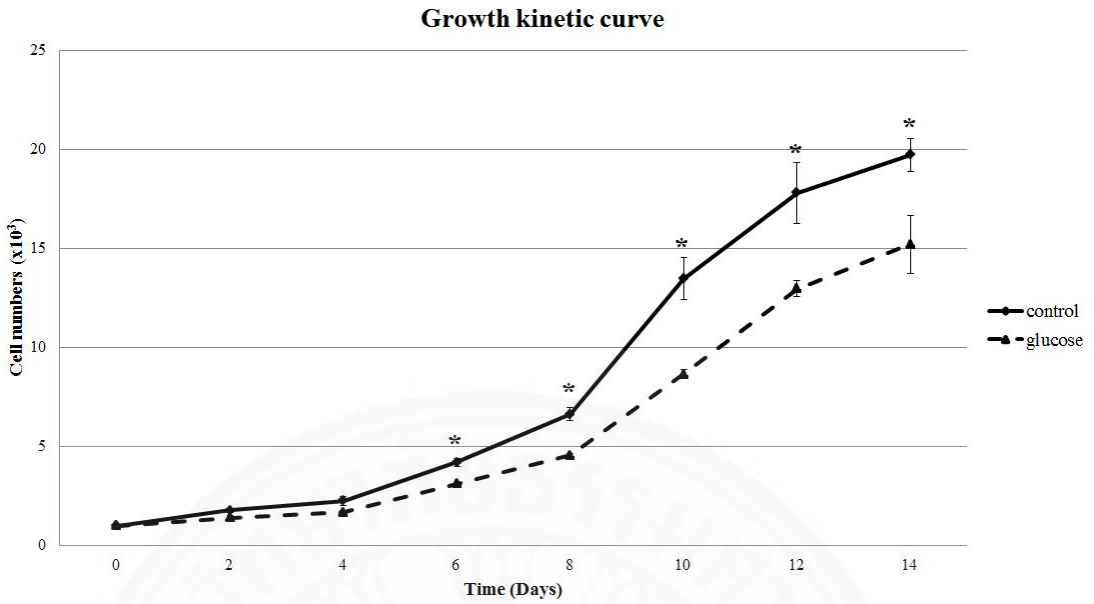

Figure 4.14 Growth kinetic curve of BM-MSCs during a 14-day culture period in high glucose condition.

$1x10^3$ 3rd passage BM-MSCs were seeded in an individual well of 24 well plate containing 500 µl complete medium supplemented with 25mM D-glucose as described in section 3.7. The cells were harvested at culture days 2, 4, 6, 8, 10, 12 and 14 to determine cell number. Bone marrow-derived MSCs cultured in complete medium without glucose supplementation serve as normal glucose control. Graph show growth kinetic curve of BM-MSCs cultured in high glucose (dashed line) and normal glucose (solid line) condition. Data were presented as mean ± standard error of the mean (SEM) from three independent experiments. *P<0.05 vs. high glucose condition.

Table 4.3 The number of CH-MSCs during a 14-day culture period in high glucose condition

Culture day	CH-MSCs	
	DMEM+10%FBS	DMEM+10%FBS +25mM D-Glucose
0	$1.00x10^3 \pm 0.00$	$1.00x10^3 \pm 0.00$
2	$1.30x10^3 \pm 0.05$	$1.05x10^3 \pm 0.03$
4	$2.75x10^3 \pm 0.05$	$1.72x10^3 \pm 0.06$
6	$4.35x10^3 \pm 0.04$	$3.61x10^3 \pm 0.03$
8	$9.64x10^3 \pm 0.15$	$5.78x10^3 \pm 0.18$
10	$13.39x10^3 \pm 0.20$	$9.75x10^3 \pm 0.25$
12	$16.69x10^3 \pm 0.31$	$13.25x10^3 \pm 0.08$
14	$18.58x10^3 \pm 0.24$	$15.55x10^3 \pm 0.32$

The Data were presented as mean ± standard error of means (SEM) from three independent experiments

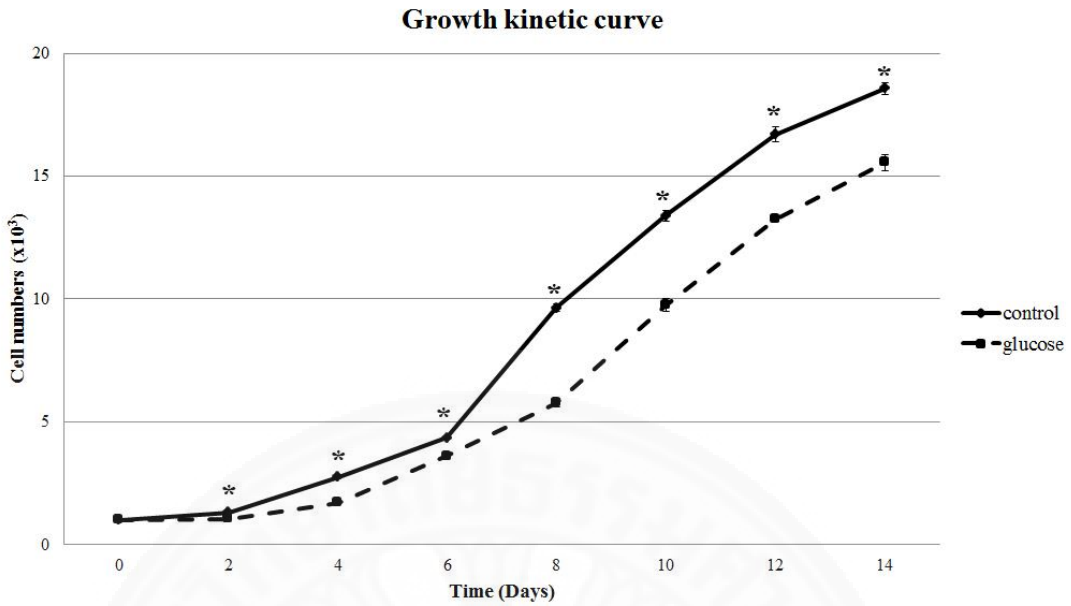

Figure 4.15 Growth kinetic curve of CH-MSCs during a 14-day culture period in high glucose condition.

$1x10^3$ 3rd passage CH-MSCs were seeded in an individual well of 24 well plate containing 500 µl complete medium supplemented with 25mM D-glucose as described in section 3.7. The cells were harvested at culture days 2, 4, 6, 8, 10, 12 and 14 to determine cell number. Chorion-derived MSCs cultured in complete medium without glucose supplementation serve as normal glucose control. Graph show growth kinetic curve of BM-MSCs cultured in high glucose (dashed line) and normal glucose (solid line) condition. Data were presented as mean ± standard error of the mean (SEM) from three independent experiments. *P<0.05 vs. high glucose condition.

Table 4.4 The number of PL-MSCs during a 14-day culture period in high glucose condition

Culture day	PL-MSCs	
	DMEM+10%FBS	DMEM+10%FBS +25mM D-Glucose
0	$1.00x10^3 \pm 0.00$	$1.00x10^3 \pm 0.00$
2	$1.58x10^3 \pm 0.21$	$1.22x10^3 \pm 0.07$
4	$2.50x10^3 \pm 0.13$	$1.86x10^3 \pm 0.18$
6	$5.25x10^3 \pm 0.05$	$3.89x10^3 \pm 0.10$
8	$8.03x10^3 \pm 0.31$	$5.00x10^3 \pm 0.24$
10	$14.17x10^3 \pm 0.63$	$9.03x10^3 \pm 0.07$
12	$18.31x10^3 \pm 0.19$	$13.58x10^3 \pm 0.13$
14	$19.53x10^3 \pm 0.53$	$15.31x10^3 \pm 0.15$

Data were presented as mean ± standard error of means (SEM) from three independent experiments

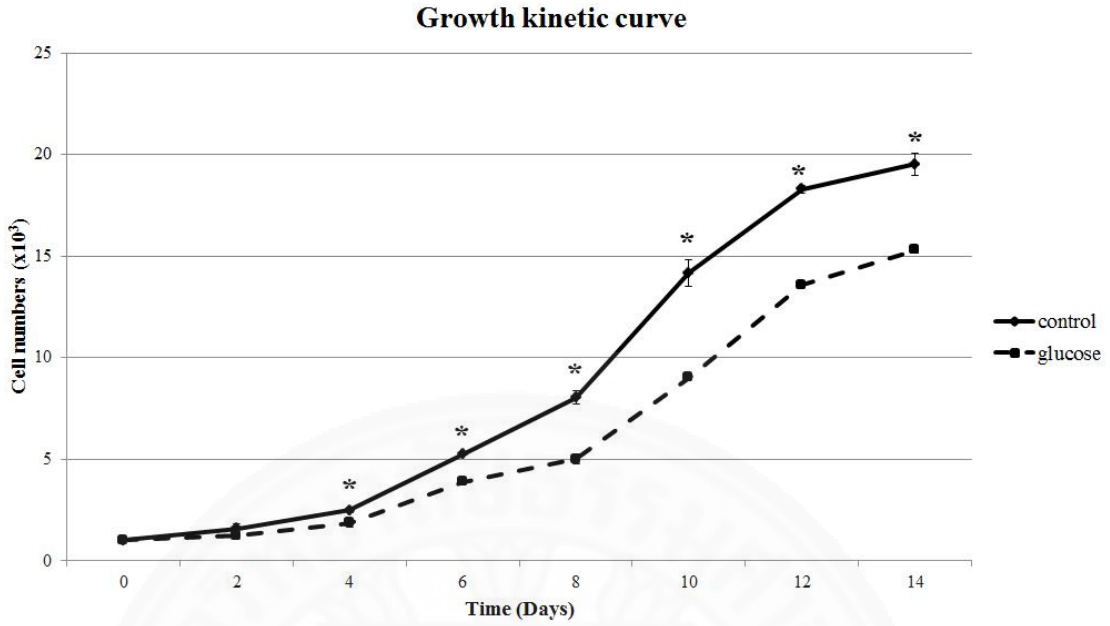

Figure 4.16 Growth kinetic curve of PL-MSCs during a 14-day culture period in high glucose condition.

$1x10^3$ 3rd passage PL-MSCs were seeded in an individual well of 24 well plate containing 500 µl complete medium supplemented with 25mM D-glucose as described in section 3.7. The cells were harvested at culture days 2, 4, 6, 8, 10, 12 and 14 to determine cell number. Placenta-derived MSCs cultured in complete medium without glucose supplementation serve as normal glucose control. Graph show growth kinetic curve of BM-MSCs cultured in high glucose (dashed line) and normal glucose (solid line) condition. Data were presented as mean ± standard error of the mean (SEM) from three independent experiments. *P<0.05 vs. high glucose condition.

Table 4.5 The number of UC-MSCs during a 14-day culture period in high glucose condition

Culture day	UC-MSCs	
	DMEM+10%FBS	DMEM+10%FBS +25mM D-Glucose
0	$1.00x10^3 \pm 0.00$	$1.00x10^3 \pm 0.00$
2	$1.44x10^3 \pm 0.03$	$1.33x10^3 \pm 0.05$
4	$2.06x10^3 \pm 0.10$	$1.75x10^3 \pm 0.05$
6	$4.03x10^3 \pm 0.26$	$2.67x10^3 \pm 0.13$
8	$6.14x10^3 \pm 0.15$	$3.42x10^3 \pm 0.22$
10	$12.58x10^3 \pm 0.22$	$6.61x10^3 \pm 0.20$
12	$18.03x10^3 \pm 0.34$	$11.47x10^3 \pm 0.27$
14	$22.00x10^3 \pm 0.25$	$16.03x10^3 \pm 0.43$

Data were presented as mean ± standard error of means (SEM) from three independent experiments

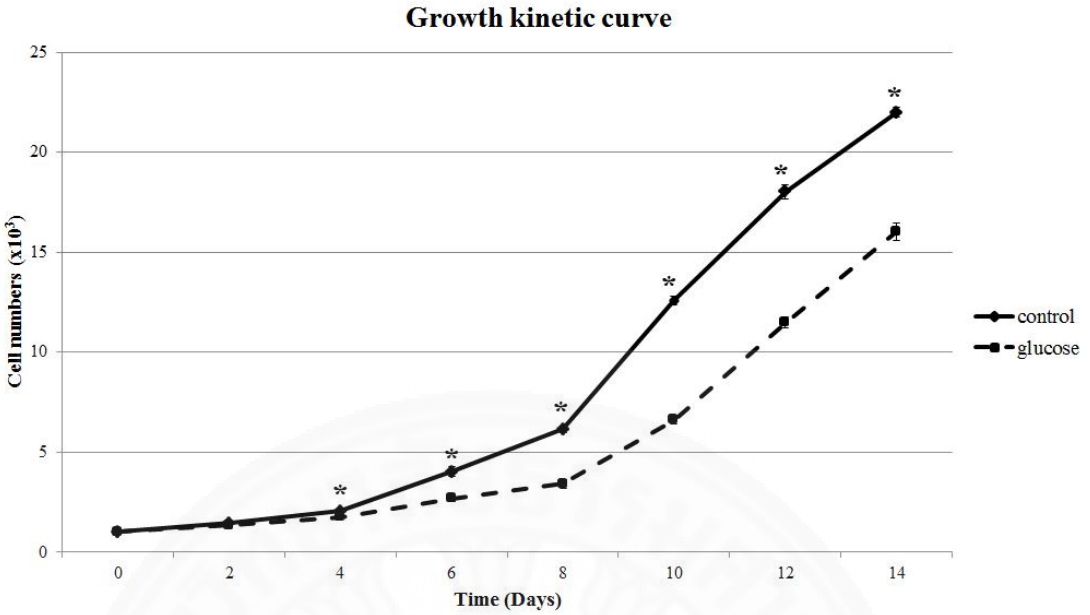

Figure 4.17 Growth kinetic curve of UC-MSCs during a 14-day culture period in high glucose condition.

$1x10^3$ 3rd passage UC-MSCs were seeded in an individual well of 24 well plate containing 500 µl complete medium supplemented with 25mM D-glucose as described in section 3.7. The cells were harvested at culture days 2, 4, 6, 8, 10, 12 and 14 to determine cell number. Umbilical cord-derived MSCs cultured in complete medium without glucose supplementation serve as normal glucose control. Graph show growth kinetic curve of BM-MSCs cultured in high glucose (dashed line) and normal glucose (solid line) condition. Data were presented as mean ± standard error of the mean (SEM) from three independent experiments. *P<0.05 vs. high glucose condition.

4.3 The effect of high glucose on adipogenic differentiation of MSCs

4.3.1 The effect of high glucose on adipogenic differentiation of BM-MSCs

To study the effect of high glucose on the adipogenic differentiation of BM-MSCs, $7.5x10^4$ 3^{rd}-5^{th} passage BM-MSCs were cultured in adipogenic differentiation medium supplemented with 25mM D-glucose. After 14 days of culture, the cells were stained with Oil Red O as described in section 3.8.1 to determine the number of adipocyte-like cells (Figure 4.18). After staining, the Oil Red O was extracted from each stained sample by isopropanol and its concentration was determined as described in section 3.8.1. The results showed that the number of adipocyte-like cells derived from BM-MSCs cultured in high glucose condition was significantly greater than those derived from BM-MSCs cultured in normal glucose condition (1308.67±28.81 vs. 879.00±75.66, * $P<0.05$) (Table 4.6 and Figure 4.19). In agreement with these results, the concentration of Oil Red O extracted from BM-MSCs cultured in high glucose was also significantly greater than that of BM-MSCs cultured in normal glucose condition (271.16±17.47 vs. * $P<0.05$) (Figure 4.20).

In addition to the number of adipocyte-like cells, the gene expression study indicates that the mRNA levels of adipogenic gene *PPARγ* and *LPL* in BM-MSCs cultured in high glucose condition were also significantly higher than those of BM-MSCs cultured in normal glucose (Table 4.8, Figure 4.21 and 4.25). In contrast to *PPARγ* and *LPL*, the mRNA levels of other adipogenic genes including *SREBP-1c*, *ADIPOQ* and *GLUT4* in BM-MSCs cultured in high glucose were not different from those of BM-MSCs cultured in normal glucose condition (Table 4.8, Figure 4.22 - 4.24).

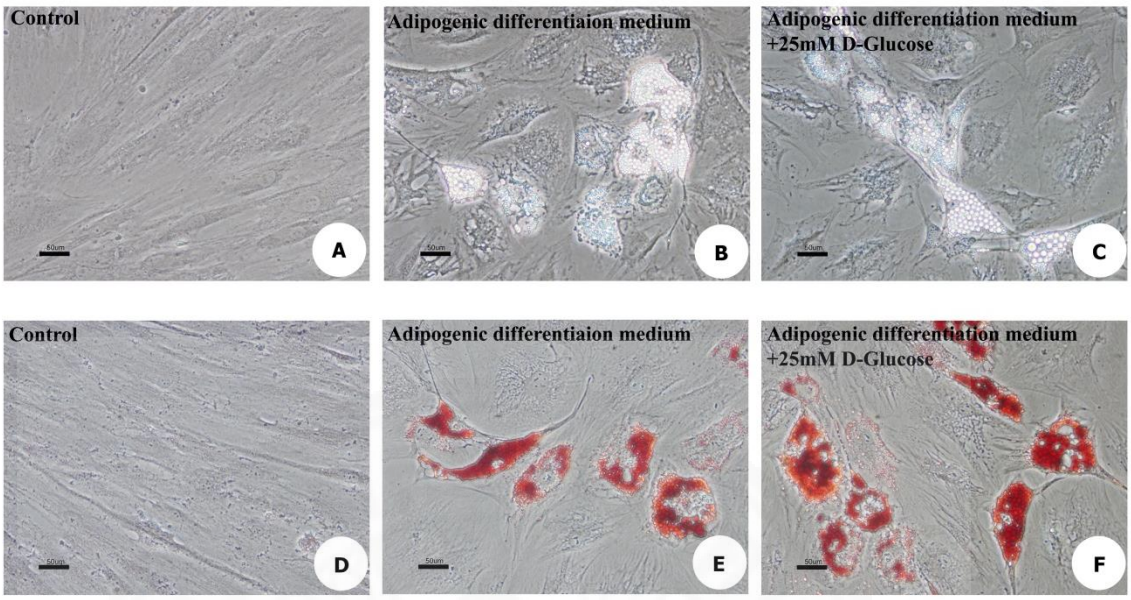

Figure 4.18 The effects of high glucose on adipogenic differentiation of BM-MSCs

$7.5x10^4$ 3^{rd}-5^{th} passage BM-MSCs were cultured in adipogenic differentiation medium supplemented with 25mM D-glucose as described in section 3.8.1. After 14 days of culture, the cells were stained with Oil Red O as described in section 3.8.1 to determine the number of adipocyte-like cells. BM-MSCs cultured in adipogenic differentiation medium without glucose supplementation serve as normal glucose controls while BM-MSCs cultured in DMEM supplemented with 10% FBS serve as non-differentiation controls

A. Morphology of BM-MSCs cultured in DMEM supplemented with 10% FBS for 14 days
B. Morphology of BM-MSCs cultured in adipogenic differentiation medium for 14 days
C. Morphology of BM-MSCs cultured in adipogenic differentiation medium supplemented with 25mM D-glucose for 14 days
D. Morphology of BM-MSCs cultured in DMEM supplemented with 10% FBS for 14 days after staining with Oil Red O
E. Morphology of BM-MSCs cultured in adipogenic differentiation medium for 14 days after staining with Oil Red O.
F. Morphology of BM-MSCs cultured in adipogenic differentiation medium supplemented with 25mM D-glucose for 14 days after staining with Oil Red O

Table 4.6 Number of adipocyte-like cells generated from BM-MSCs which were cultured in high glucose and normal glucose condition

Sample	Culture condition	Number of adipocyte-like cells (cells)
BM-MSCs	Adipogenic differentiation medium	879.00±75.66
	Adipogenic differentiation medium+25mM D-glucose	1308.67±28.81

Data were presented as mean ± standard error of means (SEM) from three independent experiments

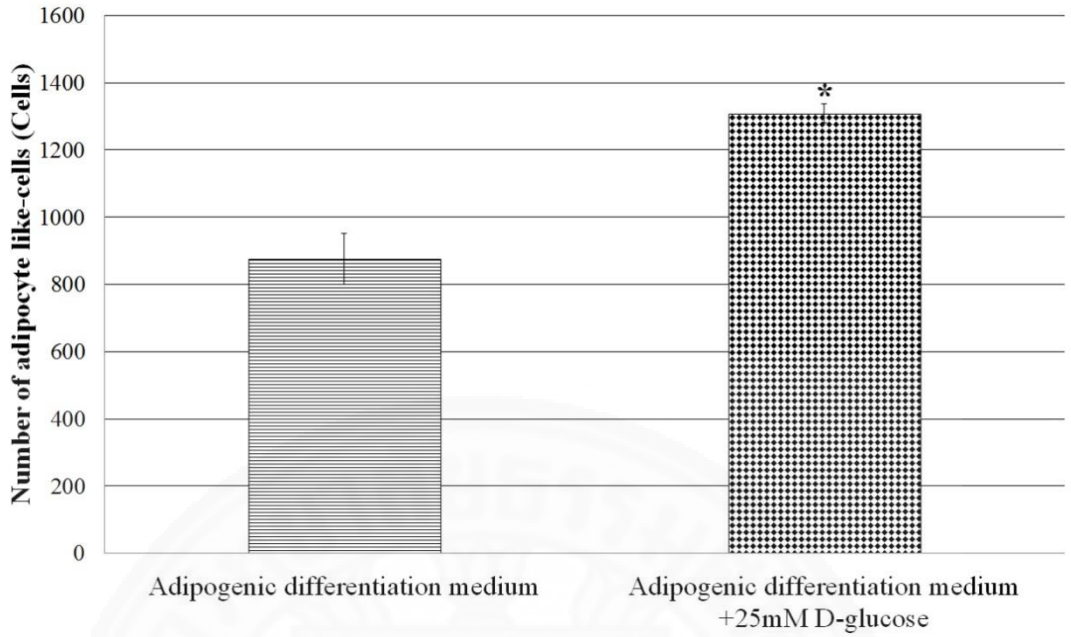

Figure 4.19 The number of adipocyte-like cells generated from BM-MSCs cultured in high glucose and normal glucose condition

Data were presented as mean ± standard error of means (SEM) from three independent experiments. * $P<0.05$ vs. adipogenic differentiation medium.

Table 4.7 The concentration of Oil Red O extracted from BM-MSCs cultured in high glucose and normal glucose condition

Sample	Culture condition	Oil Red O concentation (µg/ml)
BM-MSCs	DMEM+10%FBS	89.83±9.39
	Adipogenic differentiation medium	199.42±18.03
	Adipogenic differentiation medium+25mM D-glucose	271.16±17.47

After Oil Red O staining, the Oil Red O was extracted from BM-MSCs cultured in high glucose condition and its concentration was determined as described in section 3.8.1. BM-MSCs cultured in adipogenic differentiation medium without glucose supplementation serve as normal glucose controls while BM-MSCs cultured in DMEM supplemented with 10% FBS serve as non-differentiation controls. Data were presented as mean ± standard error of means (SEM) from three independent experiments. * $P<0.05$ vs. adipogenic differentiation medium.

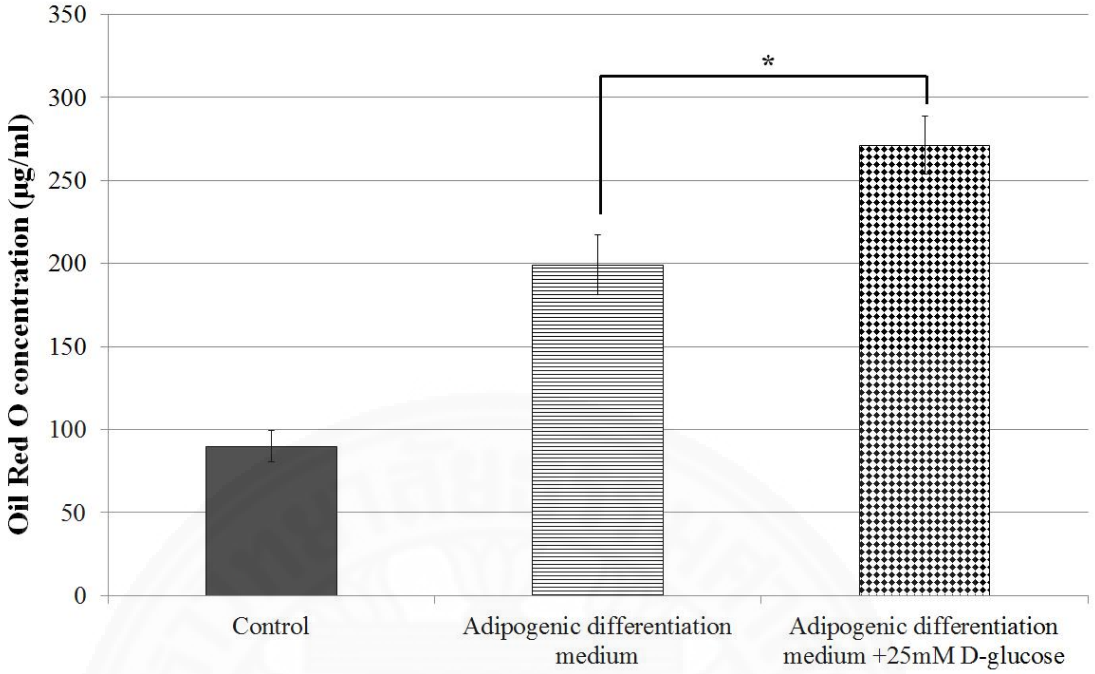

Figure 4.20 The concentration of Oil Red O extracted from BM-MSCs cultured in high glucose and normal glucose condition

Data were presented as mean ± standard error of means (SEM) from three independent experiments. * $P<0.05$ vs. adipogenic differentiation medium.

Table 4.8 Relative mRNA levels of adipogenic genes in BM-MSCs cultured in high glucose and normal glucose condition

Gene	DMEM +10%FBS	Adipogenic differentiation medium	Adipogenic differentiation medium+25mM D-glucose
PPARγ	1.00±0.00	85.07±2.58	122.84±3.87
SREBP-1c	1.00±0.00	3.47±0.11	3.67±0.07
ADIPOQ	1.00±0.00	1222.85±150.31	1595.88±241.87
GLUT4	1.00±0.00	3.13±0.10	3.51±0.16
LPL	1.00±0.00	6433.24±501.60	22281.32±1198.42

$2x10^5$ 3^{rd}-5^{th}passageBM-MSCs were cultured in adipogenic differentiation medium supplemented with 25mM D-glucose as described in section 3.10.1. After 14 days of culture, the mRNA levels of adipogenic genes *PPARγ*, *SREBP-1c*, *ADIPOQ*, *GLUT4* and *LPL* were determined by qRT-PCR as described in section 3.10.3. The mRNA levels of all adipogenic genes of BM-MSCs cultured in adipogenic differentiation medium with or without glucose supplementation were compared relative to the expression levels of those genes in BM-MSCs cultured in DMEM + 10% FBS. Data were presented as mean ± SEM of three independent experiments.

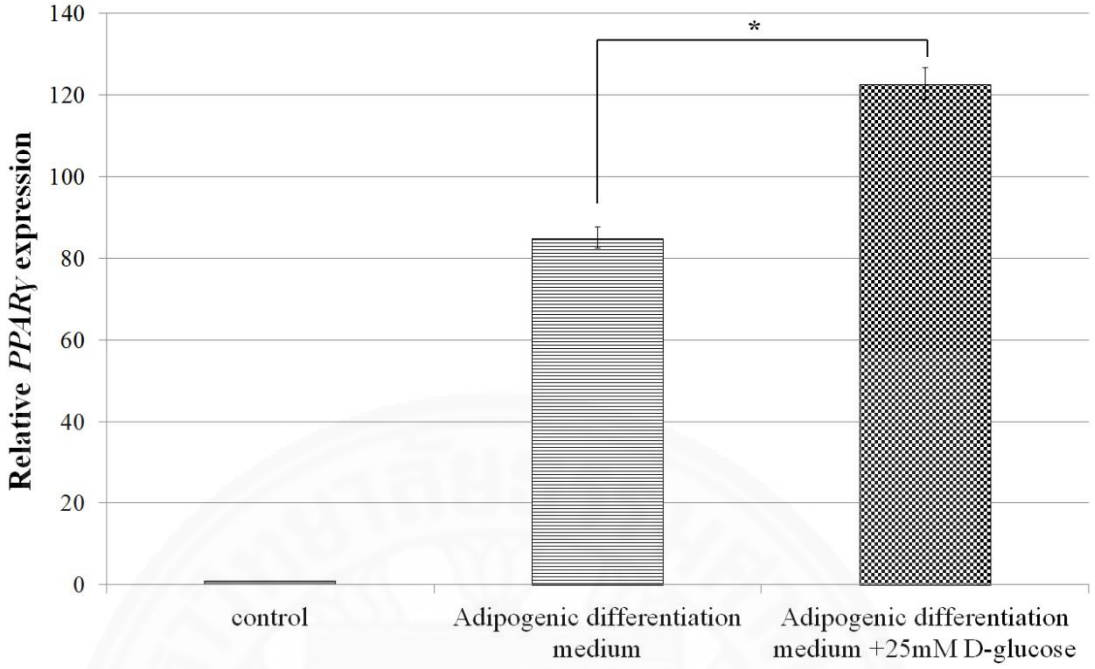

Figure 4.21 The relative mRNA levels of *PPARγ* gene in BM-MSCs cultured in high glucose and normal glucose condition

The mRNA levels of *PPARγ* gene of BM-MSCs cultured in adipogenic differentiation medium with or without glucose supplementation were compared relatively to the expression levels of *PPARγ* gene of BM-MSCs cultured in DMEM + 10% FBS. Data were presented as mean ± standard error of means (SEM) of three independent experiments. *P<0.05 vs. adipogenic differentiation medium

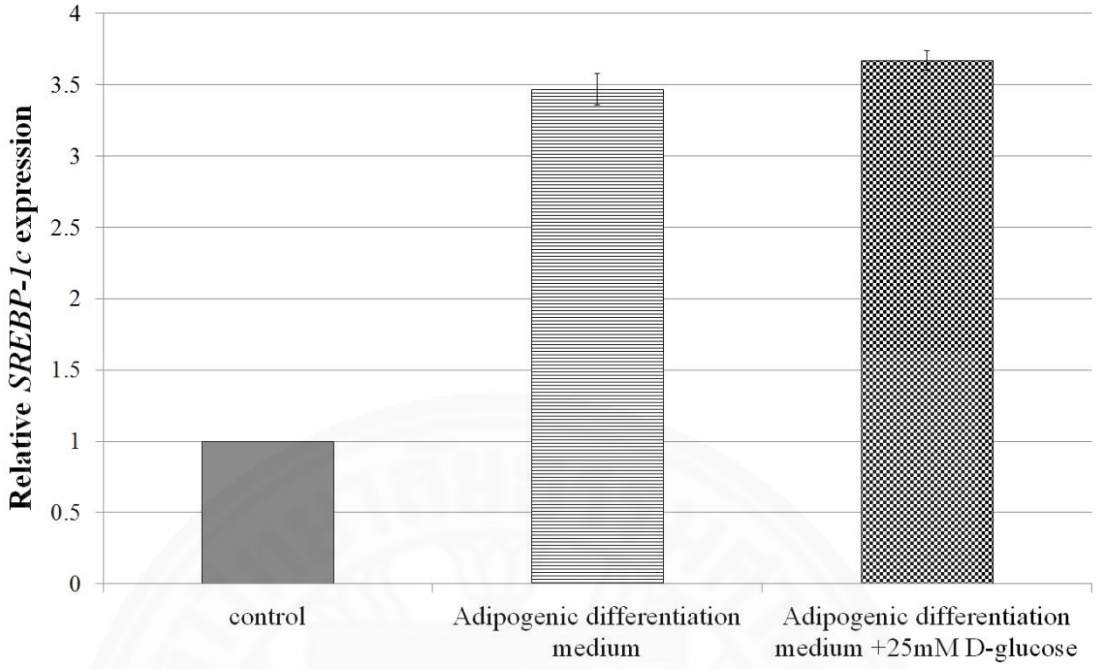

Figure 4.22 The relative mRNA levels of *SREBP-1c* gene in BM-MSCs cultured in high glucose and normal glucose condition

The mRNA levels of *SREBP-1c* gene of BM-MSCs cultured in adipogenic differentiation medium with or without glucose supplementation were compared relatively to the expression levels of *SREBP-1c* gene of BM-MSCs cultured in DMEM + 10% FBS. Data were presented as mean ± standard error of means (SEM) of three independent experiments. *P<0.05 vs. adipogenic differentiation medium

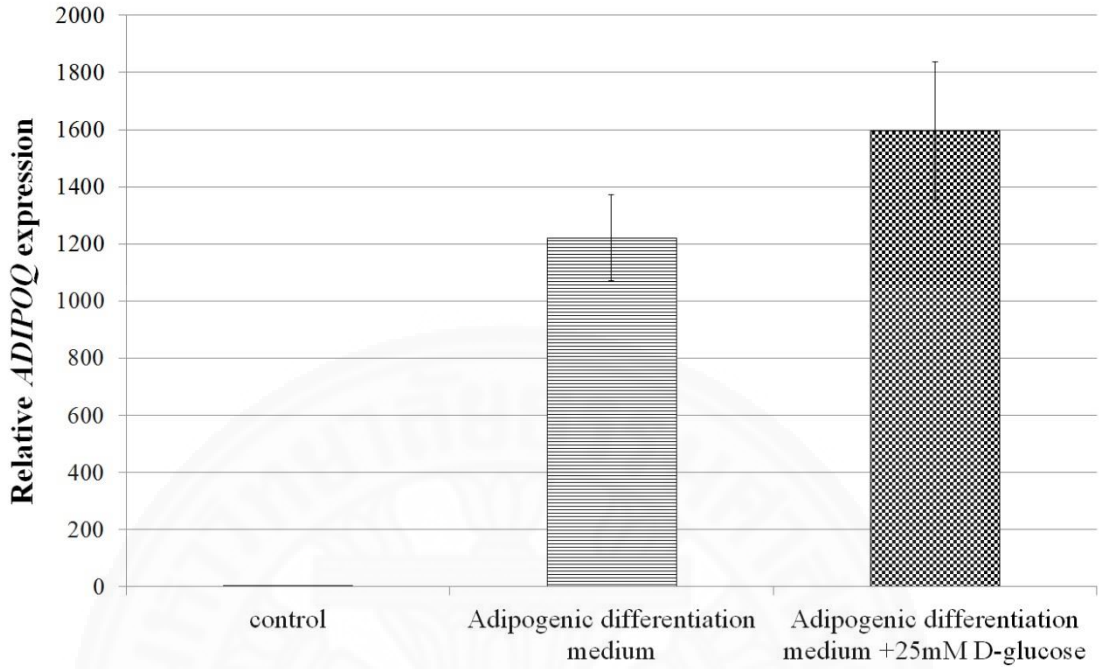

Figure 4.23 The relative mRNA levels of *ADIPOQ* gene in BM-MSCs cultured in high glucose and normal glucose condition

The mRNA levels of *ADIPOQ* gene of BM-MSCs cultured in adipogenic differentiation medium with or without glucose supplementation were compared relatively to the expression levels of *ADIPOQ* gene of BM-MSCs cultured in DMEM + 10% FBS. Data were presented as mean ± standard error of means (SEM) of three independent experiments. *P<0.05 vs. adipogenic differentiation medium

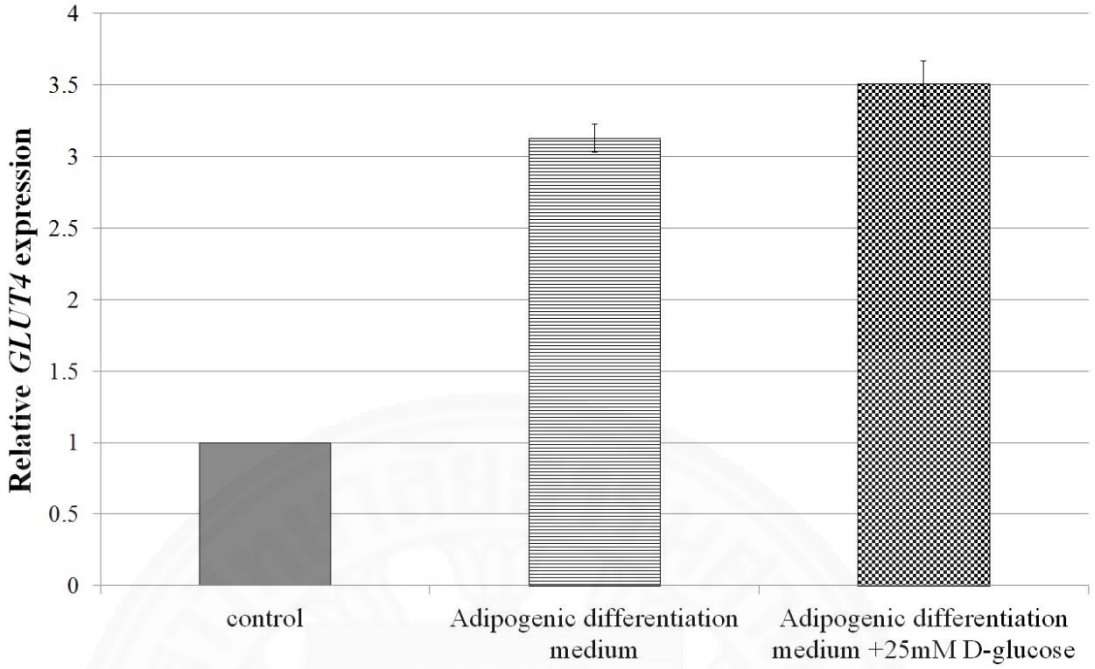

Figure 4.24 The relative mRNA levels of *GLUT4* gene in BM-MSCs cultured in high glucose and normal glucose condition

The mRNA levels of *GLUT4* gene of BM-MSCs cultured in adipogenic differentiation medium with or without glucose supplementation were compared relatively to the expression levels of *GLUT4* gene of BM-MSCs cultured in DMEM + 10% FBS. Data were presented as mean ± standard error of means (SEM) of three independent experiments. *P<0.05 vs. adipogenic differentiation medium

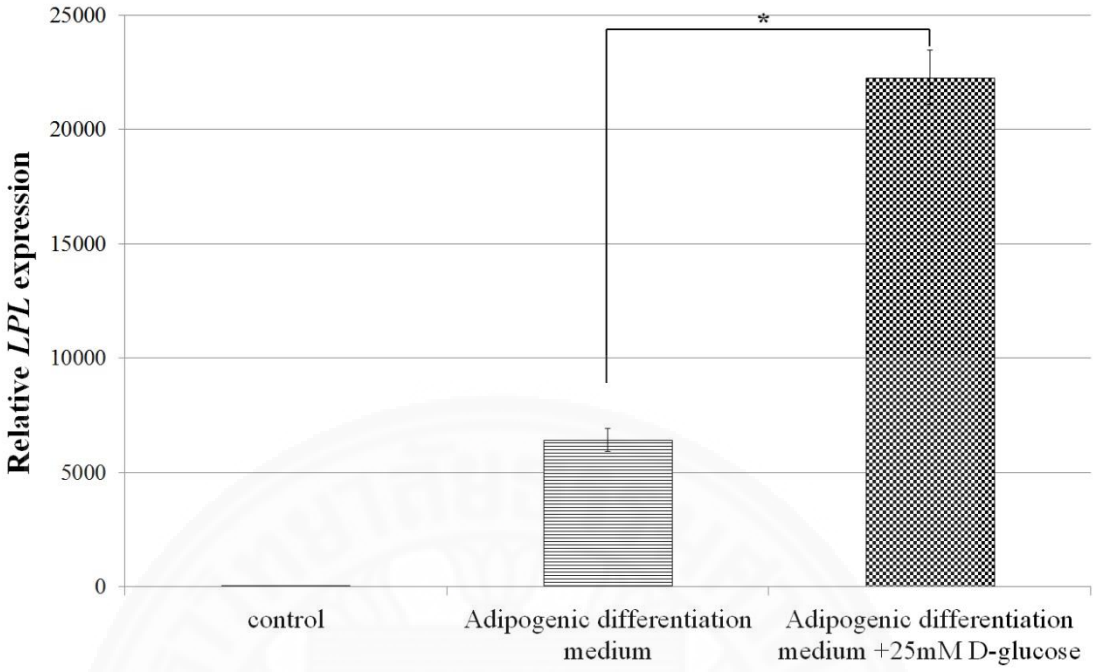

Figure 4.25 The relative mRNA levels of *LPL* gene in BM-MSCs cultured in high glucose and normal glucose condition

The mRNA levels of *LPL* gene of BM-MSCs cultured in adipogenic differentiation medium with or without glucose supplementation were compared relatively to the expression levels of *LPL* gene of BM-MSCs cultured in DMEM + 10% FBS. Data were presented as mean ± standard error of means (SEM) of three independent experiments. *P<0.05 vs. adipogenic differentiation medium

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4.3.2 The effects of high glucose on adipogenic differentiation of CH-MSCs, PL-MSCs and UC-MSCs

To study the effect of high glucose on the adipogenic differentiation of CH-MSCs, PL-MSCs and UC-MSCs, $7.5x10^4$ 3^{rd}-5^{th}MSCs from those 3 sources were cultured in adipogenic differentiation medium supplemented with 25mM D-glucose. After 28 days of culture, the cells were stained with Oil Red O as described in section 3.8.1 to determine the number of adipocyte-like cells (Figure 4.26-4.28). After staining, the Oil Red O was extracted from each stained sample by isopropanol and its concentration was determined as described in section 3.8.1.

Similar to BM-MSCs, the number of adipocyte-like cells derived from CH-MSCs, PL-MSCs and UC-MSCs cultured in high glucose condition was significantly greater than those derived from their normal glucose controls (751.67±17.63 vs. 639.00±30.20, *P<0.05 for CH-MSCs; 919.00±18.93 vs. 707.33±42.83, *P<0.05 for PL-MSCs; and 803.33±23.31 vs.698.33±11.67, *P<0.05 for UC-MSCs)(Table 4.9 and Figure 4.29). In agreement with these results, the concentration of Oil Red O extracted from CH-MSCs, PL-MSCs and UC-MSCs cultured in high glucose was also significantly greater than those of their normal glucose controls (537.08±19.95 vs. 431.50±23.59, *P<0.05 for CH-MSCs; 463.17±3.97 vs. 510.67±15.55, *P<0.05 for PL-MSCs; and 251.08±14.74 vs. 189.42±8.43, *P<0.05 for CH-MSCs) (Table 4.10 and Figure 4.30)

In addition to the number of adipocyte-like cells, the gene expression study indicates that the mRNA levels of adipogenic gene *ADIPOQ* and *LPL* in CH-MSCs and UC-MSCs cultured in high glucose condition were also significantly higher than those of their normal glucose controls (Table 4.11, Figure 4.33 and 4.35). In contrast to *ADIPOQ* and *LPL*, the mRNA levels of other adipogenic genes including *PPARɣ*, *SREBP-1c* and *GLUT4* in CH-MSCs and UC-MSCs cultured in high glucose were not different from those of their normal glucose controls (Table 4.11, Figure 4.31, 4.32 and 4.34)

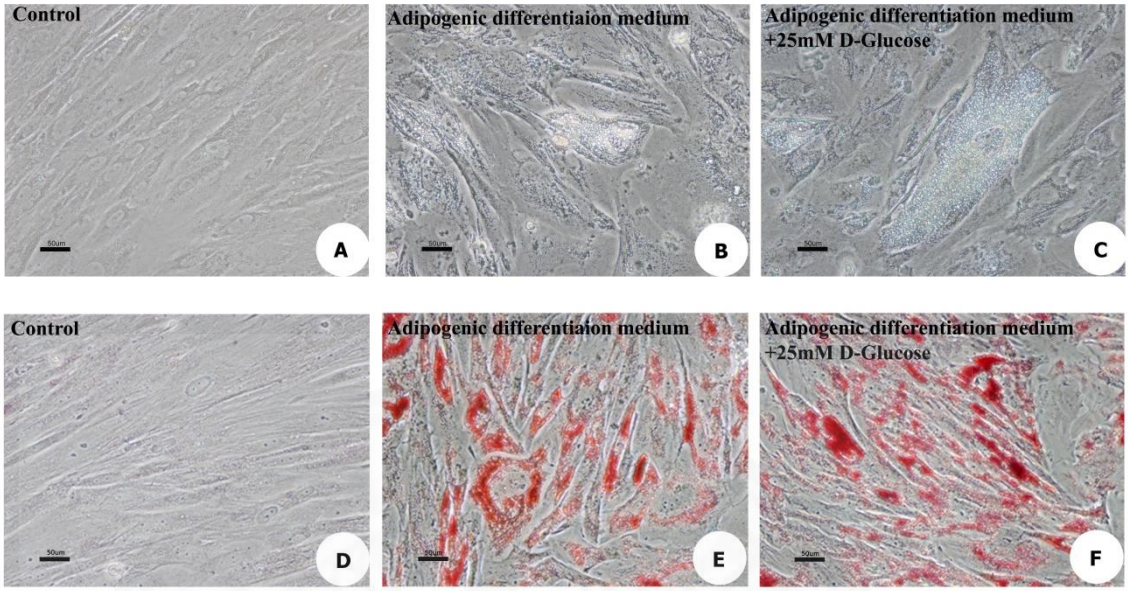

Figure 4.26 The effects of high glucose on adipogenic differentiation of CH-MSCs
$7.5x10^4$ 3^{rd}-5^{th}passageCH-MSCs were cultured in adipogenic differentiation medium supplemented with 25mM D-glucose as described in section 3.8.1. After 28 days of culture, the cells were stained with Oil Red O as described in section 3.8.1to determine the number of adipocyte-like cells. CH-MSCs cultured in adipogenic differentiation medium without glucose supplementation serve as normal glucose controls while CH-MSCs cultured in DMEM supplemented with 10% FBS serve as non-differentiation controls

A. Morphology of CH-MSCs cultured in DMEM supplemented with 10% FBS for 28 days
B. Morphology of CH-MSCs cultured in adipogenic differentiation medium for 28 days
C. Morphology of CH-MSCs cultured in adipogenic differentiation medium supplemented with 25mM D-glucose for 28 days
D. Morphology of CH-MSCs cultured in DMEM supplemented with 10% FBS for 28 days after staining with Oil Red O
E. Morphology of CH-MSCs cultured in adipogenic differentiation medium for 28 days after staining with Oil Red O.
F. Morphology of CH-MSCs cultured in adipogenic differentiation medium supplemented with 25mM D-glucose for 28 days after staining with Oil Red O

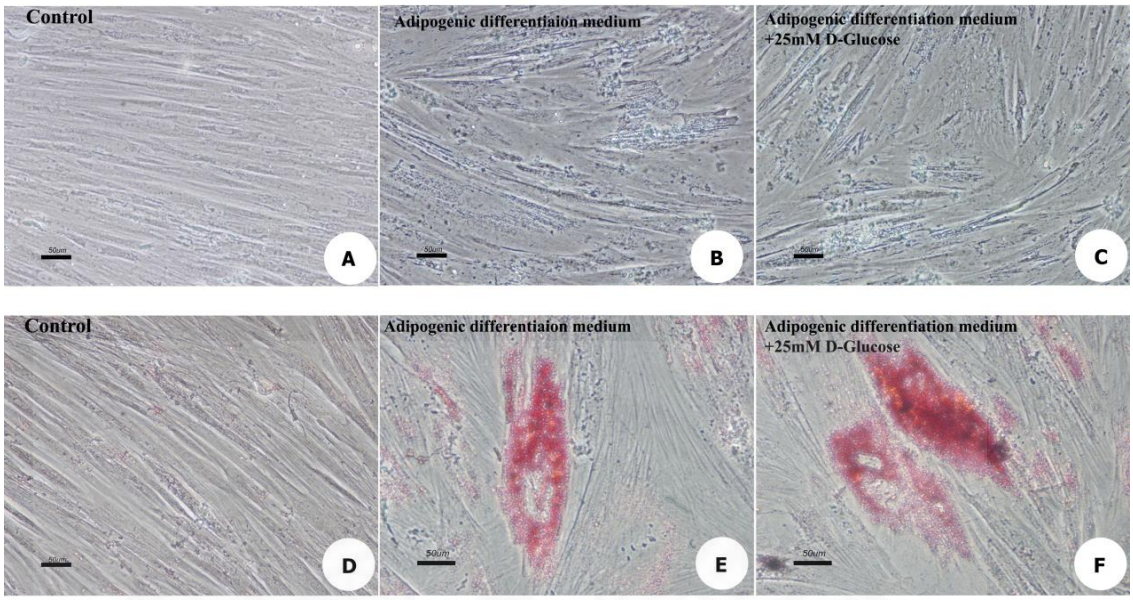

Figure 4.27 The effects of high glucose on adipogenic differentiation of PL-MSCs
$7.5x10^4$ 3^{rd}-5^{th}passagePL-MSCs were cultured in adipogenic differentiation medium supplemented with 25mM D-glucose as described in section 3.8.1. After 28 days of culture, the cells were stained with Oil Red O as described in section 3.8.1 to determine the number of adipocyte-like cells. PL-MSCs cultured in adipogenic differentiation medium without glucose supplementation serve as normal glucose controls while PL-MSCs cultured in DMEM supplemented with 10% FBS serve as non-differentiation controls

A. Morphology of PL-MSCs cultured in DMEM supplemented with 10% FBS for 28 days
B. Morphology of PL-MSCs cultured in adipogenic differentiation medium for 28 days
C. Morphology of PL-MSCs cultured in adipogenic differentiation medium supplemented with 25mM D-glucose for 28 days
D. Morphology of PL-MSCs cultured in DMEM supplemented with 10% FBS for 28 days after staining with Oil Red O
E. Morphology of PL-MSCs cultured in adipogenic differentiation medium for 28 days after staining with Oil Red O.
F. Morphology of PL-MSCs cultured in adipogenic differentiation medium supplemented with 25mM D-glucose for 28 days after staining with Oil Red O

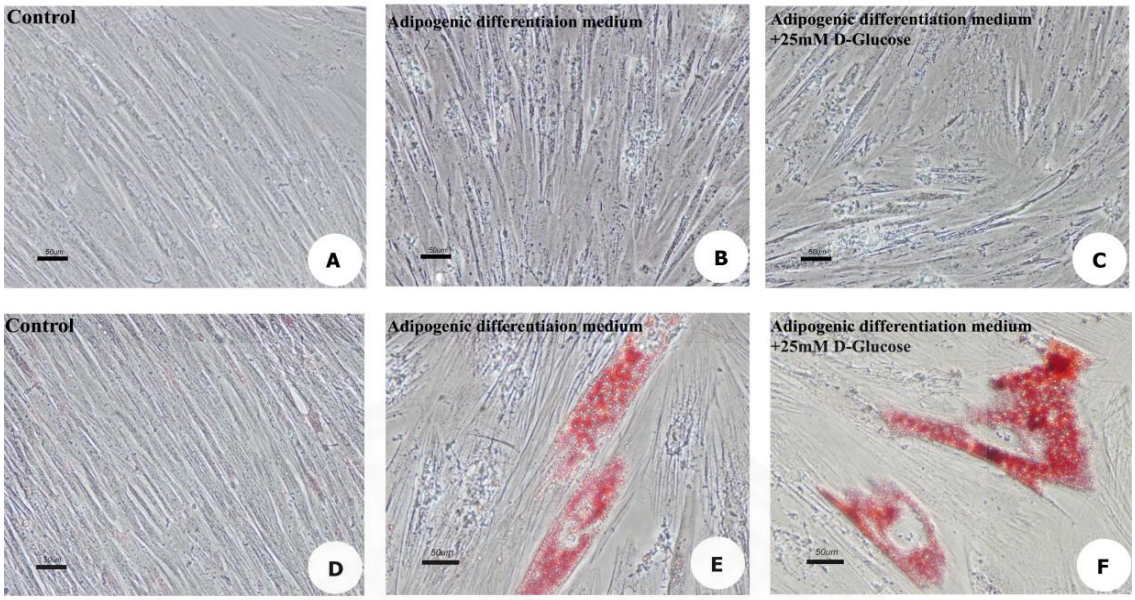

Figure 4.28 The effects of high glucose on adipogenic differentiation of UC-MSCs
$7.5x10^4$ 3^{rd}-5^{th} passage UC-MSCs were cultured in adipogenic differentiation medium supplemented with 25mM D-glucose as described in section 3.8.1. After 28 days of culture, the cells were stained with Oil Red O as described in section 3.8.1 to determine the number of adipocyte-like cells. UC-MSCs cultured in adipogenic differentiation medium without glucose supplementation serve as normal glucose controls while UC-MSCs cultured in DMEM supplemented with 10% FBS serve as non-differentiation controls

A. Morphology of UC-MSCs cultured in DMEM supplemented with 10% FBS for 28 days
B. Morphology of UC-MSCs cultured in adipogenic differentiation medium for 28 days
C. Morphology of UC-MSCs cultured in adipogenic differentiation medium supplemented with 25mM D-glucose for 28 days
D. Morphology of UC-MSCs cultured in DMEM supplemented with 10% FBS for 28 days after staining with Oil Red O
E. Morphology of UC-MSCs cultured in adipogenic differentiation medium for 28 days after staining with Oil Red O.
F. Morphology of UC-MSCs cultured in adipogenic differentiation medium supplemented with 25mM D-glucose for 28 days after staining with Oil Red O

Table 4.9 Number of adipocyte-like cells generated from CH-MSCs, PL-MSCs and UC-MSCs which were cultured in high glucose and normal glucose condition

Sample	Culture condition	Number of adipocyte like-cells (cells)
CH-MSCs	Adipogenic differentiation medium	639.00±30.20
	Adipogenic differentiation medium+25mM D-glucose	751.67±17.63
PL-MSCs	Adipogenic differentiation medium	707.33±42.83
	Adipogenic differentiation medium+25mM D-glucose	919.00±18.93
UC-MSCs	Adipogenic differentiation medium	698.33±11.67
	Adipogenic differentiation medium+25mM D-glucose	803.33±23.31

Data were presented as mean ± standard error of means (SEM) from three independent experiments

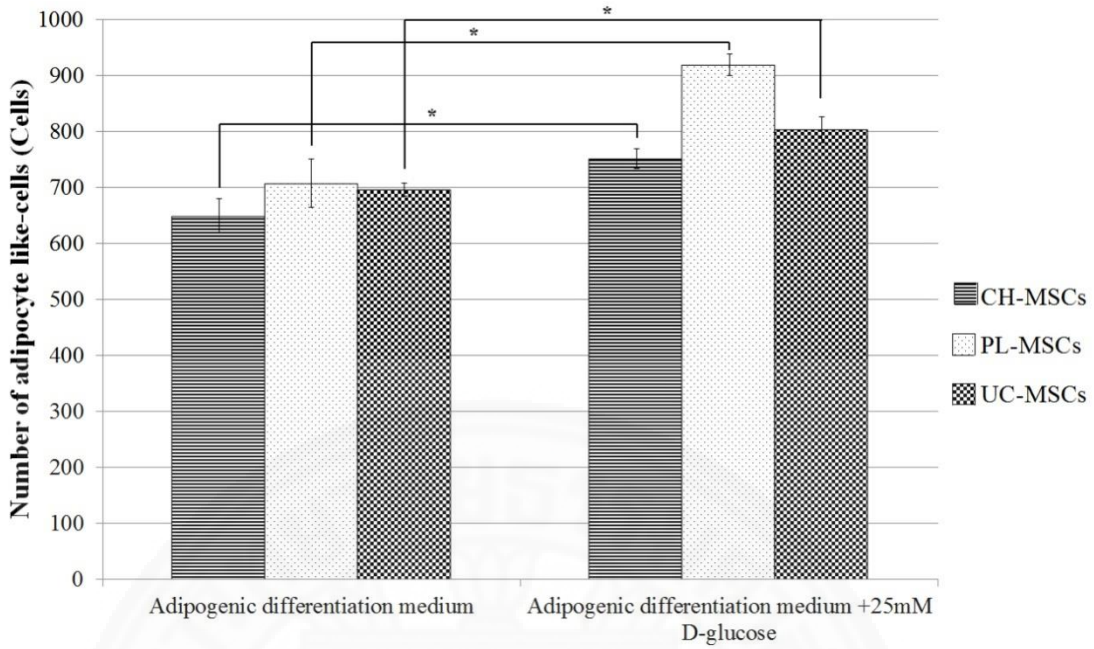

Figure 4.29 The number of adipocyte-like cells generated from CH-MSCs, PL-MSCs and UC-MSCs cultured in high glucose and normal glucose condition

Data were presented as mean ± standard error of means (SEM) from three independent experiments. * $P<0.05$ vs. adipogenic differentiation medium.

Table 4.10 The concentration of Oil Red O extracted from CH-MSCs, PL-MSCs and UC-MSCs cultured in high glucose and normal glucose condition

Sample	Culture conditions	Oil Red O concentation (µg/ml)
CH-MSCs	DMEM+10%FBS	195.42±15.66
	Adipogenic differentiation medium	431.50±23.59
	Adipogenic differentiation medium+25mM D-glucose	537.08±19.95
PL-MSCs	DMEM+10%FBS	223.58±3.70
	Adipogenic differentiation medium	463.17±3.97
	Adipogenic differentiation medium+25mM D-glucose	510.67±15.55
UC-MSCs	DMEM+10%FBS	149.42±10.64
	Adipogenic differentiation medium	189.42±8.43
	Adipogenic differentiation medium+25mM D-glucose	251.08±14.74

After Oil Red O staining, the Oil Red O was extracted from CH-MSCs, PL-MSCs and UC-MSCs cultured in high glucose condition and its concentration was determined as described in section 3.8.1. CH-MSCs, PL-MSCs and UC-MSCs cultured in adipogenic differentiation medium without glucose supplementation serve as normal glucose controls while CH-MSCs, PL-MSCs and UC-MSCs cultured in DMEM supplemented with 10% FBS serve as non-differentiation controls. Data were presented as mean ± standard error of means (SEM) from three independent experiments. * $P<0.05$ vs. adipogenic differentiation medium.

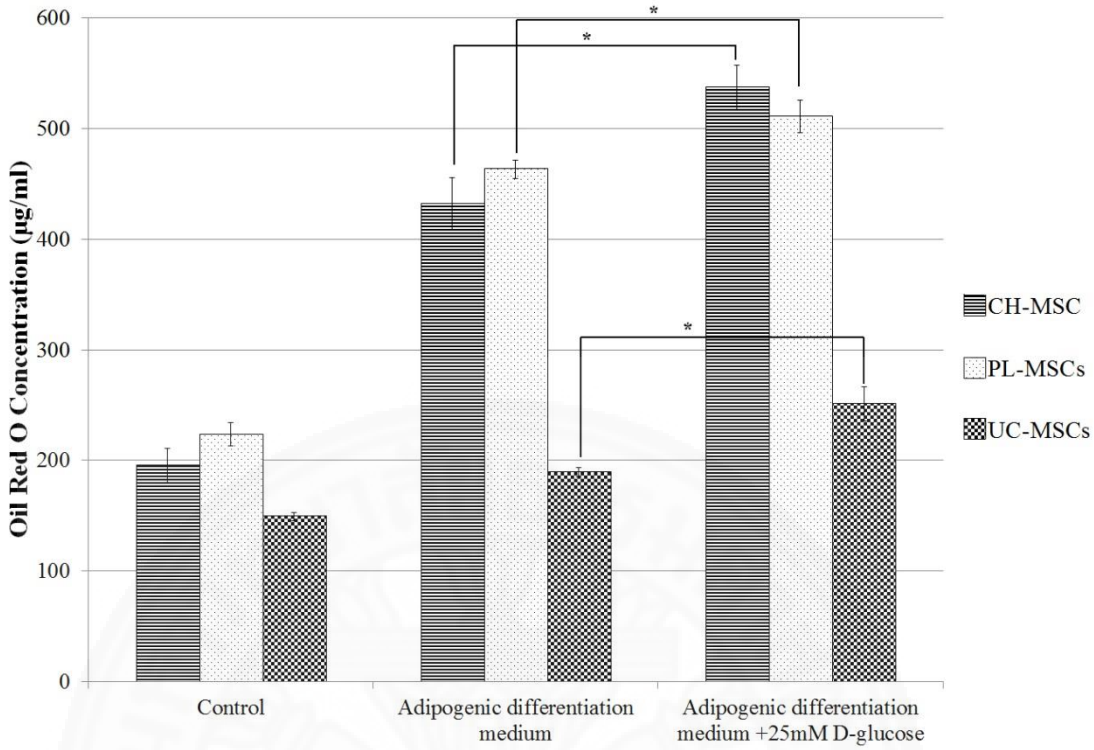

Figure 4.30The concentration of Oil Red O extracted from CH-MSCs, PL-MSCs and UC-MSCs cultured in high glucose and normal glucose condition

Data were presented as mean ± standard error of means (SEM) from three independent experiments. * $P<0.05$ vs. adipogenic differentiation medium.

Table 4.11 Relative mRNA levels of adipogenic genes in CH-MSCs, PL-MSCs and UC-MSCs cultured in high glucose and normal glucose condition

MSCs	Genes	DMEM +10%FBS	Adipogenic differentiation medium	Adipogenic differentiation medium +25mM D-glucose
CH-MSCs	*PPARg*	1.00±0.00	2.54±0.97	2.69±0.92
	SREBP-1c	1.00±0.00	1.42±0.17	2.11±0.41
	ADIPOQ	1.00±0.00	1.30±0.38	4.68±0.92
	GLUT4	1.00±0.00	0.94±0.26	2.74±0.18
	LPL	1.00±0.00	1.65±0.14	3.34±0.49
PL-MSCs	*PPARg*	1.00±0.00	0.04±0.01	0.04±0.00
	SREBP-1c	1.00±0.00	0.57±0.19	0.90±0.31
	ADIPOQ	1.00±0.00	1.01±0.54	1.75±0.72
	GLUT4	1.00±0.00	0.55±0.19	0.89±0.32
	LPL	1.00±0.00	0.63±0.34	1.20±0.30
UC-MSCs	*PPARg*	1.00±0.00	5.26±1.38	3.52±0.60
	SREBP-1c	1.00±0.00	2.47±0.12	3.85±0.58
	ADIPOQ	1.00±0.00	1.51±0.22	8.21±1.55
	GLUT4	1.00±0.00	1.57±0.22	2.55±0.80
	LPL	1.00±0.00	3.66±0.19	9.17±0.97

$2x10^5$ 3^{rd}-5^{th}CH-MSCs, PL-MSCs and UC-MSCs were cultured in adipogenic differentiation medium supplemented with 25mM D-glucose as described in section 3.10.1. After 28 days of culture, the mRNA levels of adipogenic genes *PPARɣ*, *SREBP-1c*, *ADIPOQ*, *GLUT4* and *LPL* were determined by qRT-PCR as described in section 3.10.3. The mRNA levels of all adipogenic genes of BM-MSCs cultured in adipogenic differentiation medium with or without glucose supplementation were compared relatively to the expression levels of those genes in CH-MSCs, PL-MSCs and UC-MSCs cultured in DMEM + 10% FBS. Data were presented as mean ± SEM of 3 independent experiments.

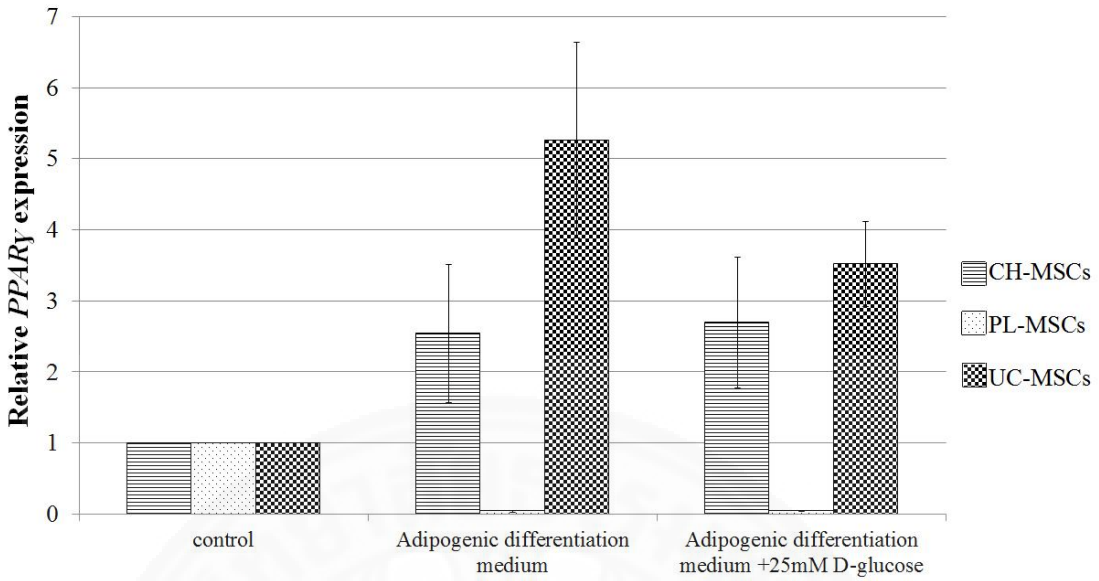

Figure 4.31The relative mRNA levels of *PPARγ* gene in CH-MSCs, PL-MSCs and UC-MSCs cultured in high glucose and normal glucose condition

The mRNA levels of *PPARγ* gene of CH-MSCs, PL-MSCs and UC-MSCs cultured in adipogenic differentiation medium with or without glucose supplementation were compared relatively to the expression levels of *PPARγ* gene of CH-MSCs, PL-MSCs and UC-MSCs cultured in DMEM + 10% FBS. Data were presented as mean ± standard error of means (SEM) of three independent experiments. *P<0.05 vs. adipogenic differentiation medium

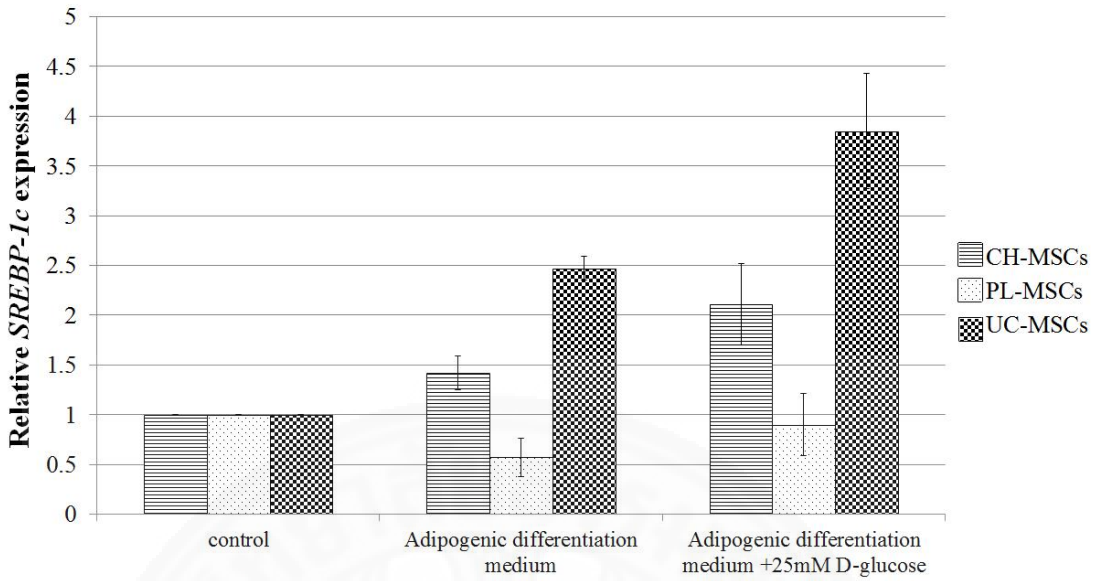

Figure 4.32The relative mRNA levels of *SREBP-1c* gene in CH-MSCs, PL-MSCs and UC-MSCs cultured in high glucose and normal glucose condition

The mRNA levels of *SREBP-1*c gene of CH-MSCs, PL-MSCs and UC-MSCs cultured in adipogenic differentiation medium with or without glucose supplementation were compared relatively to the expression levels of *SREBP-1c* gene of CH-MSCs, PL-MSCs and UC-MSCs cultured in DMEM + 10% FBS. Data were presented as mean ± standard error of means (SEM) of three independent experiments. *P<0.05 vs. adipogenic differentiation medium

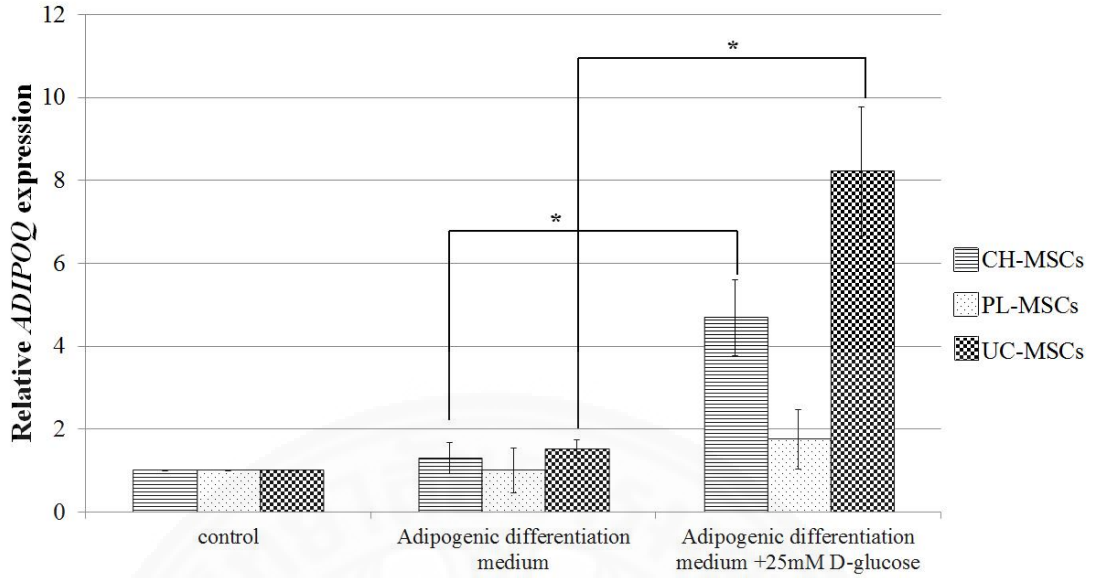

Figure 4.33 The relative mRNA levels of *ADIPOQ* gene in CH-MSCs, PL-MSCs and UC-MSCs cultured in high glucose and normal glucose condition

The mRNA levels of *ADIPOQ* gene of CH-MSCs, PL-MSCs and UC-MSCs cultured in adipogenic differentiation medium with or without glucose supplementation were compared relatively to the expression levels of *ADIPOQ* gene of CH-MSCs, PL-MSCs and UC-MSCs cultured in DMEM + 10% FBS. Data were presented as mean ± standard error of means (SEM) of three independent experiments. *P<0.05 vs. adipogenic differentiation medium

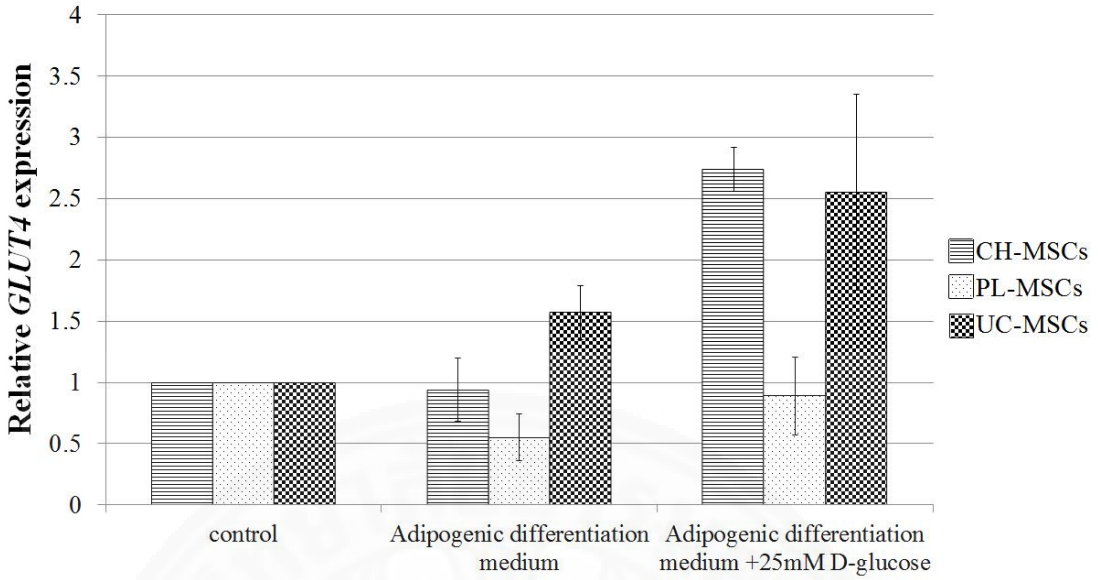

Figure 4.34The relative mRNA levels of *GLUT4* gene in CH-MSCs, PL-MSCs and UC-MSCs cultured in high glucose and normal glucose condition

The mRNA levels of *GLUT4* gene of CH-MSCs, PL-MSCs and UC-MSCs cultured in adipogenic differentiation medium with or without glucose supplementation were compared relatively to the expression levels of *GLUT4* gene of CH-MSCs, PL-MSCs and UC-MSCs cultured in DMEM + 10% FBS. Data were presented as mean ± standard error of means (SEM) of three independent experiments. *P<0.05 vs. adipogenic differentiation medium

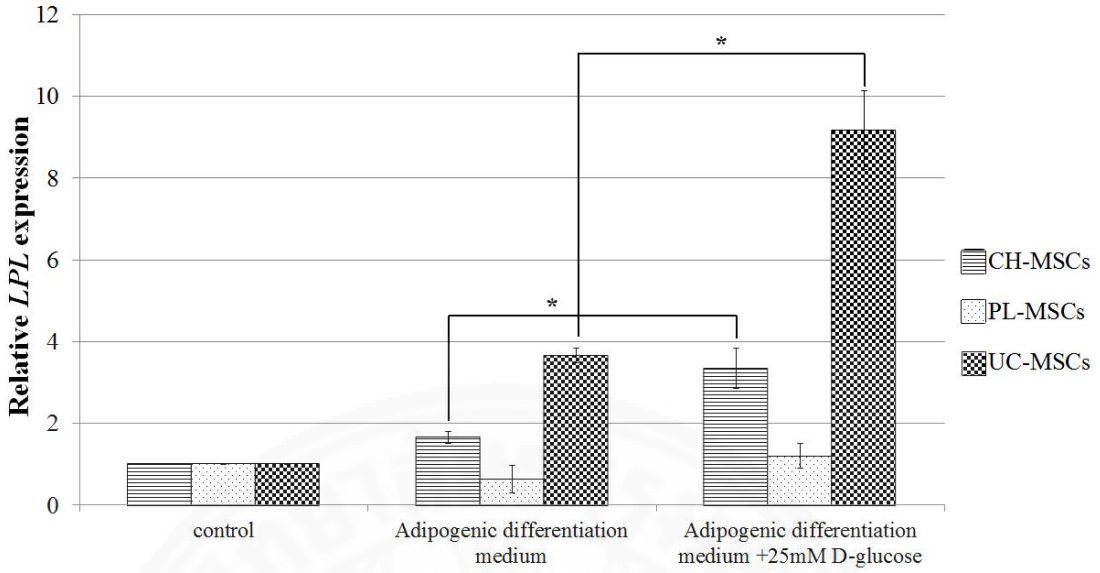

Figure 4.35The relative mRNA levels of *LPL* gene in CH-MSCs, PL-MSCs and UC-MSCs cultured in high glucose and normal glucose condition

The mRNA levels of *LPL* gene of CH-MSCs, PL-MSCs and UC-MSCs cultured in adipogenic differentiation medium with or without glucose supplementation were compared relatively to the expression levels of *LPL* gene of CH-MSCs, PL-MSCs and UC-MSCs cultured in DMEM + 10% FBS. Data were presented as mean ± standard error of means (SEM) of three independent experiments. *P<0.05 vs. adipogenic differentiation medium

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4.4 The effect of high glucose on osteogenic differentiation of MSCs

To study the effect of high glucose on the osteogenic differentiation of BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs, $2x10^5$ 3rd-5th passage MSCs from those 4 sources were cultured in osteogenic differentiation medium supplemented with 25mM D-glucose as described in section 3.10.1. At culture day 7 and 14, the expression levels of osteogenic gene *Runx2, OSX*, and *OCN* were determined by qRT-PCR. The cultures were also subjected to alkaline phosphatase assay as described in section 3.9.1 to determine the levels of osteogenic differentiation at culture day 14 (Figure 4.26-4.28).

Although, the results show that the mRNA levels of osteogenic gene *Runx2* in CH-MSCs and UC-MSCs, as well as *OSX* in CH-MSCs, PL-MSCs and UC-MSCs cultured in high glucose condition were significantly lower than those of their normal glucose controls at culture day 7 (Table 4.12 and Figure 4.40-4.41), the differences in mRNA levels of those genes could not be detected at culture day 14 (Table 4.13 and Figure 4.43-4.44). In contrast to *Runx2* and *OSX*, the mRNA levels of *OCN* in CH-MSCs, PL-MSCs and UC-MSCs cultured in high glucose were not different from those of their normal glucose controls at both time points (Table 4.12-4.13, Figure 4.43 and 4.45).

In case of BM-MSCs, there was no difference in the mRNA levels of all osteogenic gene examined at both time points, with the exception of *OCN* in which its mRNA levels in BM-MSCs cultured in high glucose condition were significantly lower than those of their normal glucose controls at culture day 14 (Table 4.12-4.13 and Figure 4.40-4.45)

In agreement with the gene expression study, the alkaline phosphatase activity assay showed that the levels of osteogenic differentiation of BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs in high glucose condition were not obviously different from those of their normal glucose counterparts (Figure 4.42-4.45)

Table 4.12 Relative mRNA levels of osteogenic genes in BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs cultured in high glucose and normal glucose condition at culture day 7

MSCs	Genes	DMEM +10%FBS	Osteogenic differentiation medium	Osteogenic differentiation medium +25mM D-glucose
BM-MSCs	*Runx2*	1.00±0.00	3.92±0.67	2.66±0.64
	OSX	1.00±0.00	1.26±0.22	1.19±0.10
	OCN	1.00±0.00	1.81±0.44	1.05±0.08
CH-MSCs	*Runx2*	1.00±0.00	2.12±0.04	1.43±0.17
	OSX	1.00±0.00	4.65±0.47	1.85±0.27
	OCN	1.00±0.00	1.22±0.72	1.32±0.41
PL-MSCs	*Runx2*	1.00±0.00	2.19±0.30	1.28±0.39
	OSX	1.00±0.00	2.58±0.22	1.25±0.10
	OCN	1.00±0.00	1.30±0.33	1.13±0.26
UC-MSCs	*Runx2*	1.00±0.00	2.89±0.43	1.49±0.21
	OSX	1.00±0.00	1.37±0.08	1.07±0.4
	OCN	1.00±0.00	1.46±0.18	1.35±0.24

$2x10^5$BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs were cultured in osteogenic differentiation medium supplemented with 25mM D-glucose as described in section 3.10.1. After 7 days of culture, the mRNA levels of osteogenic genes *Runx2*, *OSX* and *OCN* were determined by qRT-PCR as described in section3.10.3. The mRNA levels of all osteogenic genes of BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs cultured in osteogenic differentiation medium with or without glucose supplementation were compared relatively to the expression levels of those genes in CH-MSCs, PL-MSCs and UC-MSCs cultured in DMEM + 10% FBS. Data were presented as mean ± SEM of 3 independent experiments.

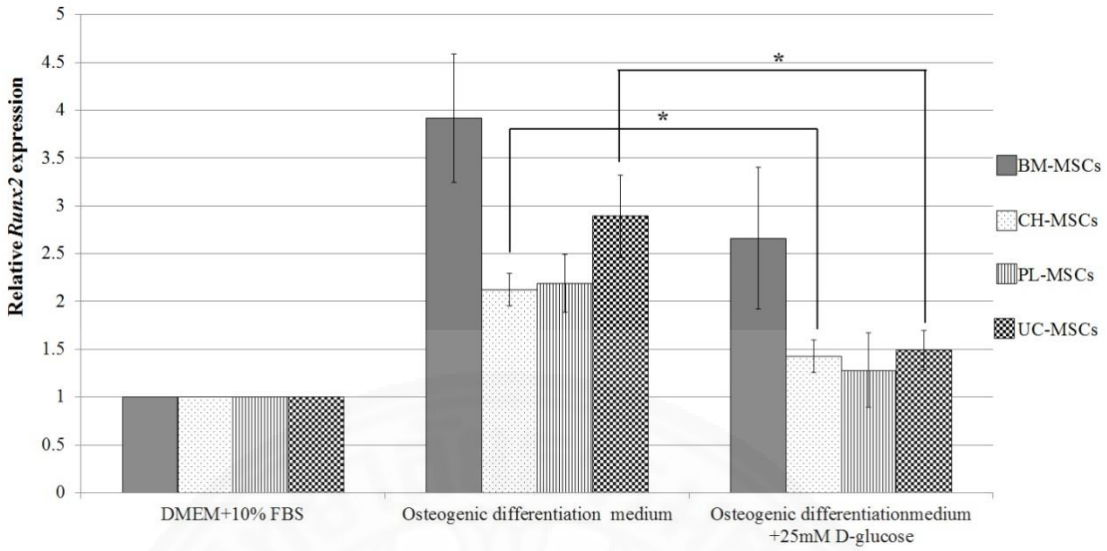

Figure 4.36The relative mRNA levels of *Runx2* gene in BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs cultured in high glucose and normal glucose condition at culture day 7

The mRNA levels of *Runx2* gene of BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs cultured in osteogenic differentiation medium with or without glucose supplementation were compared relatively to the expression levels of *Runx2* gene of BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs cultured in DMEM + 10% FBS. Data were presented as mean ± standard error of means (SEM) of three independent experiments. *P<0.05 vs. osteogenic differentiation medium

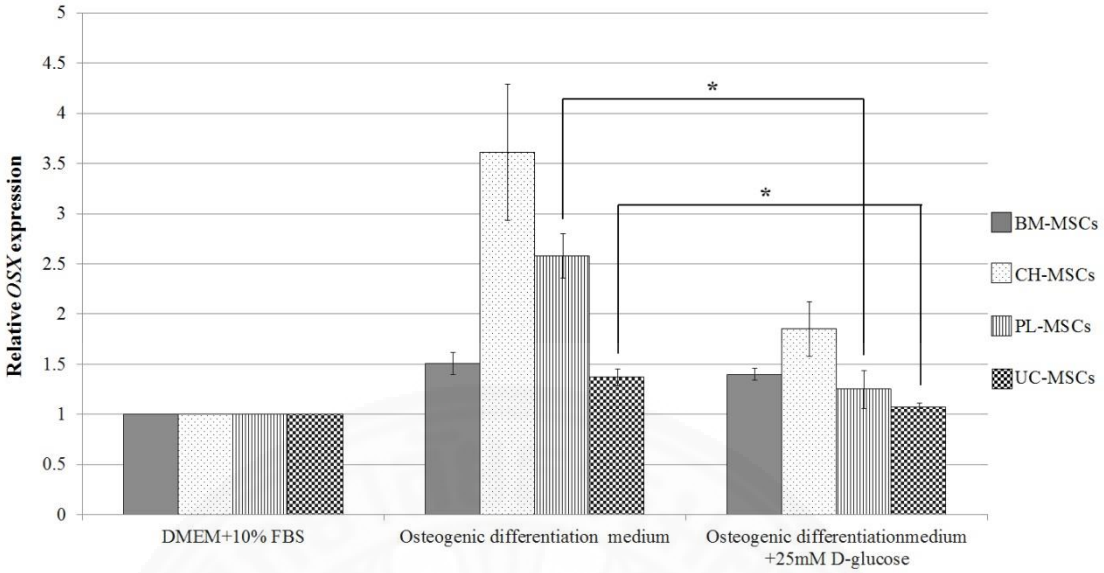

Figure 4.37The relative mRNA levels of *OSX* gene in BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs cultured in high glucose and normal glucose condition at culture day 7

The mRNA levels of *OSX* gene of BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs cultured in osteogenic differentiation medium with or without glucose supplementation were compared relatively to the expression levels of *OSX* gene of BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs cultured in DMEM + 10% FBS. Data were presented as mean ± standard error of means (SEM) of three independent experiments. *P<0.05 vs. osteogenic differentiation medium

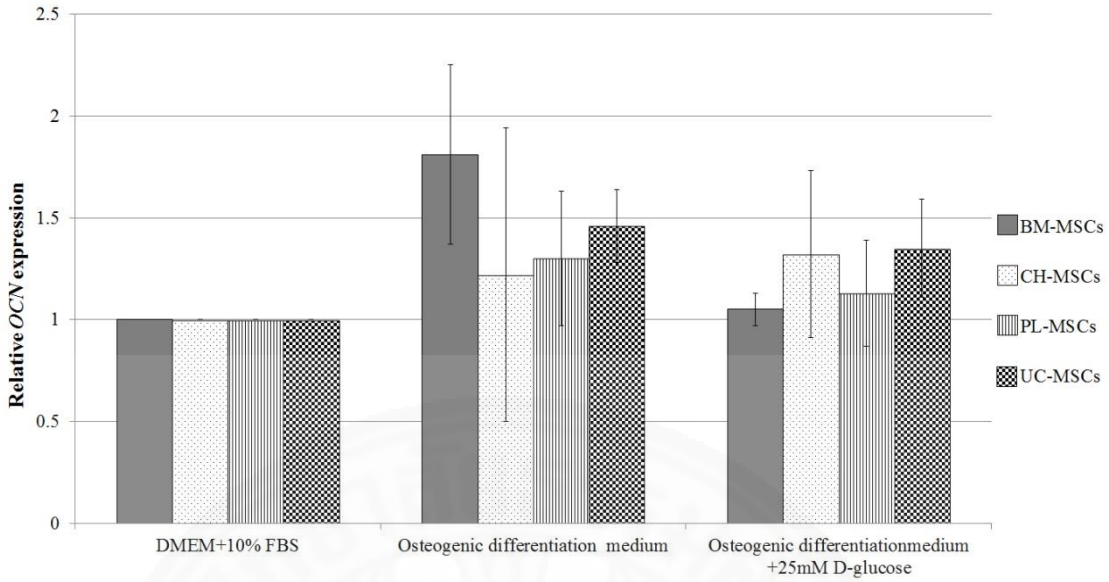

Figure 4.38The relative mRNA levels of *OCN* gene in BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs cultured in high glucose and normal glucose condition at culture day 7

The mRNA levels of *OCN* gene of BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs cultured in osteogenic differentiation medium with or without glucose supplementation were compared relatively to the expression levels of *OCN* gene of BM-MNCs, CH-MSCs, PL-MSCs and UC-MSCs cultured in DMEM + 10% FBS. Data were presented as mean ± standard error of means (SEM) of three independent experiments. *P<0.05 vs. osteogenic differentiation medium

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Table 4.13 Relative mRNA levels of osteogenic genes in BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs cultured in high glucose and normal glucose condition at culture day 14

MSCs	Genes	DMEM +10%FBS	Osteogenic differentiation medium	Osteogenic differentiation medium +25mM D-glucose
BM-MSCs	*Runx2*	1.00±0.00	5.86±0.66	5.87±0.97
	OSX	1.00±0.00	2.36±0.63	1.80±0.50
	OCN	1.00±0.00	1.65±0.15	1.01±0.15
CH-MSCs	*Runx2*	1.00±0.00	2.00±0.90	1.68±0.44
	OSX	1.00±0.00	4.18±1.14	3.69±0.70
	OCN	1.00±0.00	4.10±0.28	1.90±0.93
PL-MSCs	*Runx2*	1.00±0.00	2.19±0.19	1.61±0.36
	OSX	1.00±0.00	1.53±0.22	1.39±0.11
	OCN	1.00±0.00	1.68±0.80	1.35±0.66
UC-MSCs	*Runx2*	1.00±0.00	4.90±0.52	3.05±0.54
	OSX	1.00±0.00	5.30±1.72	3.33±1.20
	OCN	1.00±0.00	2.04±0.66	1.45±0.28

$2x10^5$BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs were cultured in osteogenic differentiation medium supplemented with 25mM D-glucose as described in section 3.10.1. After 14 days of culture, the mRNA levels of osteogenic genes *Runx2*, *OSX* and *OCN* were determined by qRT-PCR as described in section 3.10.3. The mRNA levels of all osteogenic genes of BM-MSCs CH-MSCs, PL-MSCs and UC-MSCs cultured in osteogenic differentiation medium with or without glucose supplementation were compared relative to the expression levels of those genes in BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs cultured in DMEM + 10% FBS. Data were presented as mean ± SEM of 3 independent experiments.

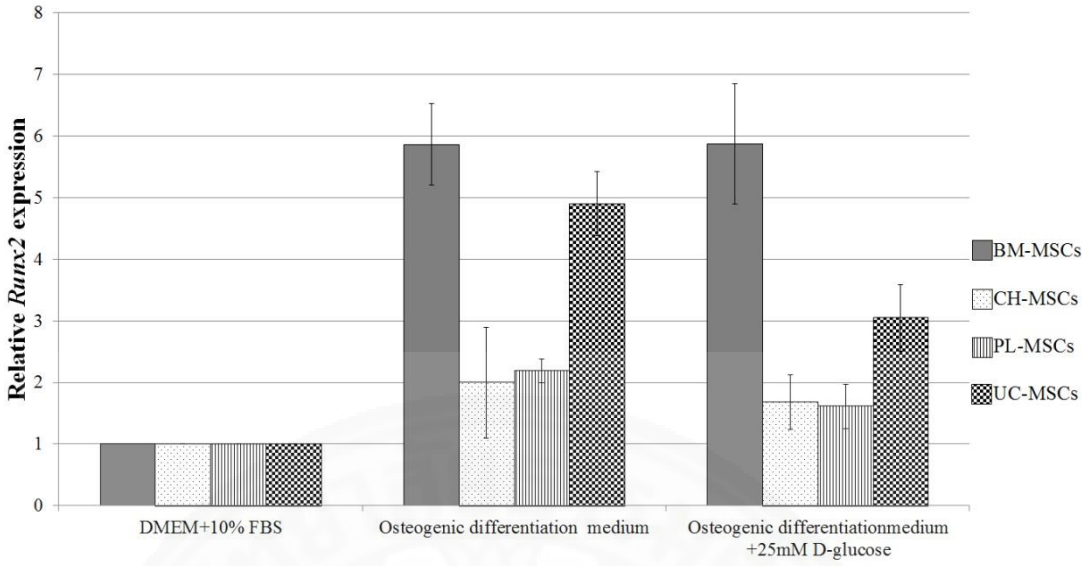

Figure 4.39The relative mRNA levels of *Runx2* gene in BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs cultured in high glucose and normal glucose condition at culture day 14

The mRNA levels of *Runx2* gene of BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs cultured in osteogenic differentiation medium with or without glucose supplementation were compared relatively to the expression levels of *Runx2*gene of BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs cultured in DMEM + 10% FBS. Data were presented as mean ± standard error of means (SEM) of three independent experiments. *P<0.05 vs. osteogenic differentiation medium

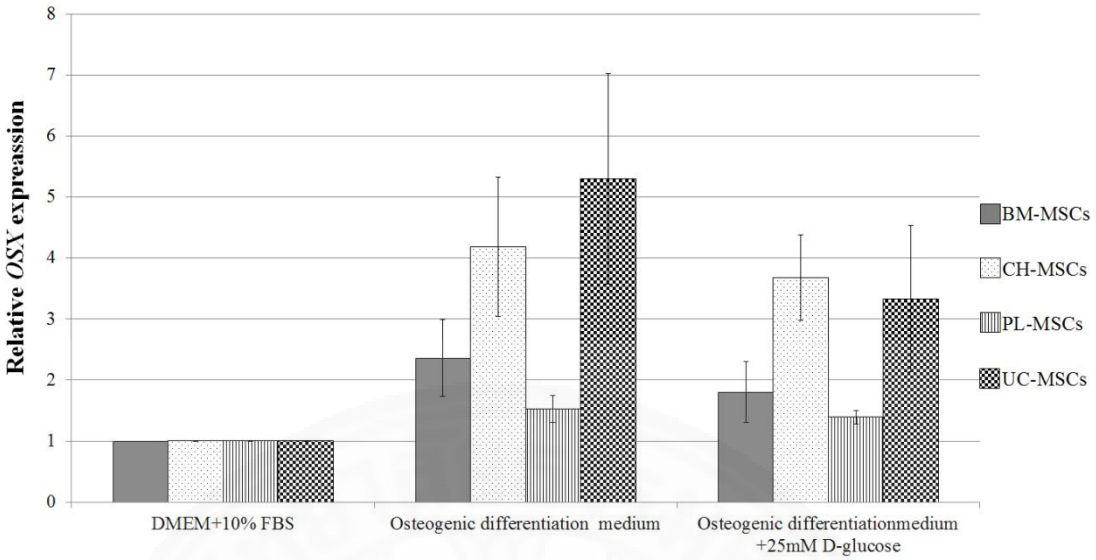

Figure 4.40The relative mRNA levels of *OSX* gene in BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs cultured in high glucose and normal glucose condition at culture day 14

The mRNA levels of *OSX* gene of BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs cultured in osteogenic differentiation medium with or without glucose supplementation were compared relative to the expression levels of *OSX* gene of BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs cultured in DMEM + 10% FBS. Data were presented as mean ± standard error of means (SEM) of three independent experiments. *P<0.05 vs. osteoogenic differentiation medium

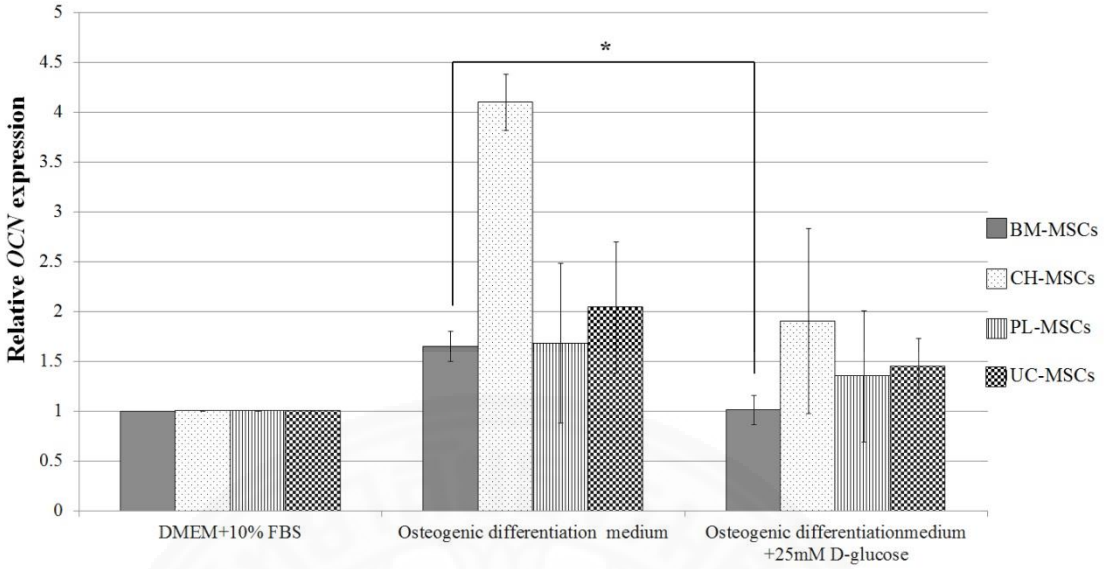

Figure 4.41The relative mRNA levels of *OCN* gene in BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs cultured in high glucose and normal glucose condition at culture day 14

The mRNA levels of *OCN* gene of BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs cultured in osteogenic differentiation medium with or without glucose supplementation were compared relatively to the expression levels of *OCN* gene of BM-MSC, CH-MSCs, PL-MSCs and UC-MSCs s cultured in DMEM + 10% FBS. Data were presented as mean ± standard error of means (SEM) of three independent experiments. *P<0.05 vs. osteogenic differentiation medium

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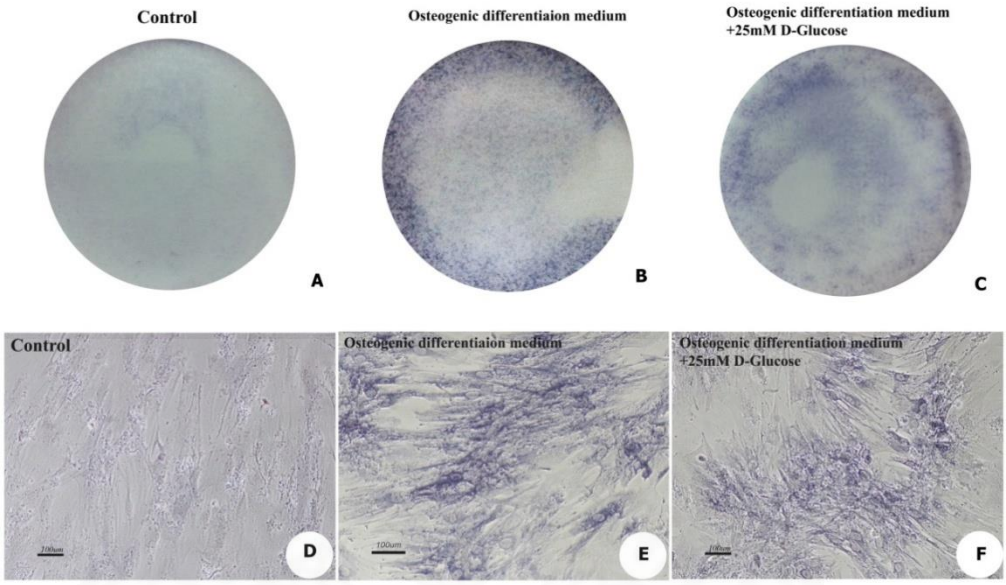

Figure 4.42 The effects of high glucose on osteogenic differentiation of BM-MSCs

$4.5x10^4$ 3rd-5th passage BM-MSCs were cultured in osteogenic differentiation medium supplemented with 25mM D-glucose. After 14 days of culture, the cells were stained subjected to alkaline phosphatase activity assay as described in section 3.9.1 to determine the levels of osteogenic differentiation. BM-MSCs cultured in osteogenic differentiation medium without glucose supplementation serve as normal glucose controls while BM-MSCs cultured in DMEM supplemented with 10% FBS serve as non-differentiation controls

A. Morphology of BM-MSCs cultured in DMEM supplemented with 10% FBS for 14 days
B. Morphology of BM-MSCs cultured in osteogenic differentiation medium for 14 days
C. Morphology of BM-MSCs cultured in osteogenic differentiation medium supplemented with 25mM D-glucose for 14 days
D. Morphology of BM-MSCs cultured in DMEM supplemented with 10% FBS for 14 days after subjected to alkaline phosphatase activity assay
E. Morphology of BM-MSCs cultured in osteogenic differentiation medium for 14 days after subjected to alkaline phosphatase activity assay
F. Morphology of BM-MSCs cultured in osteogenic differentiation medium supplemented with 25mM D-glucose for 14 days after subjected to alkaline phosphatase activity assay

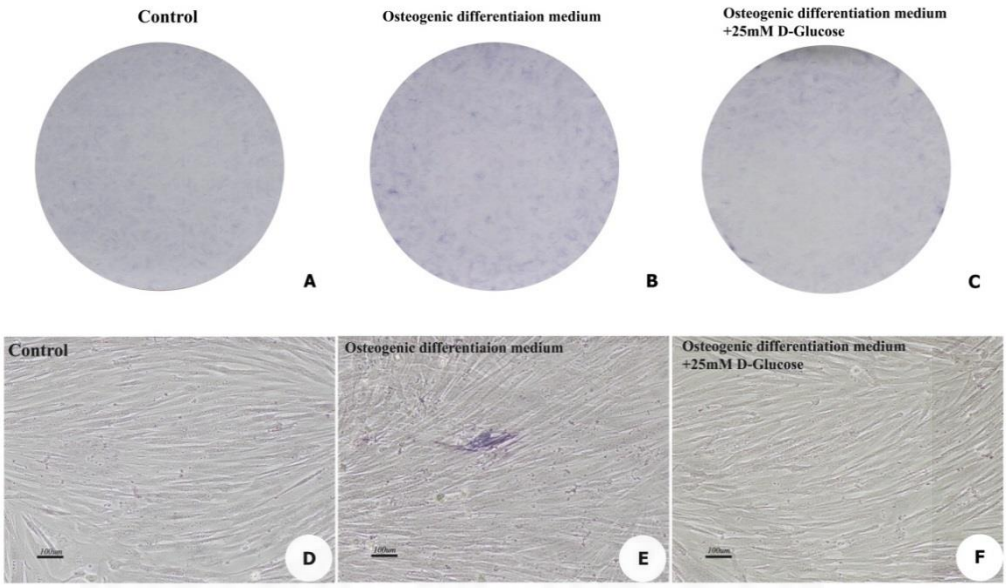

Figure 4.43The effects of high glucose on osteogenic differentiation of CH-MSCs
$4.5x10^4$3rd-5th passage CH-MSCs were cultured in osteogenic differentiation medium supplemented with 25mM D-glucose. After 14 days of culture, the cells were stained subjected to alkaline phosphatase activity assay as described in section 3.9.1 to determine the levels of osteogenic differentiation. CH-MSCs cultured in osteogenic differentiation medium without glucose supplementation serve as normal glucose controls while CH-MSCs cultured in DMEM supplemented with 10% FBS serve as non-differentiation controls

A. Morphology of CH-MSCs cultured in DMEM supplemented with 10% FBS for 14 days
B. Morphology of CH-MSCs cultured in osteogenic differentiation medium for 14 days
C. Morphology of CH-MSCs cultured in osteogenic differentiation medium supplemented with 25mM D-glucose for 14 days
D. Morphology of CH-MSCs cultured in DMEM supplemented with 10% FBS for 14 days after subjected to alkaline phosphatase activity assay
E. Morphology of CH-MSCs cultured in osteogenic differentiation medium for 14 days after subjected to alkaline phosphatase activity assay
F. Morphology of CH-MSCs cultured in osteogenic differentiation medium supplemented with 25mM D-glucose for 14 days after subjected to alkaline phosphatase activity assay

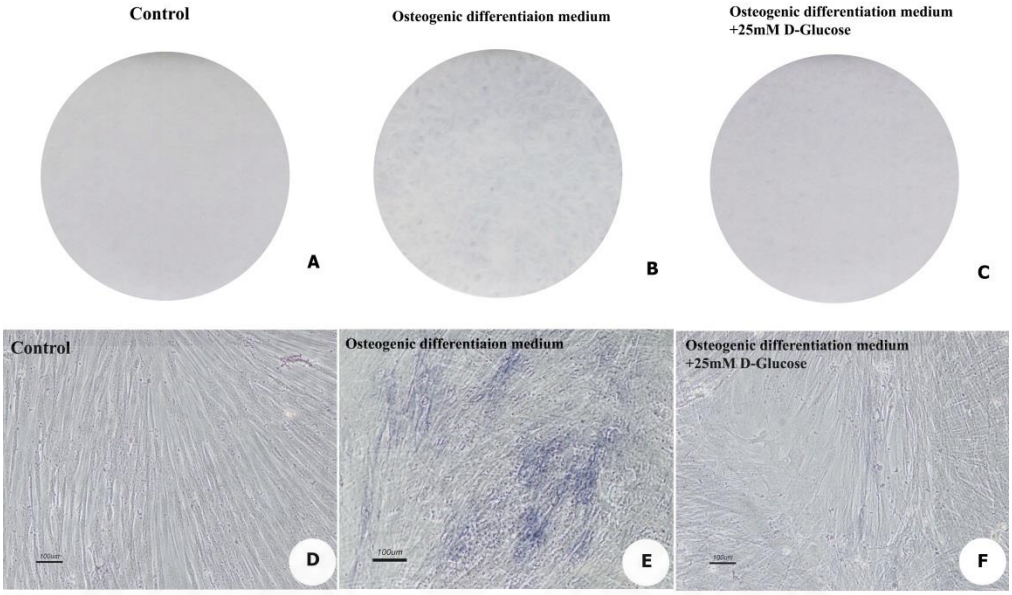

Figure 4.44The effects of high glucose on osteogenic differentiation of PL-MSCs
$4.5x10^4$3rd-5th passage PL-MSCs were cultured in osteogenic differentiation medium supplemented with 25mM D-glucose. After 14 days of culture, the cells were stained subjected to alkaline phosphatase activity assay as described in section 3.9.1 to determine the levels of osteogenic differentiation. PL-MSCs cultured in osteogenic differentiation medium without glucose supplementation serve as normal glucose controls while PL-MSCs cultured in DMEM supplemented with 10% FBS serve as non-differentiation controls

A. Morphology of PL-MSCs cultured in DMEM supplemented with 10% FBS for 14 days
B. Morphology of PL-MSCs cultured in osteogenic differentiation medium for 14 days
C. Morphology of PL-MSCs cultured in osteogenic differentiation medium supplemented with 25mM D-glucose for 14 days
D. Morphology of PL-MSCs cultured in DMEM supplemented with 10% FBS for 14 days after subjected to alkaline phosphatase activity assay
E. Morphology of PL-MSCs cultured in osteogenic differentiation medium for 14 days after subjected to alkaline phosphatase activity assay
F. Morphology of PL-MSCs cultured in osteogenic differentiation medium supplemented with 25mM D-glucose for 14 days after subjected to alkaline phosphatase activity assay

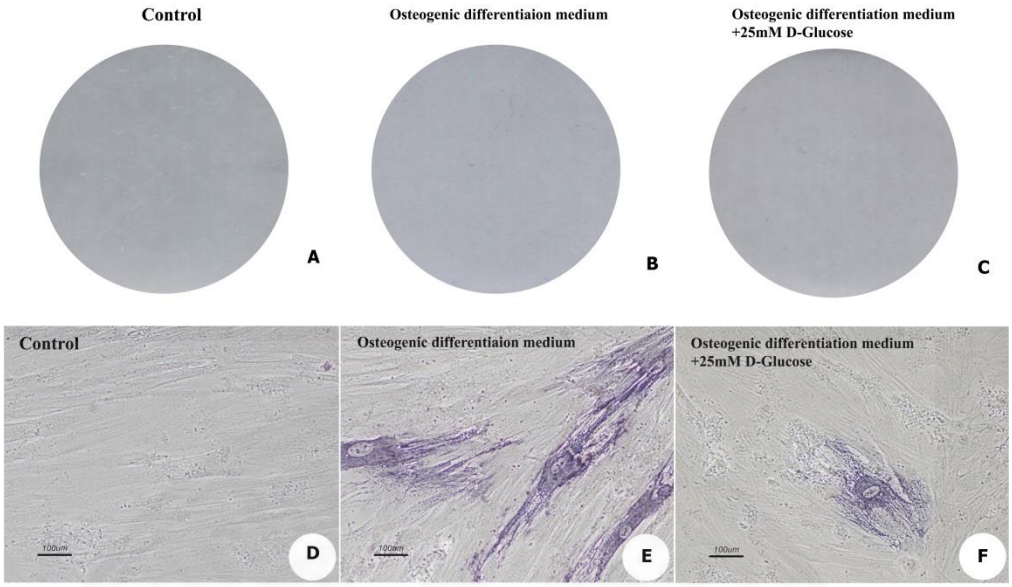

Figure 4.45 The effects of high glucose on osteogenic differentiation of UC-MSCs
$4.5x10^4$ 3rd-5th passage UC-MSCs were cultured in osteogenic differentiation medium supplemented with 25mM D-glucose. After 14 days of culture, the cells were stained subjected to alkaline phosphatase activity assay as described in section 3.9.1 to determine the levels of osteogenic differentiation. UC-MSCs cultured in osteogenic differentiation medium without glucose supplementation serve as normal glucose controls while CH-MSCs cultured in DMEM supplemented with 10% FBS serve as non-differentiation controls

A. Morphology of UC-MSCs cultured in DMEM supplemented with 10% FBS for 14 days
B. Morphology of UC-MSCs cultured in osteogenic differentiation medium for 14 days
C. Morphology of UC-MSCs cultured in osteogenic differentiation medium supplemented with 25mM D-glucose for 14 days
D. Morphology of UC-MSCs cultured in DMEM supplemented with 10% FBS for 14 days after subjected to alkaline phosphatase activity assay
E. Morphology of UC-MSCs cultured in osteogenic differentiation medium for 14 days after subjected to alkaline phosphatase activity assay
F. Morphology of UC-MSCs cultured in osteogenic differentiation medium supplemented with 25mM D-glucose for 14 days after subjected to alkaline phosphatase activity assay

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

Diabetes mellitus, a disease characterized by high blood glucose level, is usually resulted from an insufficient insulin production or a decrease responsiveness of the body to insulin. It has been estimated that the number of diabetic patients, which is approximately 382 million in 2013, will increase to at least 592 million by the end of 2035 (17). Patients with diabetes have an increased risk of developing several serious long-term complications, such as cardiovascular disease (CVD), retinopathy, nephropathy, neuropathy, diabetic foot, metabolic bone diseases and osteoporosis, making diabetes a serious health problem and a leading cause of morbidity and mortality in non-communicable diseases. Moreover, most type 2 diabetic patients are obese who have an increased percentage of visceral fat accumulated in their abdominal area. Those visceral fat release several factors including fatty acids, glycerol, hormones, pro-inflammatory cytokines that increase insulin resistance rendering diabetic treatment ineffective (22).

Mesenchymal stem cells or mesenchymal stromal cells (MSCs) were isolated for the first time from human bone marrow in 1976 by Friedenstein and colleague (4). MSCs have potential to differentiate to various mesodermal lineages, including bone, cartilage, fat and other marrow stromal connective tissues which play important roles in normal hemopoiesis. Apart from the bone marrow, MSCs are also located in the other tissue of the human body such as umbilical cord tissue (7), chorion tissue and placenta tissue (8, 9). MSCs that isolated from other tissue of human body were a popular choice for preclinical research and clinical trials of variety of diseases (10)

It has been previously reported that adipocytes which are the major cellular parts of fat tissues are derived, at least in part, from mesenchymal stem cells(3). Although the effects of high glucose on the proliferation and adipogenic differentiation of BM-MSCs have been previously reported, the effects of high glucose on the properties of gestational tissue-derived MSCs, especially their adipogenic and osteogenic differentiation capacity are currently unknown.

In the present study, we successfully isolated MSCs from several gestational tissues including chorion (CH-MSCs), placenta (PL-MSCs) and umbilical cord (UC-MSCs) tissues. Similar to BM-MSCs, these gestational tissue-derived MSCs exhibited fibroblast-like morphology, being positive for typical MSC surface marker CD73, CD90 and CD105, and being negative for hematopoietic marker CD34 and CD45. The morphology and immunophenotypes of CH-MSCs, PL-MSCs and UC-MSCs established in this study are similar to those described in the previous reports (37, 38). In agreement with those reports (6, 42-44), the CH-MSCs, PL-MSCs and UC-MSCs established in this study could differentiate to adipocytes and osteocytes when culture under appropriate conditions. However, it is worth noting that CH-MSCs, PL-MSCs and UC-MSCs took a longer period of time than BM-MSCs to differentiate to adipocytes and osteocytes and the number of adipocytes and osteocytes derived from these MSC sources were lower than those derived from BM-MSCs cultured under the same conditions. These results reflect the endogenous differences between the properties of gestational tissue-derived MSCs and BM-MSCs.

Similar to BM-MSCs, high glucose suppressed the proliferation of CH-MSCs, PL-MSCs and UC-MSCs. This result is in agreement with previous studies showing that high glucose inhibited the proliferation of mouse BM-MSCs (12, 13) and osteoblastic MG63 cells (14, 15) possibly through the modulation of JAK/STAT and p38 signaling pathway (48, 49).

Although previous studies demonstrated that high glucose enhances adipogenic differentiation of mouse BM-MSCs (7), osteoblastic MG63 cell (8) and muscle-derived stem cells (11) by upregulating the expression of several adipogenic genes (13, 14, 16), the effects of high glucose on adipogenic differentiation of gestational tissue-derived MSCs have not yet been determined. This study demonstrates that high glucose induced the expression of adipogenic gene *PPARγ and LPL* in BM-MSCs, as well as *ADIPOQ* and *LPL* in CH-MSCs and UC-MSCs. The upregulation of these key regulators of adipogenesis therefore enhanced adipogenic differentiation capacity of BM-MSCs, CH-MSCs and UC-MSCs as demonstrated by the increase in both adipocyte number and extent of Oil Red O staining in high glucose condition. These results is in agreement with the previous studies which demonstrated that high glucose upregulated the expression of several adipogenic

genes including *PPARγ, SREBP-1C, ADIPOQ, LPL* and *GLUT4* inmouse BM-MSCs, MG63 cells and muscle-derived stem cells (13, 14, 16).

With regard to the osteogenic differentiation, we found that high glucose downregulated the expression of osteogenic gene *Runx2* in CH-MSCs and UC-MSCs as well as *osterix* in CH-MSCs, PL-MSCs and UC-MSCs at culture day 7. However, the downregulation of those genes could not be detected at culture day 14. We also did not find any difference in the levels of osteogenic differentiation of CH-MSCs, PL-MSCs and UC-MSCs under high glucose condition at culture day 14. In case of BM-MSCs, high glucose only slightly reduced the expression *OCN* at culture day 14.We interpreted that the effect of high glucose on the expression of osteogenic genes in MSCs is transient and do not affect the osteogenic differentiation of gestational-tissue derived MSCs. These results are in contrast with the previous studies which showed that high glucose suppressed the expression of osteogenic gene *Runx2*, *osteocalcin*, *collagen I* and *osteonectin* in osteoblastic MG63 cells by activating cAMP/PKA/ERK pathway (14, 15). The effects of high glucose on osteogenic differentiation of bone marrow and gestational tissue-derived MSCs might use other signaling pathways such as Wnt signaling pathway or Hedgehog signaling pathway. Wnt signaling pathway plays an important role in embryonic development and maintenance and differentiation of MSCs in adulthood. Activating and inactivating aberrations of the canonical Wnt signaling pathway in osteogenesis results in sclerosteosis and osteoporosis respectively (116). In Hedgehog signaling pathway, Sonic Hedgehog (Shh) has an anti-adipogenic-osteoblastic effect on MSCs. In diabetic patients, hyperglycemia interferes with Shh signaling and Shh-induced bone regeneration; therefore, it stimulates osteoporosis. Hyperglycemia also inhibits osteogenesis through osteogenesis-related genes such as BMP4, Runx2 and osteopontin (OPN) (117). This might arise from the difference in the biological property between MG63 and gestational tissue-derived MSCs and the way they response to high glucose condition.

In conclusion, we herein reported for the first time the effects of high glucose on the proliferation, adipogenic and osteogenic differentiation of several gestational tissue-derived MSCs including CH-MSCs, PL-MSCs and UC-MSCs. Our result demonstrated that high glucose inhibit proliferation and enhance adipogenic

differentiation of BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs by upregulating the expressions of adipogenic gene *PPARγ*, *ADIPOQ* and *LPL*. In contrast to adipogenic differentiation, high glucose did not affect osteogenic differentiation of all MSCs examined in this study. The increased number of adipocyte like-cells might increase the level of insulin resistance in diabetic patients, and therefore compromise the outcome of their treatment. The knowledge gained from this study will increase our understanding on the mechanisms underlying the effects of high glucose on proliferation, adipogenic and osteogenic differentiation of MSCs and might lead to an improvement in the treatments of diabetes, obesity and metabolic syndrome in the near future.

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APPENDICES

APPENDIX A

Calibration curve

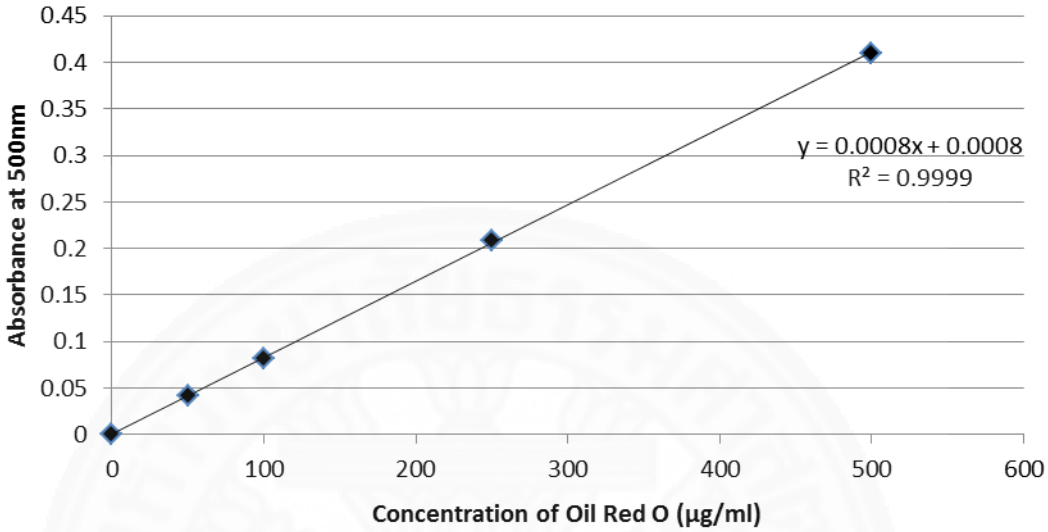

Figure A Calibration curve between Oil Red O concentrations and the absorbance at 500 nm for determination of Oil Red O concentration.

APPENDIX B

Reagent and Instrumentation

Reagent

0.25% Trypsin-EDTA	(GibcoBRL, USA)
2-Propanol	(Merck, Germany)
β-glycerophosphate	(Sigma-Aldrich, USA)
L-Ascorbic Acid	(Sigma-Aldrich, USA)
BCIP®/NBT Liquid substrate	(Sigma-Aldrich, USA)
Collagenase from Clostridium	(Sigma-Aldrich, USA)
D-glucose	(Ajax Finechem, Australia)
Deoxyribonuclease I	(Sigma-Aldrich, USA)
Dexamethasone	(Sigma-Aldrich, USA)
Dimethyl sulfoxide (DMSO)	(Sigma-Aldrich, USA)
Dulbecco's Modified Eagle's Medium (DMEM)	(GibcoBRL, USA)
Fetal bovine serum	(GibcoBRL, USA)
Ficoll-Hypaque solution	(GE Healthcare, Bio-Science AB, Sweden)
Indomethacin	(Sigma-Aldrich, USA)
Insulin from bovine pancreas	(Sigma-Aldrich, USA)
Isobutylxanthine	(Sigma-Aldrich, USA)
L-glutamine	(GibcoBRL, USA)
Oil Red O	(Sigma-Aldrich, USA)
Penicilin/Streptromycin	(GibcoBRL, USA)
PureLink™ RNA Mini kit	(Invitrogen, USA)
SuperScript® III First Strand Synthesis Kit	(Invitrogen, USA)
SYBR® Green PCR Master Mix	(Applied Biosystems, USA)

Monoclonal antibody

FITC-conjugate anti-human CD45	(BioLegend, USA)
FITC-conjugate anti-human CD90	(BioLegend, USA)
PE-conjugate anti-human CD34	(BioLegend, USA)
PE-conjugate anti-human CD73	(BioLegend, USA)
PE-conjugate anti-human CD105	(BD Bioscience, USA)

Instrumentation

0.20 µm Sterile Syringe Filter	(Corning Intercorporated, USA)
BD FACScalibur™	(Becton Dickinson, USA)
Class II biological safety carbinets	(LABCONCO, USA)
CellQuest® software	(Becton Dickinson, USA)
Centrifuge	(Hettich, Universal 320K, USA)
CO_2 Incubator	(NUAIRE TM, USA)
Inverted microscopy TS100	(Nikon, Japan)
Sunyo Biomedical Freezer	(Sunyo, Japan)
Step on plus™ Real-Time PCR system	(Applied Biosystems; ABI, USA)
Water Bath	(Julabo, USA)
Microplate reader	(BioTex, USA)
T25 Cell Culture Flask	(Corning Intercorporated, USA)
T75 Cell Culture Flask	(Corning Intercorporated, USA)
6 Well Cell Culture plate	(Corning Intercorporated, USA)
24 Well Cell Culture plate	(Corning Intercorporated, USA)
96 Well Cell Culture plate	(Corning Intercorporated, USA)
35 mm^2 dish Well Cell Culture plate	(Corning Intercorporated, USA)

APPENDIX C

Preparation of Reagent

10X Phosphate buffered saline (PBS)

NaCl	38.25 g
Na_2HPO	4 4.97g
KH_2PO_4	2.04 g
Distilled water	450 ml

Adjust pH 7.4 and volume to 500ml. The solution was sterile by autoclaving for 15 min at 121c, 151b/square inches and store at 4°c.

1X Phosphate buffered saline (PBS)

10X PBS	50 ml
Distilled water	450 ml

The solution was stored at 4°c.

16% Paraformaldehyde (500ml)

Paraformaldehyde	80 g
Distilled water	450 ml
10 N NaOH	500 µl

Apply solution heat while stirring until the solution was clears, equilibrate to pH 7.4 and add volume to 500ml. The solution was filtered stored at 4°c.

4% Paraformaldehyde (10 ml)

16%Paraformaldehyde	2.5 ml
1X PBS	7.5 ml

2.5 M D-glucose (25 ml)

D-glucose	11.26 g
Distilled water	25 ml

The solution was filtered with 0.20 µm sterile syringe filter and stored at -20°c

DMEM + 10%FBS (500ml)

FBS	50 ml
1X Penicilin/streptomycin	5 ml
L-glutamine	5 ml
DMEM solution	440 ml

The solution was mixed and stored at 4°c.

Oil Red O stock(100 ml)

Oil Red O	0.5 g
2-Propanol	100 ml

The solution was mixed and stored at 4°c.

Oil Red O working (10ml)

Oil Red O stock	6 ml
Distilled water	4 ml

The solution was filtered with Whatman #1 filter paper.

Adipogenic differentiation medium (in house)

Complete DMEM	48.4 ml
Isobutylxanthine	0.00575 g
20 mM indomethacin	500 µl
2.5 M glucose	500 µl
1 mM Dexamethasone	50 µl
1 mg/ml insulin	50 µl

The solution was filtered with 0.20 µm sterile syringe filter and stored at 4°c.

Osteogenic differentiation medium Day 1-7 (in house)

Stock DMEM	44.05 ml
FBS	4.95 ml
Penicilin/Streptromycin	500 µl
0.1µM Dexamethasone	500 µl

Add ascorbic acid 50ug/ml per well.

Osteogenic differentiation medium Day 7 up (in house)

Stock DMEM	39.60 ml
FBS	4.45 ml
Penicilin/Streptromycin	450 µl
0.1µM Dexamethasone	500 µ
10mM β-glycerophosphate	5 ml

Add ascorbic acid 50ug/ml per well.

APPENDIX D

Experiment Procedures

Isolation and culturation of MSCs from bone marrow

Reagents:

DMEM+10% FBS+2 mM L-glutamine +100 U/ml penicillin +100 µg/ml streptomycin

1X Phosphate buffered saline+100 U/ml penicillin+100 µg/ml streptomycin

Ficoll-Hypaque solotion

0.25 % trypsin EDTA

5,000 i.u./u.i./ml Heparin

Procedure

1. Aaspirate bone marrow (10 ml) from sternum and place in 50 ml Falcol tube containing 500µl of heparin (LEO 5,000 i.u./u.i./ml).
2. Dilute at 1:1 with 1X phosphate buffered saline (1XPBS) + 100 U/ml penicillin+100 µl/ml streptomycin and carefully loaded onto Ficoll-Hypaque solution.
3. Centrifuge at 2,000 rpm (Hettich, Universal 320K, USA) for 30 min at 20°C.
4. Collect mononuclear cells (MNCs) from the interphase and wash twice with 15 ml of 1XPBS + 100 U/ml penicillin+100 µl/ml streptomycin.
5. Count cells with hematocytometer.
6. BM-derived MNCs are got in culture at a density of 1 x 10^6 cells/cm^2 into 25 cm^2 tissue culture flask with DMEM supplement with 2 mM L-glutamine (GibcoBRL, USA) +10% fetal bovine serum (FBS)+ 100 U/ml penicillin+100 µg/ml streptomycin.
7. Incubation at 37 °C in humidified at containing 5 % carbon dioxide.
8. Nonadherent cells are removed and fresh medium is added to the flask every 3-4 days.
9. Fibroblastoid cells are removed between day 7 through 12 after initiated plating using 0.25% trypsin/ 2 mM EDTA (GibcoBRL, USA). Recovered all are replated at a density of 1X10^4 cell/cm^2 as passage 1 cells and thereafter.

Isolation and culturation of MSCs from chorion

Reagents:

DMEM+10% FBS +2 mM L-glutamine +100 U/ml penicillin+100 µg/ml streptomycin

1X Phosphate buffered saline+100 U/ml penicillin+100 µg/ml streptomycin

1.6 mg/ml collagenase XI

200 mg/ml Deoxyribonuclease I

0.25 % trypsin EDTA

Procedure

1. Dissect chorion tissue (diameter 5 cm) from placenta women after cesarean section and mechanically peel off from amnion
2. Rinse with 1X PBS +100 U/ml penicillin+100 µg/ml streptomycin and minced into pieces on 1-2 mm^2 in size.
3. Wash the tissue with 1X PBS + 100 U/ml penicillin+100 µg/ml streptomycin
4. Centrifuge at 2,000 rpm for 5 min the tissue are digested with 1.6 mg/ml collagenase XI (Sigma-Aldrich, USA.) and 200 mg/ml Deoxyribonuclease I (Sigma-Aldrich, USA.) for 16 hour at 37° C with shaking.
5. Wash twice with 1XPBS + 100 U/ml penicillin+100 µg/ml streptomycin, then centrifuge at 2,000 rpm for 5 min.
6. The cells and all pellet are culture in DMEM supplement with 10% FBS+2 mM L-glutamine +100 U/ml penicillin+100 µg/ml streptomycin in 25 cm^2 tissue culture flasks.
7. Cultured are maintained at 37 ℃ in a humidifiled atmosphere containing 5 % CO_2, with a change of culture medium every 3-4 days.
8. Fibroblastoid cells are removed at day 7 after initiated plating using 0.25% trypsin/ 2 mM EDTA (GibcoBRL, USA). Recovered all are replated at a density of $1x10^4$ cell/cm^2 as passage 1 cells and thereafter.

Isolation and culturation of MSCs from placenta

Reagents:

DMEM+10% FBS +2 mM L-glutamine +100 U/ml penicillin+100 µg/ml streptomycin

1X Phosphate buffered saline+100 U/ml penicillin+100 µg/ml streptomycin

1.6 mg/ml collagenase XI

200 mg/ml Deoxyribonuclease I

0.25 % trypsin EDTA

Procedure

1. Dissect placental tissue from pregnant women after cesarean section and macroscopically dissect from the central region of the maternal-facing surface of placenta under aseptic conditions (3x3 cm).
2. Rinse with 1X PBS +100 U/ml penicillin+100 µg/ml streptomycin and minced into pieces on 1-2 mm^2 in size.
3. Wash the tissue with 1X PBS + 100 U/ml penicillin+100 µg/ml streptomycin
4. Centrifuge at 2,000 rpm for 5 min the tissue are digested with 1.6 mg/ml collagenase XI and 200 mg/ml Deoxyribonuclease I for 16 hour at 37° C with shaking.
5. Wash twice with 1XPBS+100 U/ml penicillin+100 µg/ml streptomycin, then centrifuge at 2,000 rpm for 5 min.
6. The cells and all pellet are culture in DMEM supplement with 10% FBS+2 mM L-glutamine + 100 U/ml penicillin+100 µg/ml streptomycin in 25 cm^2 tissue culture flasks.
7. Cultured are maintain at 37 °C in a humidifiled atmosphere containing 5 % CO_2, with a change of culture medium every 3-4 day.
8. Fibroblastoid cells are removed at day 7 after initiated plating using 0.25% trypsin/ 2 mM EDTA. Recovered all are replated at a density of $1X10^4$ cell/cm^2 as passage 1 cells and thereafter.

Isolation and culturation of MSCs from umbilical cord

Reagents:

DMEM+10% FBS +2 mM L-glutamine +100 U/ml penicillin+100 µg/ml streptomycin

1X Phosphate buffered saline+100 U/ml penicillin+100 µg/ml streptomycin

1.6 mg/ml collagenase XI

200 mg/ml Deoxyribonuclease I

0.25 % trypsin EDTA

Procedure

1. Dissect Umbilical tissue lengths 2-4 cm obtain from pregnant women after cesarean section
2. Rinse with 1X PBS +100 U/ml penicillin + 100 µg/ml streptomycin and minced into pieces on 1-2 mm^2 in size.
3. Wash the tissue with 1X PBS containing 100 U/ml penicillin +100 µg/ml streptomycin
4. Centrifuge at 2,000 rpm for 5 min the tissue are digested with 1.6 mg/ml collagenase XI and 200 mg/ml Deoxyribonuclease I for 16 hour at 37° C with agitation.
5. Wash twice with 1XPBS + 100 U/ml penicillin+100 µg/ml streptomycin, then centrifuge at 2,000 rpm for 5 min.
6. The cells and all pellet are culture with DMEM supplement with 2 mM L-glutamine +10% FBS+ 100 U/ml penicillin+100 µg/ml streptomycin in 25 cm^2 tissue culture flasks.
7. Cultured are maintained at 37 ℃ in a humidifiled atmosphere containing 5 % CO_2, with a change of culture medium every 3-4 days.
8. Fibroblastoid cells are removed between day 7 after initiated plating using 0.25% trypsin/ 2 mM EDTA (GibcoBRL, USA). Recovered all are replated at a density of $1x10^4$ cells/cm^2 as passage 1 cells and thereafter.

Immunophenotypical characterization of culture cells

Reagents:

FITC-conjugated anti-human CD45 antibody

FITC-conjugated anti- human CD90 antibody

FITC-conjugated anti- human CD105antibody

PE-conjugated anti- human CD34 antibody

PE-conjugated anti- human CD73 antibody

1X Phosphate buffered saline (1XPBS)

0.25 % trypsin EDTA

1% paraformaldehyde in 1XPBS

Procedure

1. Primary culture (passage 3-5) are washed with 1X PBS and treated with 0.25% trypsin-EDTA at 37˚C for 3 min.
2. The harvested cells are washed twice with 1X PBS
3. 4 x 10^5 cells in 50 µl 1XPBS are incubated with 5 µl of Fluorescein isothiocyanate (FITC) or phycoerythrin(PE)-conjugated antibodies for 30 min at 4 ˚C in the dark.
4. After washing twice with 1X PBS fixed for 15 min with 1% paraformadehyde in 1X PBS.
5. The label cells acquire and analyze using flow cytometer (FACS caliber, Becton Dickinson) and Cell Quest software.

Adipogenic differentiation assay of culture cells

Reagents:

Adipogenic differentiation medium (in house)

1X Phosphate buffer saline

0.5% Oil Red O

Distilled water

40% formalin

Procedure

1. Culture-expand MSCs passage 3 from bone marrow, amnion, placenta and umbilical cord are seed at a density of $7.5x10^4$ cell in 35 mm^2 dish and allowed adherent cell overnight.
2. Wash with 1xPBS and change to adipogenic differentiation medium.
3. The cells are cultured at 37°C in humidifiled atmosphere containing 5 % CO_2
4. The medium are replaced every 3 days.
5. After 2 weeks of culture, the cells are washing with 1XPBS and fix with 40% formalin vapor for 10 min at room temperature.
6. After that the cells are washing twice with distilled water and stain with 0.5% Oil Red O in 60% 2-Propanol for 20 min at room temperature.
7. The cells are washed twice with distilled water.
8. The positive cells are observed under inverted microscope (Nikon TS100, Japan.).

Osteogenic differentiation assay of culture cells

Reagents:

Osteogenic differentiation medium (in house)

1X Phosphate buffer saline

BCIP®/NBT Liquid substrate

Distilled water

4% formaldehyde

Procedure

1. Culture-expand MSCs passage 3 from bone marrow, amnion, placenta and umbilical cord are seed at a density of $4.5x10^4$ cell in 35 mm^2 dish and allowed adherent cell overnight.
2. Washing with 1XPBS and change to osteogenic differentiation medium.
3. The cells are cultured at 37°C in humidifiled atmosphere containing 5 % CO_2.
4. The medium are replaced every 3 days.
5. After 2 weeks of culture, the cells are washed with 1XPBS and fix with 4% formaldehyde for 5 min at 4 °C.
6. To visualize osteogenic differentiation, cells are stained for alkaline phosphatase (AP) activity. For AP expression cells are washed with PBS and incubate for 10 min with substrate solution (BCIP®/NBT Liquid substrate (Sigma-Aldrich, USA)).
7. The positive cells are observed under inverted microscope (Nikon TS100, Japan).

Proliferation assay of culture cells(high glucose effect)

DMEM+10% FBS +2 mM L-glutamine +100 U/ml penicillin+100 µg/ml streptomycin

25mM D-glucose

1X Phosphate buffered saline+100 U/ml penicillin+100 µg/ml streptomycin

0.25 % trypsin EDTA

Procedure

1. Culture MSCs (passage3) derived from bone marrow, chrion, placenta and umbilical cord were seeded at $1x10^3$ cells in 24 wells plate containing 500 µl of complete medium with or without 25mM D-glucose in triplicates.
2. The cultures were maintained at 37°C in a humidified tissue culture incubator with 5% carbon dioxide for several intervals (0, 2, 4, 6, 8, 10, 12, and 14 days).
3. MSCs were treated with 0.25% trypsin-EDTA and cell numbers were determined using hematocytometer.
4. The mean numbers of cells were calculated and plotted against culture time to generate a growth curve.

Adipogenic differentiation assay of culture cells (high glucose effect)

Reagents:

Adipogenic differentiation medium

25mM D-glucose

1X Phosphate buffer saline

40% formalin

0.5% Oil Red O

Distilled water

2-Propanol

Procedure

1. Culture-expand MSCs passage 3 from bone marrow, amnion, placenta and umbilical cord are seed at a density of $7.5x10^4$ cells in 35 mm^2 dish and allowed adherent cell overnight.
2. Wash with 1xPBS and change to adipogenic differentiation medium with or without 25mM D-glucose.
3. The cells are cultured at 37°C in humidifiled atmosphere containing 5 % CO_2
4. The medium are replaced every 3 days.
5. After 14 and 28 days of culture, the cells are washing with 1XPBS and fix with 40% formalin vapor for 10 min at room temperature.
6. After that the cells are washing twice with distilled water and stain with 0.5% Oil Red O in 60% 2-Propanol for 20 min at room temperature.
7. The cells are washed twice with distilled water.
8. The positive cells are counted under inverted microscope (Nikon TS100, Japan.).
9. The Oil Red O presented in each stained culture plates were extracted by incubation with 1ml 2-Propanol for 5 minute at room temperature with shaking.
10. The optical density (OD) of each extracted Oil Red O sample was measured by microplate reader at the wavelength of 500 nm.
11. The amount of extracted Oil Red O in each sample were calculated by comparing the measure OD values against a standard curve generated from 0 µg/ml to 500 µg/ml of Oil Red O.

The expression levels of adipogenic genes (high glucose effect)

Reagents:

DMEM+10%FBS+100 U/ml penicillin + 100 µg/ml streptomycin

Adipogenic differentiation medium (in house)

25mM D-glucose

0.25 % trypsin EDTA

PureLink™ RNA Mini Kit

SYBR® Green PCR Master Mix

Procedure

1. MSCs (2×10^5 cells) derived from bone marrow, chorion, placenta and umbilical cord were cultured in $25cm^2$ tissue culture flasks (Costa, Corning, USA) with complete medium and allowed to adhere to the plate overnight.
2. The medium were removed and adipogenic differentiation medium with or without 25mM D-glucose. The cultures of MSCs in DMEM medium were used control.
3. After 14 days in BM-MSCs and 28 days in CH-MSCs, PL-MSCs and UC-MSCs of incubation were isolated with PureLink™ RNA Mini Kit (Invitrogen, USA).
4. Messenger RNA was reverse transcribed to cDNA using SuperScript® III First Strand Synthesis Kit (Invitrogen, USA)
5. Expression of adipogenic genes are determined by Real-Time PCR, using SYBR® Green PCR Master Mix (Applied Biosystems, USA)

The expression levels of osteogenic genes (high glucose effect)

Reagents:

DMEM+10%FBS+100 U/ml penicillin + 100 µg/ml streptomycin

Osteogenic differentiation medium (in house)

25mM D-glucose

0.25 % trypsin EDTA

PureLink™ RNA Mini Kit

SYBR® Green PCR Master Mix

Procedure

1. MSCs (2×10^5 cells) derived from bone marrow, chorion, placenta and umbilical cord were cultured in $25cm^2$ tissue culture flasks (Costa, Corning, USA) with complete medium and allowed to adhere to the plate overnight.
2. The medium were removed and osteogenic differentiation medium with or without 25mM D-glucose. The cultures of MSCs in DMEM medium were used control.
3. After 7 and 14 days, BM-MSCs, CH-MSCs, PL-MSCs and UC-MSCs of incubation were isolated with PureLink™ RNA Mini Kit (Invitrogen, USA).
4. Messenger RNA was reverse transcribed to cDNA using SuperScript® III First Strand Synthesis Kit (Invitrogen, USA)
5. Expresstion of osteogenic genes are determined by Real-Time PCR, using SYBR® Green PCR Master Mix (Applied Biosystems, USA)

BIOGRAPHY

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Publication

1. Weerawan Hankamolsiri, Sirikul Manochantr, Chairat Tantrawatpan, Duangrat Tantikanlayaporn, Pairath Tapanadechopone, Pakpoom Kheolamai. Adipogenic differentiation capacity of human bone marrow-derived mesenchymal stem cells in high glucose condition. Thammasat Medical Journal 2014; 14(3): 319-329.

Poster presentation

1. Weerawan Hankamolsiri, Sirikul Manochantr, Chairat Tantrawatpan, Duangrat Tantikanlayaporn, Pairath Tapanadechopone, Pakpoom Kheolamai. Adipogenic differentiation capacity of mesenchymal stem cells in high glucose condition. Annual meeting of Faculty of Medicine, July 16-18, 2014, Faculty of Medicine, Thammasat University, Thailand.