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นายโชคดี วงศ์บริสุทธิ์

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

REDUCTION IN OSTEOGENIC COMMITMENT OF MESENCHYMAL STEM CELLS FROM THALASSEMIC PATIENTS

Mister Chokdee Wongborisut

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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จุฬาลงกรณ์มหาวิทยาลัย

โชคดี วงศ์บริสุทธิ์:การลดลงของการเจริญพัฒนามีเซนไคมอลสเตมเซลล์ไปเป็นกระดูกในผู้ป่วยธาลัสซีเมีย. (REDUCTION IN OSTEOGENIC COMMITMENT OF MESENCHYMAL STEM CELLS FROM THALASSEMIC PATIENTS) อ. ที่ปรึกษา:ศาสตราจารย์แพทย์หญิงนวพรรณ จารุรักษ์, อ.ที่ปรึกษาร่วม : รองศาสตราจารย์นายแพทย์สุรเดช หงส์อิง, 78หน้า. ISBN 974-53-2930-4.

การเปลี่ยนแปลงของกระดูกในผู้ป่วยธาลัสซีเมียมีความสัมพันธ์โดยตรงกับการสร้างเม็ดเลือดแดงของไขกระดูก ในภาวะปรกติกระดูกจะมีการสลายกระดูกเก่าและสร้างกระดูกใหม่ซึ่งมีความสมดุลย์กันตลอดเวลา ผู้ป่วยธาลัสซีเมียที่มีอาการทางคลินิกรุนแรงต้องเร่งสร้างเม็ดเลือดแดงในไขกระดูกเพิ่มเป็น15-20เท่า การเพิ่มของมวลไขกระดูกนี้ทำให้โพรงกระดูกกว้างขึ้นและกระดูกเสียรูปทรงกระดูกบางเปราะและหักง่าย มีเซนไคมอลสเตมเซลล์เป็นเซลล์ต้นกำเนิดที่สามารถเปลี่ยนแปลงไปเป็นเนื้อเยื่อชนิดต่างๆเช่น กระดูก กระดูกอ่อน เซลล์ไขมัน เซลล์กล้ามเนื้อ เป็นต้น ปัจจัยที่ควบคุมการเพิ่มจำนวนและพัฒนาการของมีเซนไคมอลสเตมเซลล์นั้นมีหลายปัจจัยเช่น ไซโตไค โกรทแฟคเตอร์ ฮอร์โมน โดยปัจจัยเหล่านี้ถูกสร้างมาจากเซลล์ชนิดต่างๆที่อยู่ภายในไขกระดูก ดังนั้นอาจเป็นไปได้ว่าภาวะที่มีการสร้างเม็ดเลือดแดงที่มากกว่าปรกติในผู้ป่วยธาลัสซีเมียอาจส่งผลกระทบต่อการเจริญและเปลี่ยนแปลงของมีเซนไคมอลสเตมเซลล์ในระดับการแสดงออกของยีนที่จะพัฒนาไปเป็นเซลล์กระดูก ดังนั้นในการศึกษานี้ได้เปรียบเทียบการแสดงออกของยีนที่จะพัฒนาไปเป็นเซลล์กระดูกในมีเซนไคมอลสเตมเซลล์และเซลล์ต้นกำเนิดเม็ดเลือดเปรียบเทียบกับคนปรกติ ผลการทดลองพบว่าการแสดงออกของยีน *Cbfa1*, *Osterix*, *BMP-2*, *Collagen type I*, *Alkaline phosphatase* และ *Osteocalcin* ในมีเซนไคมอลสเตมเซลล์ลดลงเมื่อเปรียบเทียบกับคนปรกติ การแสดงออกของยีน *Cbfa1*, *Osterix*, *Osteocalcin* ในเซลล์ต้นกำเนิดเม็ดเลือดลดลงเมื่อเปรียบเทียบกับคนปรกติ และไม่พบการแสดงออกของยีน *PPARγ2* ซึ่งเป็นยีนที่ใช้พัฒนาไปเป็นเซลล์ไขมันทั้งในมีเซนไคมอลสเตมเซลล์และเซลล์ต้นกำเนิดเม็ดเลือดและไม่พบการแสดงออกของยีน*BMP-2*, *Alkaline phosphatase* ในเซลล์ต้นกำเนิดเม็ดเลือดทั้งในคนปรกติและผู้ป่วยธาลัสซีเมีย โดยสรุปการศึกษาครั้งนี้พบว่าการสร้างเม็ดเลือดแดงในไขกระดูกที่เพิ่มขึ้นในผู้ป่วยธาลัสซีเมียส่งผลโดยลดการแสดงออกของยีนที่จะพัฒนาไปเป็นเซลล์กระดูกในมีเซนไคมอลสเตมเซลล์

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จุฬาลงกรณ์มหาวิทยาลัย

สาขาวิชา Medical Science
ปีการศึกษา 2005

ลายมือชื่อนิสิต..
ลายมือชื่ออาจารย์ที่ปรึกษา..
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม..

4575216230 : MAJOR MEDICAL SCIENCE
KEY WORD: MESENCHYMAL STEM CELLS / OSTEOPOROSIS / OSTEOBLAST

CHOKDEE WONGBORISUT: REDUCTION IN OSTEOGENIC COMMITMENT OF MESENCHYMAL STEM CELLS FROM THALASSEMIC PATIENTS.THESIS ADVISOR :PROFESSOR NAVAPUN CHARURUKS M.D.,THESIS COADVISOR : ASSOCIATE PROFESSOR SURADEJ HONGENG M.D.,78pp. ISBN 974-53-2930-4.

Osteoporosis is presented in severe thalassemia due to ineffective in erythropoiesis. More than 15-20 times comparing to normal individual expansion of bone marrow causes bone loss and osteoporosis. The patients may be suffered from bone change depending on the degree of osteoporosis.. Osteoblasts are originate from mesenchymal stem cells, which have the capacity to differentiate into osteoblast , adipocytes , chondrocytes , myoblasts or fibroblast. The commitment proliferation and differentiation of mesenchymal stem cells are regulated by multiple factors, including cytokines, growth factors, systemic hormones and transcription regulators. Bone marrow cells are responsible for the production of many paracrine and autocrine bone regulatory factors and the stromal cells population are essential for normal bone formation. Hence, we hypothesize that bone marrow expansion may interrupt on mesenchymal stem cells differentiate into bone cells that lead to osteoporosis in severe thalassemic patients. Thus, in this study we investigate the expression level of a transcription factor genes and bone differentiation genes for mesenchymal stem cells in severe thalassemic patients compare with normal by using relative quantification real time RT-PCR. Our data show that the expression of *Cbfa1* , *Osterix* , *BMP-2* , *Collagen type I* , *Alkaline phosphatase* , *Osteocalcin* was downregulated in MSCs from the patients. In addition the expression of *Cbfa1*, *Osterix* , *Osteocalcin* was downregulated in HSCs from the patients. Interestingly, the mRNA of PPARγ2 was not detected from both of mesenchymal stem cells and hematopoietic stem cells from healthy donor and thalassemic patients. Moreover, the mRNA of BMP-2, Alkaline Phosphatase was not detected from hematopoietic stem cells. In conclusion, the expansion of bone marrow interrupt MSCs differentiation by downregulate expression of transcription factor genes and bone differentiation genes.

Field of study Medical science
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Student's signature................................
Advisor's signature................................
Co-advisor's signature................................

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TABLE OF CONTENTS

Page

Abstract (Thai)....................iv

Abstract (English)....................v

Acknowledgments....................vi

Table of contents....................vii

List of Tables....................viii

List of Figures....................ix

List of Abbreviation....................x

Chapter

I. Introduction....................1

II. Review of Related Literatures....................6

III. Materials and Methods....................35

IV. Results....................42

V. Discussion and Conclusion....................57

References....................62

Appendices....................75

Biography....................78

LIST OF TABLE

Table Page

1 Data of thalassemic patients……………………………………………………………..42

2 Data of healthy donor…………………………………………………………………..43

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF FIGURES

Figure		Page
1.	Schematic of the osteoblast differentiation.	19
2.	Culture of mesenchymal stem cells.	45
3.	Isolated MSCs (CD105 positive cells)	46
4.	Isolated HSCs (CD133 positive cells)	47
5.	Phenotypic analysis characterization of MSCs.	48
6.	Phenotypic analysis characterization of HSCs after positive selection.	49
7.	Relative gene expression of MSCs from thalassemic patients.	50
8.	Relative gene expression of HSCs from thalassemic patients.	51
9.	Relative gene expression of Cbfa1 from MSCs.	51
10.	Relative gene expression of Osterix from MSCs.	52
11.	Relative gene expression of BMP-2 from MSCs.	52
12.	Relative gene expression of Type I Collagen from MSCs.	53
13.	Relative gene expression of Alkaline Phosphatase from MSCs.	53
14.	Relative gene expression of Osteocalcin from MSCs.	54
15.	Relative gene expression of Cbfa1 from HSCs.	54
16.	Relative gene expression of Osterix from HSCs.	55
17.	Relative gene expression of Type I Collagen from HSCs.	55
18.	Relative gene expression of Osteocalcin from HSCs.	56

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF ABBREVIATIONS

ALCAM	=	Activated leukocyte cell adhesion molecule
ALP	=	Alkaline phosphatase
AP-1	=	Activator protein-1
AsAp	=	L-Aspartic-2-phosphate
BMP-2	=	Bone morphogenetic protein-2
BSP	=	Bone sialoprotie
Cbfa1	=	Core binding factor alpha 1
CD	=	Cluster of differentiation
cDNA	=	Complementary deoxyribonucleic acid
Dex	=	Dexamethazone
Dlx5	=	a homolog of the distal-less in Drosophila
DMEM	=	Dulbecco's Modified Eagle Medium
EDTA	=	Etylene diamine teracetate
ES cell	=	Embryonic stem cell
FBS	=	Fetal bovine serum
FGF	=	Fibroblast growth factor
FITC	=	Fluorescein isothiocyanate

สถาบันวิทยบริการ
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LIST OF ABBREVIATIONS

G-CSF	=	Granulocyte-colony stimulating factor
GM-CSF	=	Granulocyte macrophage- colony stimulating factor
HSC	=	Hematopoietic stem cell
ICAM	=	Intracellular adhesion molecule
IFN	=	Interferon
IL	=	Interleukin
Ihh	=	Indian hedgehog
LIF	=	Leukemia inhibitory factor
LRPs	=	Low density lipoprotein receptor related proteins
MAP kinase	=	Mitogen-activated protein kinase
M-CSF	=	Macrophage-colony stimulating factor
Msx2	=	Muscle segment homeobox gene
mg	=	Milligram
ml	=	Milliliter
MMP	=	Muscle morphogenetic protein
MSC	=	Mesenchymal stem cell
MRNA	=	Messenger ribonucleic acid
NFAT	=	Nuclear factor of activated T cell
NPY	=	Neuropeptide Y

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF ABBREVIATIONS

NTX	=	N-telopeptides of collagen type I
OPG	=	Osteoprotegerin
OSE2	=	Osteoblast specific cis-acting element
Osx	=	Osterix
PBS	=	Phosphate buffer saline
PCR	=	Polymerase Chain Reaction
PE	=	Phycoerythrin
PECAM	=	Platelet endothelial cell adhesion molecule
PDGFR	=	Platelet-derived growth factor receptor
PPARγ	=	Peroxisome proliferator activated receptor γ
pRb	=	Retinoblastoma tumor suppressor protein
PTHrP	=	Parathyroid hormone-related protein
RANK	=	Receptor activator of nuclear factor kappa B
RANKL	=	Receptor activator of nuclear factor kappa B ligand
RT-PCR	=	Reverse transcriptase polymerase chain reaction
SCF	=	Stem cell factor
Shh	=	Sonic hedgehog
Sox9	=	Sex determining region Y related box gene 9
TGF	=	Transforming growth factor

สถาบันวิทยบริการ
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LIST OF ABBREVIATIONS

TNF	=	Tumor necrosis factor
Tob	=	Transducer of ErbB-2

สถาบันวิทยบริการ
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CHAPTER I

INTRODUCTION

Background and rationale

Thalassemia is an inherited single gene disorders anemia characterized by defects in synthesis of one or more globin chain subunits of hemoglobin tetramer. Clinical disease of thalassemia arised from the combined consequence of inadequate hemoglobin accumulation and unbalanced accumulation of globin subunits. Inadequate hemoglobin accumulation cause hypochromia and microcytosis. Unbalanced accumulation of globin subunits caused ineffective erythropoiesis and hemolytic anemia.

Osteoporosis is presented in severe thalassemia due to ineffective in erythropoiesis. More than 15-20 times comparing to normal individual expansion of bone marrow causes bone loss and osteoporosis. The patients may be suffered from bone change depending on the degree of osteoporosis. These defect could be observed in the radiological changing in the skull, facial bone, and other extremities.

Iron overload is not a primary cause of osteoporosis in severe thalassemia, but seem to be the secondary causes, due to the high level of iron contributing to the deposition in many organs such as thyroid gland, parathyroid gland, and others, causing the organ damage. In the case of parathyroid gland damage causing hypoparathyroidism, then involving osteoporosis in these patients. These evidence suggest that iron overload may play a minority role in osteoporosis in severe thalassemia.

Vitamin D metabolism in severe thalassemia patients is not different from normal individual. By the report of Rioja et al. In 1990, they underwent measuring 25 hydroxyvitamin D in severe thalassemia children patients comparing with the normal age matched with the controlling of diet intake in both groups. They found that the levels of 25 hydroxyvitamin D in these patients and control groups were not different. These data

suggested that vitamin D metabolism may be not related to the pathophysiology of osteoporosis in severe thalassemic individual.

In 1968 Friendenstein et al, described the colony forming unit of fibroblast (CFU-F) from the bone marrow culture by noticing that the small number of spindle-shaped adherent cells began to multiply rapidly after several passages in culture the adherent cells became more uniformly spindle-shaped in appearance and they had the ability to differentiate into colonies that resembles small deposit of bone and cartilage[1].

This initial observation had led Caplan et al, to delineate these specialized population of cells which reside in the bone marrow. They hypothesized that besides the hematopoietic process that occurs in the bone marrow, the mesengenic process also occurs at this site.[2]

The mesengenic process of mesenchymal tissue differentiation and development. MSCs, an initiated cells of mesenchymal tissues origin, have the capability of commitment and differentiation into mesenchymal lineages including osteoblastic, tendogenic, chondrogenic, adipocytic, myogenic, and other lineages[2-4]. The transition from MSCs to each lineage are controlled by many signaling pathways such as cell-to-cell contact, cytokines and growth factors, and pathologic condition.[2,4]

MSCs reside in a variety organs including bone marrow, cord blood, muscle,dermal, and other tissues. These could explain the supplied mesenchymal cells during tissue injury or regeneration of new organs.

Erices et al, reportes the plenty of MSCs and hematopoietic stem cells in umbilical cord blood. Based on these findings, umbilical cord blood-derived MSCs can be visualized as attractive targets for cellular and gene therapy strategies.[5]

Research Question

Are bone marrow expansions reduce the osteogenic commitment of mesenchymal stem cells in thalassemic patients ?

Objective

To determine the expression level of a transcription factor genes and bone differentiation genes for mesenchymal stem cells in thalassemic patients compare with normal by using relative quantification real time RT-PCR.

Hypothesis

Effect of bone marrow expansion may interrupt on mesenchymal stem cells differentiation by downregulation of transcription factor that involve in osteogenesis that lead to osteoporosis in severe thalassemic patients.

Assumption

In this study bone marrow samples were collected from thalassemic children patients after an informed consent at Ramathibodi hospital.

Conceptual Framework

Bone marrow expansion in thalasemic patients

↓

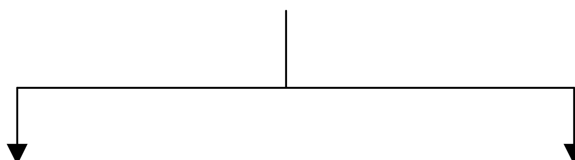
Osteoporosis

↓

Are bone marrow expansions reduce the osteogenic commitment of mesenchymal stem cells in thalassemic patients ?

↓

Mesenchymal stem cells study



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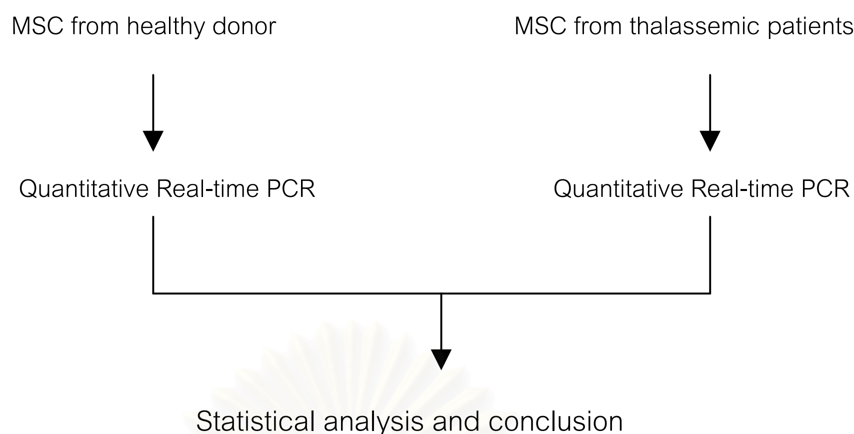

Limitation

There is no limitation

Operational Definition

1.Mesenchymal stem cells also know as marrow stromal cells or mesenchymal progenitor cells are defined as self-renewalble , multipotent progenitor cells with the capacity to differentiate into several distinct mesenchymal lineages such as bone , cartilage , fat , tendon , and muscle tissue. MSCs reside in a variety organs including bone marrow, cord blood, muscle,dermal, and other tissues. These could explain the supplied mesenchymal cells during tissue injury or regeneration of new organs

2. Osteoporosis is a skeletal disorder characterized by low bone mass and disturbance of the microarchitecture of the bone tissue. This pathologic process results in enhanced bone fragility and consequent increase in fracture risk.

Expected Benefit

The result from this study may uses to be a basic knowledge of molecular regulation of mesenchymal stem cells differentiation and applied to regenerative medicine , tissue engineering and cells therapy.

Research Methodology

1.sample collection

Bone marrow samples were obtained using a bone marrow biopsy needle inserted through posterior iliac crest of the 10 severe thalassemic children patients (age 1-15 years) and 10 healthy bone marrow donor (age 1-15 years) after an informed consent. The total 20-25 milliliters of bone marrow cells were heparinized and gently mixed for clot preventing.

2. process of study

2.1 Bone marrow collection

2.2 Mononuclear cells gradient centrifugation

2.3 Flow cytometry

2.4 Stem cells isolation

2.5 RNA extraction

2.6 Reverse transcriptase reaction

2.7 Quantitative real-time PCR

3.Data collection and analysis.

Results are presented as mean ± SEM. Student *t* test was used to compare group means of two samples. The difference was considered significant at *P* value below 0.05.

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CHAPTER II

REVIEW OF RELATED LITRATURE

In postnatal, hematopoiesis occurs chiefly in the bone marrow providing the sufficient variety of functional hematopoietic cells throughout life-long. The majority of structural components of bone marrow are composed of hematopoietic tissues, non-hematopoietic tissues and extracellular matrix.[6-7] Hematopoietic cells seeding in the bone marrow are comprised of erythrocytic, myelocytic, lymphocytic, and megakaryocytic linages. Erythrocyte is the terminal differentiated cells in the erythrocytic lineages. Neutrophil, monocyte, macrophage, dendritic cell, are the most differentiated cell in myelocytic lineagse. T and B lymphocyte and NK cell are the most differentiated cells in the lymphocytic lineages, and platelets for the megakaryocytic lineages.[8]

All of the hematopoietic cells were first originated from the embryonic stem cells in the embryonic stage. These embryonic stem cells subsequently differentiated into endodermal layers, mesodermal layers, and ectodermal layers. The tissues of the mesodermal layers were committed to differentiated into the multi-pluripotential cells termed hematopoietic stem cells (HSC). HSCs were characterized by self-renewal capacity (the potential to give rise to new identical daughter HSCs), commitment capacity (the potential to commit to each hematopoietic lineages), and differentiation and maturation capacity.[8-9]

HSCs reside in the bone marrow in steady quiescent stage are called cells G0 cells. Upon receiving the appropriate signals the HSCs exist the quiescent stage, increase the mitotic activity, and undergo differentiation and maturation processes through each lineage. The transition from a primitive HSCs to functional hematopoietic cells occurs via a number of intermediate stages characterized by progression of self-renewal and lineage restriction.[10-11] These hematopoietic processes require the nutrients, cytokines, and growth factors supplied by the non-hematopoietic tissues termed stromal cells which play a crucial role in supporting hematopoiesis by cell-to-cell contract with HSCs and releasing a bulk of cytokines and growth factors.[12-14] The bone marrow stromal cells comprise a variety of cells from hematopoietic and non-

hematopoietic lineages such as macrophages, osteoclasts, fibroblasts, adipocytes, reticular cells, osteoblasts, and endothelial cells.[15-17] These non-hematopoietic cells are given rise from the stem-like cells of mesenchymal tissues called mesenchymal stem cells (MSCs).[18-22]

1. Mesenchymal Stem Cells (MSCs)

In 1968 Friendenstein et al, described the colony forming unit of fibroblast (CFU-F) from the bone marrow culture by noticing that the small number of spindle-shaped adherent cells began to multiply rapidly after several passages in culture the adherent cells became more uniformly spindle-shaped in appearance and they had the ability to differentiate into colonies that resembles small deposit of bone and cartilage.[1]

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2.Characterization of Mesenchymal Stem Cells

Morphology of Mesenchymal Stem Cells

Less amount of human mesenchymal stem cells reside in bone marrow with the estimation rate of 1 MSC in 10,000 bone marrow mononuclear cells. MSCs can be isolated by density gradient centrifugation of 1.073 g/ml Percoll. Cultivation and expansion to doubling a grate number of human MSCs in vitro are easily possessed by culturing in the culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in 5% CO_2 at 37 $^{\circ}$C. The MSCs grow as adherent fibroblastic cells that developed into visible symmetric colonies during 5 to 7 days after initial plating, and expanded more than 20 passages without changing their ability to differentiate throughout each mesenchymal tissue lineage.[23]

Cell Surface Markers of Mesenchymal Stem Cells

Phenotypic analysis of cell surface markers of MSCs by conventional flow cytometry reveal that these culture-expanded MSCs uniformly positive for SH-2, SH-3, SH-4, SB-10, CD29, CD44, CD71, CD90, CD106, CD120a, CD124, and many other surface proteins. They are negative for other markers of hematopoietic lineages including CD14, CD34, CD45, CD41, T and B cell markers. In contrast, the mature mesenchymal cells such as fibroblasts or endothelial cells remain positive for CD34 surface antigen.[24]

Barry et al, reported the monoclonal antibody SH-2 against human marrow mesenchymal stem cells. They found that SH-2 monoclonal antibody recognized an epitope on endoglin (CD105), the transforming growth factor-β (TGF-β) receptor III presenting on endothelial cells, syncytiotrophoblasts, macrophages, and stromal cells. Endoglin on mesenchymal stem cells potentially plays a crucial role in TGF-β signaling in controlling of chondrogenic differentiation of mesenchymal stem cells, and also mediating interactions between mesenchymal stem cells and hematopoietic cells in the bone marrow microenvironment.[25]

Bruder et al, reported that the SB-10 antigen on mesenchymal stem cells surface corresponded to Activated Leukocyte Cell Adhesion Molecules (ALCAM).

Molecular cloning of full length cDNA from SB-10 antigen on mesenchymal stem cells expression library demonstrated the nucleotide sequence identity with ALCAM . SB-10 antigen was expressed on human mesenchymal stem cells but was lost during their developmental progression through mature mesenchymal lineages. The addition of antibody to SB-10 to human mesenchymal stem cells in vitro accelerated the osteogenic differentiation process. These results provided evidence that ALCAM plays a crucial role in the differentiation of mesenchymal tissues.[26]

Cytokine Expression of Mesenchymal Stem Cells

Bone marrow stroma is a multifunctional connective tissues composed of a heterogeneous population of cells. The stroma provides the bone marrow microenvironment that control hematopoiesis. Cells of the marrow stroma provide signal to the hematopoietic stem cells and its progeny through a variety of molecular mechanism, including cell-to-cell contract, secreted cytokines, and cell-matrix interaction. In addition, cells from the bone marrow stroma have been reported to include undifferentiated cells of mesenchymal origin, termed MSCs, which have the potential to give rise into multiple phenotypes of mesenchymal tissues including osteocyte, adipocyte, myocyte, fibroblast, and other mesenchymal phenotypes.[27-28]

MSCs are believed to differentiate along variety lineage pathways in response to signals they encounter in their microenvironment. However, the signals that induce mesenchymal stem cells entrance to progression along mesenchymal lineage pathways are not well understood.[27]

Heynesworth et al, reported the phenotypic characterization of human MSCs by establishing through the identification of cytokine expression profile under standardized growth medium condition and in the presence of regulator of osteogenic and stromal cell lineages. Under standardized growth medium, human MSCs constitutively express cytokines including granulocyte colony stimulating factor (G-CSF) , stem cell factor (SCF), leukemia inhibitory factor (LIF), interleukin-6 (IL-6), interleukin-11 (IL-11), while granulocyte-macrophage-colony stimulating factor (GM-CSF),interleukin-3 (IL-3), and transforming growth factor-beta (TGF-β) were not detected in the growth medium.[28]

Interleukin-1 (IL-1α), a cytokine released by many cell types during inflamatory response in duce MSCs to increase production of G-CSF, GM-CSF, LIF, M-CSF, IL-6, IL-11.GM-CSF and M-CSF are cytokines that support granulocytic lineage. LIF, IL-6, M-CSF, GM-CSF, IL-11 are cytokines that support monocyte/osteoblastic differentiation. IL-11 is the cytokine that support megakaryocytic lineage.[28]

Majumdar et al, showed the RT-PCR analysis of cytokine and growth factor mRNA in human mesenchymal stem cells and human bone marrow stromal cells. They reported the similar pattern of mRNAof IL-6, IL-7, IL-8, IL-11, IL-12, IL-14, IL-15, M-CSF, Flt3L, and SCF in the population of mesenchymal stem cells and bone marrow stromal cells. From these data, they extrapolated that MSCs could support hematopoiesis and represent an important cellular component of bone marrow microenvironment.[29]

3.Differentiation of Mesenchymal Stem Cells

Osteogenic Differentiation

MSCs involve in the bone formation in embryo and during adult fracture repair by continuously replicate themselves and differentiate into osteoprogenitor cells, while a portion become committed to mesenchymal cell lineage. The osteoprogenitor cells differentiate along the osteogenic lineages during osteoblast,secretory osteoblasts, and osteocyte. The transition from osteoprogenitor cells to each differentiated stages are controlled by cytokines and growth factor environment of the osteogenic lineage.[30]

Osteogenic differentiation of mesenchymal stem cells in vitro had been reported to occur in response of many bioactive factors including bone morphogenetic protein-2 (BMP-2)[30-31], osteogenic growth peptides[32], and synthetic glucocorticoid dexamethazone.[33]

Since endogenous glucocorticoids are involved in bone formation and bone remodeling.[34] Jaiswal et al , used dexamethazone to induce osteogenic differentiation of mesenchymal stem cells. They cultured human MSCs in the presence of osteogenic inductive elements including Dexamethazone (Dex) , L-ascorbic-2-phosphate or ascorbic acid (AsAp) ,and beta-glecerophosphate for 16 days. This

cultured cells performed the osteogenic differentiation by expression of alkaline phosphatase activity, modulation of osteocalsin mRNA production , and formation of mineralized matrix.[30]

Hanada et al , showed that fibroblast growth factor-β (FGF-β) and bone morphogenetic protein-2 (BMP-2) had been involved in the osteogenic differentiation by virtue of their mitogenic and differentiation activities. FGF-β greatly enhanced the mitogenic and osteogenic activities in dexamethazone-treated MACs more than BMP-2. Combining treatment with FGF-β and BMP-2 synergistically the osteogenic potency of FGF-β in marrow mesenchymal stem cells.[35]

D'Ippolito et al , explaned the decreasing number of osteogenitor cells in human after maturation by cultivation of human MSCs from various age subjects ranging from 4 to 88 years without metabolic diseases. They found that a number of alkaline phosphatase-positive CFU-Fs decreased with ages. These findings suggest that individual may caused by decreased number of osteoprogenitor cells in the bone marrow while rapid healing of fracture repair in children may caused by numerous number of osteoprogenitor cells.[36]

Chondrogenic Differentiation

Differentiation of MSCs along the chondrogenic lineage is a multi-transitory stages exemplified by the MSC committed to the chondrogenic progenitor , differentiated to be chondroblast , chondrocyte, hypertrophic chondrocyte, and calcifying hypertrophic chondrocyte, respectively.[37]

In vitro chondrogenic differentiation of human MSCs was reported by Yoo et al , in 1998. The MSCs were cultured in aggregate culture system in the presence of TGF-β. By the fourteenth day of cultivation , type II and X collagen were detected. Immunohistochemical staning with monoclonal antibodies specific for chondroitin4-sulfate and keratan sulfate demonstrated a uniformly distribution of proteoglycans throughout the extracellular matrix of the cell aggregation.[38]

Ranger et al , reported that the nuclear factor of activated T cells (NFAT) transcription factor NFATp (NFATc2) is a repressor of chondrogenesis. NFATp

expression is regulated in MSCs , induced differentiation along the chondrogenic pathway. Lack of NFATp resulted in increasing the expression of cartilage marker.[39-40]

Adipogenic Differentiation

MSCs differentiate along the adipogenic lineage by cultivation in the presence of adipogenic induction for 48 to 72 hours. The adipogenic induction consists of 1μM dexamethazone, 0.5 mM methy-isobutylxanthine , 10 g/ml insulin , 100 mM indomethacin , and 10% fetal bovine serum. After the incubation period , the adipogenic differentiation will be observed by the accumulation of lipid vesicles and by the expression of adipose-specific genes. These adipocytes remain healthy in culture at least 3 months.

Jaiswal et al , showed that mitogen-activated protein kinase (MAP kinase) activation contributes to osteogenic differentiation in mesenchymal stem cells, while blockage of MAP kinase induced the differentiation along adipogenic lineage explained by the expression of adipose-specific gene mRNA peroxisome proliferator γ2 , Ap2, and lipoprotein lipase. These data suggest that MAP kinase play a vital role in balancing the differentiation of osteoblastic and adipogenic lineage from human MSCs and relevant to the understanding mechanism of osteoporosis.[41]

Myogenic Differentiation

5-azacytidine, the analogue of cytidine, cause hypomethylation of some cytosine in DNA which may involve in activation of phenotype-specific genes, has the capacity to induce differentiation of MSCs along the myogenic pathway.[42] 5-azacytidine act as an activator of myogenic-specific genes involve MyoD1.[43] The observed myogenic cells exhibit multinucleated morphology of myoyubes, spontaneously contraction, and stained positively with a monoclonal antibody to muscle-specific myosin.[42]

Roger et al , reported that muscle morphogenetic protein (MMP) has the capability to induce differentiation of MSC through myogenic lineage. Adding MMP to the mesenchymal stem cell culture induces the expression of mypgenic regulatory gene

including MyoD1 and Myogenin suggesting that bone marrow provides the myogenic progenitor cells which can function in clinically myogenic regeneration.[44]

Cardiomyocytes can be generated from bone marrow MSCs by treatment with 5-azacytidine. About 30% of 5-azacytidine treated cells differentiate into cardiomyocyte in vitro.[45]

4. Mesenchymal Stem Cells in Clinical Application

Mesenchymal Stem Cells Support Hematopoiesis

MSCs characterized by having the potential to give rise to multiple mesenchymal tissue lineage, release a variety of cytokine and growth factors including IL-6, IL-7, IL-8 , IL-11 , IL-12 , IL-14 , IL-15 , Flt3L , LIF , M-CSF , and SCF.[27-28] Upon the releasing of a bulk of cytokines and growth factors resemble to the bone marrow stromal cells determine the hematopoiesis supporting capability of these culture-expanded MSCs.

Koc et al , reported in vivo hematopoiesis supporting capability of MSCs. They underwent expanding human MSCs and subsequently co-infusion with autologous hematopoietic stem cells in advanced breast cancer patients receiving high dose chemotherapy. They found the rapid recovery of hematopoietic cells in these patients, especially the megakaryocytic lineage. These data suggest that infusion of mesenchymal stem cells with hematopoietic stem cells after high-dose chemotherapy may have a positive impact on hematopoiesis process.[46]

There was evidence that some of mature mesenchymal stem cells could support hematopoiesis. Taichman et al , reported that osteoblast supported human hematopoietic stem cells in vitro bone marrow cell cultures by production of a variety of cytokines and growth factors.[47]

MSCs seem to support osteoclast differentiation from CD34 positive bone marrow hematopoietic progenitor cells, explained by the secreted cytokined such as IL-6 , IL-11 , LIF , which are the inductive cytokines for osteoclast differentiation.[48]

Mesenchymal Stem Cells and Stem Cell Transplantation

Transplantation of human MSCs was reported in 1995 by Lazarus et al , they determined the feasibility of ex vivo expansion and intravenous infusion of human marrow-derived MSCs in cancer patients. There are no toxicity and adverse reactions observed after infusion of these culture-expanded MSCs.[49] Transplantation of allogenic bone marrow with the donor stromal microenvironment improve engraftment of hematopoietic stem cells in the bone marrow.[50] These could be explained by the MSCs capacity to homing the bone marrow , engraft , and provide the suitable microenvironment for engraftment of hematopoietic stem cells.[51]

Homing and engraftment of the MSCs in the bone marrow provides a new therapeutic strategy to fight some of diseases such as genetic disorder, e.g. osteogenesis imperfecta , bone marrow transplantation could cure osteogenesis imperfecta by normal collagen type I production of engrafted donor mesenchymal stem cells in host bone.

Mesenchymal Stem Cell in Regenerative Medicine and Tissue Engineering

MSCs can be isolated , cryopreserved , and culture-expanded a several times and still retain multilineage potential. MSCs have been used to implant into the defect tissues such as bone , cartilage.[52]

A very promising study , using autologous MSCs enriched with chondrocytes implanted into surgically induced defect in chicks, resulted in a 100% success rate. MSCs also isolated from rabbits , expanded in culture, suspended in collagen gel and implanted into surgically induced defects in the patelar tendon.[53] Compare with the cell-free control , MSCs could repair the tissue defect and shoed good integration with surrounding tissues.[54]

Expanded human MSCs in a porous implant are capable of regenerating bone in rats and dogs in large segmental defects.[54]

Mesenchymal Stem Cell and Cell Therapy

MSCs have been used to support hematopoiesis by co-culture with $CD34^+$ hematopoietic stem cells. Transduction of $CD34^+$ cells in MSC culture condition is superior to bone marrow stromal cell culture condition.[55]

MSCs can be used to replace marrow microenvironment damaged following high-dose chemotherapy in order to improve hematopoietic recovery from myeloablative chemotherapy or to treat late failure or delay platelet engraftment.[55] MSCs are attractive vehicles for gene therapy in that expected not to be lost through differentiation comparing to hematopoietic stem cells. Examples of diseases in which mesenchymal stem cells-mediated gene therapy might be appropriate include factor VIII and factor IX deficiencies and the various lysosomal storage diseases.[56]

5. Osteoporosis

Osteoporosis is a skeletal disorder characterized by low bone mass and disturbance of the microarchitecture of the bone tissue. This pathologic process results in enhanced bone fragility and consequent increase in fracture risk.[57]

6. Pathophysiology of Osteoporosis

Males and Females will increase their bone mass with growth, achieving a peak bone mass by the age of 25. Thereafter, bone will be lost at a slow rate for men. Women have a markedly drop occurring around menopause, but after 60 years of age their rate is identical to bone loss in men.[58]

Bone constantly undergoes remodeling and repairing. These processes can be explained by osteoclastic resorption and then following with the resorption pit developed. The repair process initiated by growing of osteoblasts replacing the resorption pit.[58] Every remodeling cycle leaves a small deficit of bone. The unbalancing of the rate of bone resorption and bone formation lead to the onset of osteoporosis.[59]

Bone has a variety of function exemplified by providing structure support for human, repository of calcium which 98% of calcium are maintained in bone. And also the site of hematopoiesis.

Vitamin D is produced in the skin , converted to 25-hydroxyvitamin D in the liver. The 25-hydroxyvitamin D has 3 day half-life and still inactive. It can be degraded by P450 hydroxylase enzymes in the liver. When the calcium level is low, parathyroid hormone is released , stimulates the kidney to convert inactive 25-hydroxyvitamin D to the active 1,25-dihydroxyvitamin D. The kidney retains calcium from the glomerular filtrate. 1,25-dihydroxyvitamin D works with parathyroid hormone ultimately leads to the resorption of bone. The continuing of this process results in the elevation of serum calcium. Calcium deficiency often leads to the bone resorption.[59]

Bone is extremely sensitive to exercise and mechanical load. High loads will remodel bone to withstand the new load, low loads will maintain bone , no loads cause bone lost. Very high loads will lead to the failure of bone.

7. Osteoporosis therapy

There are numerous agents that have been developed to treat osteoporosis such as estrogen which acts as antiresorptive agents, the selective estrogen receptor modulators, calcitonin, and bisphosphonate. The general recommendation for all patients with osteoporosis is to ingest a physiologic level of calcium and vitamin D, having an appropriate exercise program.

Calcium and vitamin D decrease the bone resorption. The osteoporosis patients who receive calcium and vitamin D supplement have higher bone mass and low fracture rate.[60-61]

Estrogen has been used for prevention of osteoporosis, decrease bone resorption and increase bone mass. Estrogen has many effects, including a favorable modification of the cardiolipid profile which has been related to the decreasing risk for heart disease. Estrogen improve the genitourinary physiology lead to less urinary tract infection and better vaginal functions. There is some evidence that estrogen may be effect on improving of cognitive functioning in Alzheimer’s disease.[62]

Calcitonin, a non-sex, non steroid hormone play a role in skeleton development in the embryo and fetus. It has been used in patients with hypercalcemia. Calcitonin increase bone mass in the osteoporotic patients.

Bisphosphonate functions by binding the osteoclast-resorbing surface and acts as a non-degradable shied, and inhibits the osteoclast activity subsequent prevent bone loss.[63]

8. Osteoporosis in severe thalassemia

Thalassemia is an inherited single gene disorders anemia characterized by defects in synthesis of one or more globin chain subunits of hemoglobin tetramer. Clinical disease of thalassemia arised from the combined consequence of inadequate hemoglobin accumulation and unbalanced accumulation of globin subunits. Inadequate hemoglobin accumulation cause hypochromia and microcytosis. Unbalanced accumulation of globin subunits caused ineffective erythropoiesis and hemolytic anemia.

Osteoporosis is presented in severe thalassemia due to ineffective in erythropoiesis. More than 15-20 times comparing to normal individual expansion of bone marrow causes bone loss and osteoporosis. The patients may be suffered from bone change depending on the degree of osteoporosis. These defect could be observed in the radiological changing in the skull, facial bone, and other extremities.

Iron overload is not a primary cause of osteoporosis in severe thalassemia, but seem to be the secondary causes, due to the high level of iron contributing to the deposition in many organs such as thyroid gland, parathyroid gland, and others, causing the organ damage. In the case of parathyroid gland damage causing hypoparathyroidism, then involving osteoporosis in these patients. These evidence suggest that iron overload may play a minority role in osteoporosis in severe thalassemia.

Vitamin D metabolism in severe thalassemia patients is not different from normal individual. By the report of Rioja et al. In 1990, they underwent measuring 25 hydroxyvitamin D in severe thalassemia children patients comparing with the normal age matched with the controlling of diet intake in both groups. They found that the levels of 25 hydroxyvitamin D in these patients and control groups were not different. These data suggested that vitamin D metabolism may be not related to the pathophysiology of osteoporosis in severe thalassemic individual.[64]

9. Molecular Regulation of Osteoblast Differentiation

Bone, the major component of the skeleton, is formed by two distinct ossification processes, intramembranous and endochondral. Intramembranous bone arises directly from mesenchymal stem cells condensing at ossification centers and transforming directly into osteoblasts. This form of ossification give rise to flat bones of the skull, parts of the clavicle, and the periosteal surface of long bones. Endochondral ossification differs from the intramembranous component in that it is formed in the presence of a cartilaginous blastema. It is a complex, multistep process requiring the sequential formation and degradation of cartilaginous structures that serve as templates for developing axial and appendicular bones. This formation of calcified bone on a cartilage scaffold occurs not only during skeletogenesis but is an integral part of postnatal growth and fracture repair.

At the onset of skeletal development, undifferentiated mesenchymal stem cells come together to form condensation that have the shape of the skeletal elements they prefigure, followed by overt differerntiation along either the oateoblastic (intramembranous) or the chondrocytic (endochodral) pathway. Chondrocytes deposit an extracellular matrix composed of type IIb IX,and XI collagen and several other matrix proteins that are cartilage-specific. Following proliferation, these cells exit the cell cycle and undergo further differentiation to a hypertrophic form, characterized by the decreased expression of the type II collagen, expression of type X collagen, calcification of the extracellular matrix, and apoptotic death. Formation of mineralized cartilage is vital as it favors the vascular invasion of previously avascular cartilaginous anlage from the perichondrium. Osteoblast, which originate from mesenchymal stem cells, and osteoclast , which are derived from the hematopoietic compartment, also enter the zone of hypertrophy along with blood vessel. Osteoclast procees to degrade the calcified cartilage matrix, while osteoblast begin depositing the bone matrix , that consists primarily of the type I collagen, with the cartilage matrix being used as a scaffold.

10. The Osteoblast Differentiation Program

Skeletal cells are derived from three distinct embryonic cell lineage: neural crest cells contribute to the craniofacial skeleton; sclerotome cells frome somites give rise the axial skeleton; and lateral plate mesoderm cells form the appendicular skeleton.[65] Osteoblasts originate from immature mesenchymal cells, which could also give rise to chondrocytes, muscle, fat, ligament, and tendon cells.[66-67] These mesenchymal stem cells need to undergo several transitional steps, the exact number remaining imprecise at present, before becoming mature osteoblasts. Each transition requires the activation or suppression of critical molecular elements for the progression of differentiation to occur (Figure 1) . As will become apparent, the pathways of chondrocyte and osteoblast differentiation are intertwined number of genes are recruited to alter a precursor cells ability to commit to a particular cell lineage.

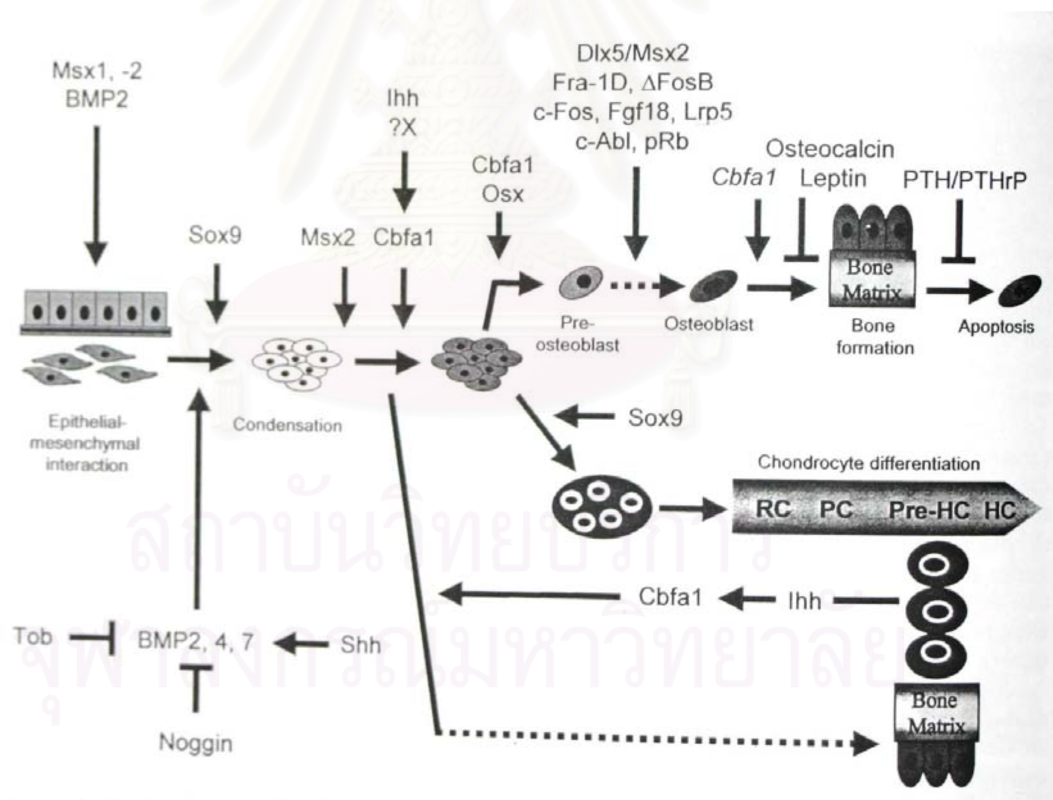

Figure1. Schematic representation of the network of signaling factors that control the formation of mesenchymal condensations their subsequent transition through the chondrocytic and osteoblastic differentiation programs.

The BMPs are members of the transforming growth factor-β (TGF-β) superfamily of proteins that act as morphogens to influence fundamental processes such as mesoderm patterning, left-right asymmetry, neurogenesis, development of the kidney, gut, lung, and teeth, as well as bone formation.[68] BMPs are said to control the commitment of mesenchymal pluripotent cells to the osteoblast phenotype and to induce ectopic bone formation in vivo.[69] The molecular signaling of BMPs is through heteromeric complexs of transmembrane type I and type II Ser/Thr kinase receptors that then propagate signals to the Smad proteins, which mediate BMP-induce signals from the cell surface to the nucleus.

Whatever in vivo evidence exists that BMPs influence osteoblast proliferation is indirect and stems from studies on Tob (transducer of ErbB-2), a member of a novel antiproliferative protein family (Tob/BTG proteins) that suppresses cell growth.[70] Overexpression of Tob mRNA in osteoblast lineage cells has indicated that the protein plays a prominent role in the biology of these cells. Moreover, the observation that mice carrying a targented deletion of the Tob gene have increased numbers of osteoblasts and greater bone mass has identified this protein as a negative regulator of BMP/Smad signaling in osteoblasts.[71]

How does Tob modify the process of osteoblastogenesis ? Tob represses BMP-induced, Smad-dependent transcription in osteoblast through its physical association with Smad4, as well as Smad1,5, and 8 , thereby initiating a negative feedback mechanism to allow a precise and timely regulation of BMP signaling and proper bone formation. In conclusion, although there is no direct evidence that BMPs specifically alter osteoblast proliferation , the findings in Tob deficient mice favor some role for these proteins in osteoblast proliferation.

The two vertebrate homologs of the Drosophila segment polarity gene hedgehog (hh), Sonic(Shh) , and Indian (Ihh), encode secreted proteins implicated in a variety of developmental process including skeletogenesis. Signaling to target cells is mediated by a receptor that consists of two subunits, Patched (Ptc), a12-trasmembrane protein which is the binding subunit,[72-73] and Smoothened (Smo) , a seven-transmembrane protein which is the signaling subunit. In the absence of hh, Ptc associates with Smo and inhibits its activities. In contrast, binding of hh to Ptc relives the

Ptc- dependent inhibition of Smo.[74] Signaling then ensues as the Gli family of transcriptonal activators. The three Gli genes (Gli, Gli2, and Gli3) encode a family of DNA-binding zing finger proteins with related target sequence specificities.

Shh induces expression of osteoblastic markers and ectopic bone formation when injected into muscle and commits C3H10T1/2 mesenchymal cells toward the osteoblastic lineage.[75] It appears that Shh increases the responsiveness of the cells to BMP-2 signaling and hence induction of osteoblast differentiation. Interestingly, only mature mesenchymal stem cells respond to Shh-potentiated BMP-2 signaling , whereas the more differentiated preosteoblastic MC3T3 cells do not.

One of the earliest transcription factors expressed during skeletogenesis is Sox9 (SRY (sex-determining region Y) –related, high-mobility-group (HMP)-box gene 9). BMP and Shh signaling induce and sustain Sox9 expression,[76] although there is no evidence that these factors act directly to activate Sox9 transcription. The protein is characterized by an HMG-box that binds to a specific sequence in the minor groove of DNA and, notably, bends and unwinds the DNA double helix. A potent inducer of genes required for cartilage formation, such as type2a1, 9a2, 11a2 collagen, and Aggrecan , Sox9 is initially expressed in mesenchymal progenitor cells , reaches high level in differentiated in chondrocytes, but its expression in turned off in hypertrophic chondrocytes.

Cells deficient in Sox9 are excluded from all cartilage, but are present as a juxtaposed mesenchymal that does not express the chondrocyte specific markers.[77] This exclusion occurs at the condensing mesenchyme stage of chondrogenesis, suggesting that Sox9 also controls expression of cell surface proteins needed for mesenchymal stem cells condensation , and thereby identifies Sox9 as a transcription factor essential for this transition step.

Sox9 is expressed in osteoblasts. Nevertheless , recent studies indicate that cells in mesenchymal condensation of both endochondral and membranous skeletal elements which, based on Cbfa1 expression, have committed to the osteoblast lineage, continue to express Sox9 and other chondrocytic markers (Sox5 and Sox6) when further differentiation is blocked.[78] These early progenitors, therefore, are bipotential and possess the ability to differentiation into osteoblasts and chondrocytes.

What processes in these common precursor cells dictates the final transition toward the osteoblast lineage remains unclear. The novel zinc finger-containing transcription factor Osx may act as the specific switch, perhaps functioning as a negative regulator of Sox9 expression that prevents these cells from choosing a chondrocyte differentiation program.

Msx2, a mammalian homologue of the Drosophila muscle segment homeobox gene (msx), has also been implicated in the regulation of early osteoblast development. Msx2 null mice have reduced numbers of osteoblasts and ossification defects, both intramembranous and endocondral, resulting from decreased proliferation of osteoblast progenitor cells.[79] These mice also show decreased level of Cbfa1 and Osteocalcin transcripts. In addition, studies in vitro have shown increased msx2 level at day 7 of osteoblast differentiation, which is prior to the appearance of Cbfa1. These finding suggest that msx2 is an upstream factor that coordinates the events of osteoblast formation, perhaps by influencing the transcriptional efficiency of Cbfa1 in particular target tissues. However, msx2 represses transcription of osteocalcin directly by binding to the OC box, a homeodomain motif in the osteocalcin promoter region. Because osteocalcin is expressed late in osteoblast differentiation and is potentially regulated by msx2, this homeobox-containing transcription factor may have two distinct roles: independently coordinating both early (transcription of Cbfa1) and late (transcription of osteoclacin) differentiation events.

Dlx5, a homologue of the distal-less (Dll) in Drosophila, is a BMP-inducible homeoprotein whose transcriptional activity is engaged in osteoblast differentiation. While overexpression of Dlx5 in osteoblastic cell lines leads to increased alkaline phosphatase activity, osteocalcin production, and accelerated maturation of the mineralized matrix, Cbfa1 expression is unaltered.[80] The generation of Dlx5 null mice has provided additional experimental support for a role of the transcription factor in osteoblast biology.[81] These mice exhibit only craniofacial defects,a delayed ossification of the roof of the skull, and abnormal osteogenesis, while no overt abnormalities are observed in the limbs, consistent with expression of Dlx5 in the cranial neural crest. Cbfa1 expression remain unaffected in these mice, suggesting that the two proteins use independent pathways to regulate osteoblast differentiation.

From this discussion, it is apparent that the transcriptional properties of msx and Dlx proteins display reciprocal action : Msx2 act as a transcriptional repressor, while Dlx5 operates as a transcriptional activator. Additionally, Msx2 and Dlx5 proteins in combination counteract each other's transcriptional activities.[82] Based on these observation, it has been proposed that functional antagonism through hererodimer formation is the mechanism for regulating the transcriptional actions of Msx and Dlx homeoproteins in vivo.

Ihh, the other member of the Hh family of proteins that partake in endochondral ossification, play a more prominent role by regulating the balance between growth and ossification of the developing bones. In the growth plate, Ihh is expressed in prehypertrophic chondrocytes. Studies of Ihh overexpression and misexpression in the chick developing cartilage[83] and of targeted disruption of Ihh[84] have shown that Ihh impedes the differentiation of chondrocytes to undergo hypertrophy. This process is mediated by the parathyroid hormone-related protein (PTHrP), the secretion of which plays a pivotal role in delaying the transition.[85-86] These observation have led to the suggestion that Ihh promotes chodrocyte proliferation[84] and, by signaling to cells in the perichondrium that in turn relay signals to upregulate PTHrP expression in the growth plate, indirectly delays chodrocyte differentiation.[83,87] Recent work has identified TGF-β2 as the intermediary signal between Ihh and PTHrP in the regulation of cartilage hypertrophic differentiation.[88] Ihh–deficient mice lack differentiated osteoblast in endochondral bones. They also exhibit complete absence Cbfa1 expression in the perichondrial/periosteal region. Thus, the absence of Ihh signaling affects osteoblast development in the endochondral skeleton, which at birth contains no mature osteoblasts. The presence of mature osteoblasts in membranous bones of the Ihh mutant is an argument for the existence of two distinct pathways for osteoblast development and indicates that formation of the bone collar, a process often described as similar to intamembranous ossification, is in fact genetically distinct. To summarize, by providing key local signals from prehypertrophic chondrocytes to both chondrocytes and preosteoblasts, Ihh couples chondrogenesis to osteogenesis in endochondral bone development.

11. The Osteoblast Lineage

The genes and corresponding protein products discussed thus far have been implicated in osteoblastogenesis because, in general, their null allele seems to delay or block the mesenchymal precursor cells from differentiating into osteoblast. Alternatively, these genes and their protein products may downregulate other molecular markers that define the osteoblast phenotype. Nevertheless, none appears to control osteoblast formation. While differentiation is coordinated by transcription factors that either up-or downregulate gene expression in response to local signals, lineage-specific transcription factors play pivotal roles in determining the fate of each cell type. Over the past five years, much effort has been placed on defining the role of Cbfa1 (core binding factor 1 ; Runx2) as the master ' switch' of osteoblast differentiation. This mammalian homologue of the Drosophila runt family of transcription factors is conserved from Caenorhabditis elegans to humans and contains a hallmark 128 amino acid DNA binding domain, called the runt domain, that is present in all transcription factors belonging to runt family. The CBF proteins bind the enhancer core TGYGGT, a sequence motif identified in transcriptional enhancers of many genes expressed in cells of hematopoietic origin.

Cbfa1 was first identified as the nuclear protein that binds to an osteoblast-specific cis-acting element (OSE2)[89] and activates expression of osteoblast-specific gene, Osteocalcin (OG2)[90] . it is the earliest and most specific marker of osteogenesis identified to date. In the mouse, it is first expressed in the lateral plate mesoderm at embryonic day 10.5 (E10.5) in region that prefigure cartilaginous condensation of the developing skull and the axial and appendicular skeleton, but not in earlier undifferentiated mesenchymal stem cells. Between E10.5 andE12.5, Cbfa1 expression is evident in mesenchymal condensation representing cells that are bipotential: i.e.,they have the capacity to differentiate into chondrocytes and osteoblasts. As bone formation begins (E14.5), Cbfa1 transcripts are restricted to osteoblast while in chondrocytes expression is localized to prehypertrophic chondrocytes. Postnatally, Cbfa1 becomes undetectablle in these cells and is expressed only in osteoblasts.[91]

Cbfa1 can induce osteoblast-specific gene expression from non-osteoblast cells in the culture.This indicates that Cbfa1 is necessary, but perhaps not

sufficient, for mesenchymal cells to differentiate into osteoblasts.[90] Additional functions for Cbfa1 may include a role in osteoblast function. In chondrocytes, Cbfa1 is required for skeletogenesis, including formation of joints, permanent cartilage, and endochondral bones.[92]

The ultimate demonstration that Cbfa1 is a transcription factor essential for regulation of osteoblast differentiation has come from genetic studies in mice and humans. Mice lacking Cbfa1 have a skeleton that is entirely cartilaginous, failing to undergo endochrodral and intramembranous bone formation.[93-94] This is because osteoblast differentiation is arrested as early as E12.5; this also indicates that there is no alternative molecular pathway to compensate for this defect. Mice heterozygous for Cbfa1 –null allele display a phenotype that is characterized by hypoplastic clavicles and a delay in fontanelle closure.[93] This condition resembles the human disorder cleidocranial dysplasia (CCD) , transmitted by autosomal dominant gene with complete penetrance. CCD patients exhibit short stature and delayed skeletal development. They have hypoplastic clavicle or ,in extreme cases,clavicle are completely absent. Their fontanelles are open and they have dental anomalies such as supernumerary teeth. Molecular analysis of genomic DNA from patients with CCD has indicated the existence of deletions, insertions, nonsense,or missense mutations dispered throughout the Cbfa1 gene.[95-96] Sequencing of the two Cbfa1 alleles in several CCD patients showed that one copy of the gene was consistently mutated. This demonstrates that CCD caused by Cbfa1 haploinsufficiency. More than 50 independent mutations in the Cbfa1 gene have been reported so far, all leading to CCD with varying degrees of clinical severity. Most of the mutations appear to affect the runt domain, with missense mutations abolishing the ability of Cbfa1 to bind DNA. The linkage with CCD, a haploinsufficiency of Cbfa1 emphasizes the important role played by this transcription factor in osteoblast differentiation.

How does Cbfa1 induce the osteoblast phenotype? As already mentioned, Cbfa1 was first identified because of its capacity to bind to OSE2 in the Osteocalcin promoter.[89-90] OG1, the other murine Osteocalcin gene, Bone sialoprotein (BSP) , Osteopontin , and type α1(I) collagen gene also contain in their promoter regions OSE2 elements that mediate their transcriptional regulation by Cbfa1 , in vitro

and in vivo. For instance, addition of Cbfa1 antisense oligonucleotides to osteoblast cultures specifically decreases the expression of these genes. In both Osteocalcin and type α1 (I) collagen promoters, more than one functional OSE has been identified. In fact, there appears to be a hierarchy among the OSE regions that confer a greater ability to activate transcription of the particular gene.[89] These elements likely work synergistically to transcribe osteoblast genes in a temporal fashion so as to promote proper maturation of the osteoblast phenotype.

Once it was established that Cbfa1 is the molecular switch for the induction of osteoblast formation, attention switched to defining what controls its regulation. Cbfa1 expression and hence function must be tightly controlled since appropriate activation and repression of its transcription during osteoblast differentiation would be essential for proper program execution. Several growth and transcription factors have been identified as potential regulators of Cbfa1. Regulation of this master ' switch' likely occurs at several levels, perhaps one not excluding the other, such as transcription enhancement or repression of specific growth factors that alter Cbfa1 expression, that activate or disable Cbfa1 function post-translationally or make available cofactors that alter Cbfa1 affinity for DNA, and thereby its transcriptional activity.

To date, at least three genes have been reported to encode transcription factors that regulate Cbfa1 expression: Msx2 whose inactivation in mice lead to a downregulation of Cbfa1[79]; Bapx, a gene encoding a homeobox protein required for axial skeleton formation, which may activate Cbfa1,[97] and Hoxa-2. The latter encodes another homeobox protein that inhibits Cbfa1 expression in the second branchial arch.[98] However, none of these transcription factors, other than Cbfa1 itself, have been shown to bind upstream regulatory elements of the Cbfa1 promoter. This unique mechanism of gene auto-regulation has also been described in the case of Cbfa2, raising the possibility that that feedback on itself is common property of the Cbfa family of transcription factors. There are at least three Cbfa1 recognition motifs in the rat Cbfa1 promoter and three tandemly repeated Cbfa1 sites within the 5'UTR. As a result, forced expression of Cbfa1 protein downregulates Cbfa1 promoter activity.[99] This negative autoregulation may allow for immediate adjustments in the transcriptional level of Cbfa1,thereby avoiding generation of excess gene transcripts. Such rigorous control is

undoubtedly necessary for optimal and precise regulation of a key homeostatic function such as skeletal ossification.

In vitro studies have indicated that Cbfa1 expression is under the control of BMP, TGF-β, and their effectors, the Smad proteins.[100] Osteoblasts are a source of the TGFβ that is found in the bone matrix, and are also modulated by TGFβ in an autocrine fashion.[101] Although TGFβ promotes proliferation, early osteoblast differentiation, and extracellular matrix production, it inhibits transcription of the Cbfa1 and Osteocalcin genes, whose expression is controlled by Cbfa1 in osteoblast-like cell lines.[102] TGFβ requires the presence of Cbfa1 in order to downregulate transcription. Down regulation occurs through Smad3, wich interacts physically with Cbfa1 and represses its transcriptional activity at the Cbfa1-binding OSE2 promoter sequence.

Cbfa1 has also been reported to interact with the basic helix-loop-helix transcription factor HES1, a mammalian counterpart of the Drosophila hairy and enhancer of split proteins. This interaction is mediated by the carboxyl terminal domains of Cbfa1 and HES1.[103] HES1 can antagonize the binding of Cbfa1 to mammalian transcriptional corepressors of the Groucho family. Moreover , HES1 can potentiate Cbfa1-mediated transactivation in transfected cells. Taken together , these finding implicate HES1 in the transcriptional activity of Cbfa1 and suggest that the concerted activities of Groucho and HES proteins modulate the functions of Cbfa1.

Several lines of evidence now implicate the retinoblastoma tumor suppressor protein (pRb) in osteoblastogenesis.pRb inhibits cell cycle progression and promotes differentiation by acting as a corepressor of the E2F family of transcription factors. These proteins transactivate genes important for G1 to S phase transition.[104] pRb itself is frequently inactivated in a subset of human tumors, including retinoblastomas, osteosarcomas, small cell lung carcinomas, and bladder carcinomas. Concerning osteoblastogenesis, viral oncoproteins that target the core domain, referred to as the pRb pocket, inhibit osteoblast differentiation. Continued differentiation in contingent upon deactivation of the oncoprotein.[105] Furthermore, reexpression of pRb in SAOS2 osteosarcoma cells induces markers suggestive of bone differentiation[106]. How does pRb promote osteoblast differentiation? it physically interacts with the osteoblast transcription factor Cbfa1 and, in a Cbfa1-dependent fashion, associates with

osteoblast-specific promoters in vivo.[107] Hence, pRb binds to full length Cbfa1 with both its C terminus and the pocket domain. For Cbfa1, the C-terminal 141 amino acids are required for the interaction. This region contains domains that are important for nuclear matrix tethering and binding to transcriptional modulators.[108] Consequently, association of pRbwith Cbfa1 and promoter sequences results in synergistic transactivation of an osteoblast specific reporter and promotion of osteoblast differentiation by facilitating expression of late markers of the process.pRb,thereby, participates in two function , one linked to cell cycle exit and the other to differentiation control. These two function can be dissociated genetically and mechanistically.

A novel zinc finger-containing transcription factor called osterix (Osx) has recently been cloned and appears vital for osteoblast differentiation.[78] Osx null mice fail to from bone, as cells in the periosteum and membranous skeletal elements of the cranium cannot differentiate into osteoblasts. Similarly, mesenchymal stem cells, along with osteoclasts and blood vessels, invade the mineralized matrix. However, the mesenchymal stem cells fail to express bone matrix. Interestingly, these cell express Cbfa1, but Osx is not expressed in Cbfa1 null osteoblasts ; this suggests that Osx acts downstream of Cbfa1. Lack of bone formation appears to be caused entirely by the inability of osteoblasts to differentiate. Interestingly, Osx null preosteoblasts also express chondrocyte marker genes (Sox9, Sox5, Typeα1 (I) collagen , and Ihh) ; this adds credence to the hypothesis that osteoblasts and chondrocytes derive from a common precursor cell. From these finding, a model of osteoblast differentiation emerges in which mesenchymal stem cells condensation first differentiate into osteo/chondroblasts, a process that absolutely dependent on Cbfa1 expression. These cells do not express osteoblast markers except type α1 (I) collagen, but are bipotential. It is likely that Cbfa1 and Osx collaborate to activate osteoblast-specific matkers and produce the bone specific matrix.

Members of the activator protein-1 (AP-1) family have also been shown to take part in the regulation of osteoblast differentiation. AP-1 is a dimeric complex of Fos and Jun proteins that belongs to the bZIP transcription factor. Early studies have shoen that c-Fos overexpression in transgenic mice leads to osteosarcoma development[109].; this data suggest that c-FOS is a regulator of osteoblast proliferation in

vivo. On the other hand, overexpression of ΔfosB, a truncated from of FosB[110], and of Fra-1[39], leads to severe osteosclerosis in transgenic mice due to enhanced progenitor differentiation and increased activity of osteoblasts. Apparently, this is not a true physiological function of these proteins since FosB null[112] and Fra-1 null[111] mice have normal bone formation. Osteosclerosis in two mouse models may arise from the interaction of the two factors through heterodimerization either to an activator of osteoblast differentiation to increase its transcriptional activity or to an inhibitor of osteoblast differentiation and thereby relive inhibition of a normal physiological process.[113]

One of the most unanticipated developments in the field of osteoblast biology has come from the investigation of low-density lipoprotein receptor-related proteins (LRPs). These are cell surface receptors characterized by the presence of cysteine-rich complement-type ligand-binding domains. The LRPs are larger than, but structurally similar to, other members of the LDL receptor gene family. Whereas the LDL receptor, the founding member of this family, acts solely in lipoprotein metabolism, the LRPs appear to have other distinct function including homeostasis of proteinases and proteinases inhibitor, cellular entry of viruses and toxins, activation of lysosomal enzymes, cellular signal transduction and neurotransmission.[114]

In recent work, LRP5 has been shown to transduce the canonical Wnt signal by fucntioning as a component of the Wnt receptor complex.[115] Wnt proteins control early events during skeletal development such as limb patterning and joint formation. However, the recent finding that LRP5 is inactivated in osteoporosis-pseudoglioma syndrome patients, and is mutated in patients with high bone mass syndrome,[116] strongly point to a role that Wnt proteins may play later during development and postnatally. In vitro and In vivo analysis of mice with targeted disruption of Lrp5 has established that the protein is expressed in osteoblasts and required for optimal Wnt signaling. Moreover, the low bone mass phenotype that becomes evident postnatally is secondary to decreased osteoblast proliferation, but functions in a Cbfa1-independent manner.[117]

c-Abl, the product of the c-Abl protooncogene, is a widely expressed non-receptor tyrosine kinase found in moderately high levels in osteoblasts. Mice

homozygous for the disrupted c-Abl gene display thinner cortical bone and reduced trabecular bone volume.[118] The osteoporotic phenotype in these animal is not due to accelerated bone turnover, but rather to dysfunctional osteoblasts, which show delayed maturation in vitro as measured by expression of alkaline phosphatase activity, induction of Osteocalcin mRNA and mineral deposition. Cell adhesion regulates the kinase activity and subcellular localization of c-Abl.[119] Upon cell adhesion to fibronectin, c-Abl is exported from the nucleus into the cytoplasm where it is activated and thereby can transmit integrin signals to the nucleus. There, it functions to integrate adhesion and cell cycle signals. Lack of c-ABL kinase in the mutant osteoblasts is therefore likely to interfere with the integrin signaling required for proper differentiation.

The gene encoding Fgf18 is normally expressed in the perichondrium and negatively regulates Ihh levels. When disrupted, there follows increased Ihh expression and a delay in periosteal osteoblast differentiation. Yet, the levels of Cbfa1 expression in the perichondrium/periosteum of Fgf18-null mice are normal ; this suggests the presence of an appropriate number of osteoprogenitor cells.[120] Therefore, in contrast to Ihh, which is required for the early specification of the osteogenic cell lineage, Fgf18, signaling through Fgfr2 in the perichondrium/periosteum functions to promote osteoblast maturation/proliferation.

Krox20, originally identified as a serum response immediate-early gene, encodes a three C2H2 zinc finger transcription factor that is expressed in chondrocytes, and in endosteal and periosteal osteoblasts. Krox20 first appears at E14.5 (2 day following the appearance of Cbfa1) and is limited to the periphery of the diaphyseal part of the cartilage model. This observation suggest that Krox20 is involved in differentiation, once commitment by mesenchymal precursors to the osteoblast lineage has been established. It occurs in all sites undergoing endochondral ossification and is completely absent in areas that undergo intramembranous ossification. Krox 20 null mice have defects in endochondral bone formation. The defects are characterized by shorter and thinner long bones, markedly diminished trabecular bone, and severe porosity.[121] Although at present there is little understanding of the proteins function and mode of action, its expression in hypertrophic chondrocytes and differentiating osteoblasts has led to the postulate that Krox20 acts to further differentiate these

chondrocytes into " osteoblast-like cells " , a hypothetical last step of chondrocyte differentiation.[122] Hence, in Krox20 null mice chondrocyte differentiation and proper bone formation would be disrupted, this in turn leading to a decrease in trabecular bone mass.

12. Osteoblast Function

Osteocalcin , a predominant non collagenous protein of bone, is expressed late in osteoblast maturation, well after the induction of differentiation , at the onset of extracellular matrix mineralization. Osteocalcin is not expresses in non-osseous cells, nor is it transcribed in osteo-progenitor cells or early stage proliferating osteoblasts. However, transcript levels increase significantly following the postproliferative onset of osteoblast differentiation.

Examination of the upstream regulatory elements of the Osteocalcin promoter has provided insight into the regulation of osteoblast differentiation by uncovering novel upstream regulating factors.[123] The Osteocalcin promoter encompasses several cis-acting upstream elements, including the OC box, a 24 nulcleotide sequence within the promoter domain that is a protein/DNA interaction site for the Cbfa1 transcription factor. Another element binds the MSX family of homeobox genes, which negatively regulate transcription. Other upstream elements in the osteocalcin promoter are C/EBD and AP-1 sites, a TGF-β response element, a vitamin D response element, glucocorticoid response elements and matrix protein binding sites. This large repertoire of cis-acting elements is a clear indication of the precise and rigorous regulatory control that ultimately dictates the level of Osteocalcin gene expression. What than is the role of osteocalcin in osteoblast biology ? Analysis of genetically engineered osteocalcin deficient mice reveals an accelerated rate of bone formation without changes in osteoblast or osteoclast number; this suggest that the protein functions to inhibit bone formation. The highest level of osteocalcin expression is in the osteocyte. Because these cells appear at least partly responsible for promoting mineral maturation , this osteocyte function may be defective in the knockout mice. This defect in turn strengthens the inference that osteocalcin is required to stimulate bone mineral maturation.[124]

A role in osteoblast function, beyond cellular differentiation, has also been described for the pleiotropic regulator of skeletogenesis, Cbfa1. Such a function was documented by the generation of the transgenic mice that express a dominant-negative variant of Cbfa1 under the control of the Osteocalcin promoter.[125] These mice have a normal skeleton at birth, but develop an osteopenic phenotype thereafter. Histomorphometric studies have shown that this phenotype is the result of a major decrease in the rate of bone formation. Yet, the number of osteoblasts is normal. This indicates that once osteoblasts are differentiated, Cbfa1 regulates their function, i.e., the production of the bone extracellular matrix. Additional confirmation for Cbfa1 role in osteoblast function has come from the observation that its overexpression in osteoblasts inhibits their maturation and causes osteopenia with multiple fractures.[126]

The observation that bone remodeling occurs simultaneously at multiple skeletal sites hints at endocrine regulation of this process. This was first uncovered by studies evaluating the effects of sex steroid hormones and PTH on osteoclast differentiation and resulting bone resorption. This led to the suggest that bone formation also is under hormone influence.

Leptin , because of its recognized influence in several different but potentially related pathways, may be such an endocrine factor.[127] Leptin, the product of ob gene, is a small polypeptide produces primarily by white adipose tissue. It influences body weight maintenance by modulating food intake and energy expenditure through negative feedback on hypothalamic nuclei.[128] Ob/ob mice (animal model of leptin deficiency) and leptin receptor negative (db/db) mice are obese and exhibit a high bone volume , notwithstanding concurrent hypogonadism and hypercortisolism, two strong proresorptive signals that would normally lead to low bone mass. This relationship between high bone mass and obesity is specific to the leptin pathway, since, for example, the Agouti yellow mouse, which is genetically obese, does not show increases in bone mass. Moreover, high bone mass in the ob/ob mice precedes the development of obesity. Finally, transgenic mice that lack most of the white adipose tissue due to adipocyte-specific expression of a dominant negative form of a protein that prevents the DNA binding of B-ZIP transcription factors,[129] have lower leptin levels but have a high bone mass. It is therefore the absence of leptin signaling, rather than a

secondary, adipocyte-derived signal arising from leptin deficiency, that favors bone formation. Together , these observation raise the likelihood that leptin is directly involved in the regulation of bone metabolism.

The question then arises by what mechanism leptin coordinates skeletal metabolism. Histomorphometric analysis suggested that leptin exerts its effect by inhibiting bone formation, rather than stimulating its resorption. In ob/ob mice, there is an increase in functional osteoclasts, likely as a consequence of the concomitant hypogonadism. On the other hand, bone formation rate is twice as high as that of wild type mice, while the number of osteoblasts remains normal, suggesting that leptin action on bone mass is through inhibition of osteoblast function.

Once it had been established that leptin deficiency causes a change in osteoblast function and not number, then the focus switched to determining whether leptin mode of action is autocrine/paracine,endocrine,or neuroendocrine. Studies by Ducy and coworkers failed to show that leptin or ObRb, the functional form of the leptin receptor, is expressed in primary osteoblasts. Moreover, leptin did not induce early gene expression (Tis11 and c-fos) through phosphorylation of Stat3. This suggest that osteoblasts are not a direct target of leptin. On the other hand, Thomas and colleagues showed that HMS2-12 cells, a bipotential human bone marrow stromal cell line that can differentiate into either osteoblasts or adipocytes, express the ObRb leptin receptor and are therefore targets of leptin action[130]. Leptin mRNA expression and leptin immunoreactivity have also been observed in human primary osteoblast cultures, and notable amounts of leptin were released into the culture medium following prolonged incubation.[131]

Are the intermediary coponents of this relay neuronal or endocrine ? Neuropeptide Y (NPY) is a downstream modulator of the leptin action[132], possibly at the level of the arcuate nucleus where NPY neurons are known to express both leptin receptors and Y2 receptors. Y2 receptor-deficient mice exhibit a two-fold increase in the trabecular bone volume, as well as greater trabecular number and thickness, compared with control mice.[133] Central Y2 receptors are crucial for this process, since selective deletion of the hypothalamic Y2 receptors in mature conditional Y2 knockout mice results in an identical increase in the trabecular bone volume. This hypothalamus-

สถาบันวิทยบริการ จุฬาลงกรณ์มหาวิทยาลัย

specific Y2 receptor deletion stimulates osteoblast activity and increases the rate of bone mineralization and formation, with no effect on osteoblast or osteoclast surface measurements.There were no changes in plasma calcium,leptin,or hypothalamopituitary corticotropic, thyrotropic, somatotropic,or gonadotropic hormone output. This indicates that Y2 receptors do not modulate bone formation by humoral mechanisms. Rather, alteration of autonomic function, and specifically , the sympathetic nervous system, may be the mechanism by which central NPY, and consequently leptin , can influence bone formation. In fact, recent studies have indicated that leptin deficiency results in low sympathetic tone, and genetic or pharmacological ablation of adrenergic signaling leads to a leptin-resistant high bone mass.[134] Moreover, β-adrenergic receptors on osteoblasts appear to regulate their proliferation, and administration of β-adrenergic antagonists increases bone mass in wild type and ovariectomized mice.

Taken together , the findings strongly favor a role leptin in the control of bone mass. The evidence so far indicates that leptin, acting via a hypothalamic relay involving NPY and the sympathetic nervous system, limits the amount of bone matrix that osteoblasts can make. A direct action of leptin on osteoblasts is an alternate, as yet undocumented mechanism.

CHAPTER III

MATERIALS AND METHODS

Research Instruments

1. Optical 96-well reaction plate (Applied Biosystems, USA.)
2. MS separation column (Miltenyi Biotec GmbH, Germany)
3. Separation filter (Miltenyi Biotec GmbH, Germany)
4. Magnetic stand with column (Miltenyi Biotec GmbH, Germany)
5. Hemocytometer (Bright-line, USA.)
6. Centrifugator (Eppendorf, France)
7. 15 and 50 ml. tube (Falcon, USA.)
8. Conical tube (Falcon, USA.)
9. Optical adhesive cover starter kit (Applied Biosystems, USA)
10. 1.5 ml. microcentrifuge tube (Falcon, USA.)
11. 0.2, 1.0 , 1.5 ml microtube (Axygen scientific, USA.)
12. 1, 5, 10, 25 ml. and transfer pipette (Falcon, USA.)
13. 10, 100, 1000 μl tip (Axygen scientific, USA.)
14. 12-well culture plate (Falcon, USA.)
15. Light microscope (Olympus, Japan)
16. Inverted microscope (Nicon, Japan)
17. Slide (Sail brand, China)

18. Cytospin (Shandon, USA.)

1. Automatic adjustable micropipette : P2 (0.1-2 μl), P10 (0.5-10 μl), P20 (5-20 μl), P100 (20-100 μl), P1000 (0.1-1 ml) (Gilson, France)

20. Pipetette boy (Drummond, Japan)

21. Vortex (Scientific industry, USA.)

22. Parafilm (Pechiney, USA)

23. Waterbath (Memmert, Germany)

24. CO_2 incubator (Lab-lines, USA.)

25. Laminar flow (Holten LaminAir, Denmark)

26. FAScan (Becton-Dickinson, USA)

27. Camera set (Olympus, Japan)

28. Refrigerator 4 ^{0}C (Frigidarire, USA.)

29. Deep freeze -20 ^{0}C, -80 ^{0}C (SHARP, Japan)

30. Autoclave (Stermite, Japan)

31. Mastercycler gradient (Eeppendorf, France)

32. ABI Prism 7000 sequence detection system (Applied Biosystems, USA)

Reagents

1. Phosphate buffer saline; PBS (GIBCO, USA)

2. Tryphane blue (Merck, USA.)

3. Giemsa and giemsa buffer (Merck, USA.)

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

4. Water Nuclease-free (Hyclone, USA.)

5. Ficoll Hypaque (Robbin scientific, USA)

6. CD133 cell isolation kit (Miltenyi Biotec GmbH, Germany)

7. CD 105 cell isolation kit(Miltenyi Biotec GmbH, Germany)

8. Dulbecco's modified eagles medium-high glucose ; DMEM-HG (BioWhittaker, USA)

9. Fetal calf serum; FCS (BioWhittaker, USA)

10. L-glutamine (GIBCO, USA)

11. Trypsin-EDTA (GIBCO, USA)

12. Gentamycin (M&H Manufacturing, Thailand)

13. CD133/2 conjugated with PE (Miltenyi Biotec GmbH, Germany)

14. CD105 conjugated with PE (Serotec, UK)

15. CD34 conjugated with FITC (Caltag Laboratories, USA)

16. CD38 conjugated with FITC (Caltag Laboratories, USA)

17. CD45 conjugated with TriColor (Caltag Laboratories, USA)

18. Isoton II sheath fluid (Immunotech-Coulter, USA)

19. 1% paraformaldehyde

20. AquaPure RNA isolation kit (Bio-Rad, USA)

21. Taqman Reverse Transcription System(Applied biosystems,USA)

22. SYBR Green PCR mastermix (Applied Biosystems, USA)

23. Primer (Pacificscience, USA)

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Procedure

1. Bone marrow collection

Bone marrow samples were obtained using a bone marrow biopsy needle inserted through posterior iliac crest of the 10 severe thalassemic children patients (age 1-15 years) and 10 healthy bone marrow donor (age 1-15 years) after an informed consent. The total 20-25 milliliters of bone marrow cells were heparinized and gently mixed for clot preventing.

2. Separation of bone marrow mononuclear cells

Bone marrow mononuclear cells were separated by density gradient centrifugation with Ficoll-Paque. Briefly, 20 ml of heparinized bone marrow mononuclear cells were mixed in equal volume of Dulbecco's Modified Eagle's Medium (DMEM) (Bio Whittaker ,USA) and centrifuged at 900g for 10 minutes at room temperature. The washed cells were resuspended in DMEM at a density of 4 x 10^7 cells/ml, and 10 ml aliquot was layered over Ficoll-Paque and centrifuged at 1,300g for 20 minutes at room temperature in a swing-bucket rotor without brake. The interface mononuclear cells were collected and washed with DMEM twice. Finally, counting and viability checking were performed by 0.2% trypan blue exclusion.

3. Cultivation of mesenchymal stem cells

The 2 x 10^4 cells/ml of isolated mesenchymal stem cells were transferred to the 12-well plate and cultured in DMEM supplemented with 10% fetal bovine serum, and 1 μM Penicillin-streptomycin (M&H manufacturing, Thailand). The cultured cells were incubated at 37°C 5% CO_2 in CO_2 incubator. On day 3 of cultivation, the non-adherent cells were eliminated by discarding and replacing with fresh-warmed DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics. Medium changing was performed every 4 days after that. The initial plating called passage 0 cells. Trypsinization and passaging were performed when the cultured cells reached 90% confluent.

4. Trypsinization of Mesenchymal Stem Cells

When the cultured cells were reached 90% confluent, the MSCs were trypsinized by 0.05% trypsin (Gibco BRL, USA). In brief, the medium was discarded from tissue culture flask, and replaced with 2 ml of 2% EDTA/PBS solution and incubated at 37°C for 2 minutes. After that , 2% EDTA/PBS solution was removed and replaced with 1 ml of 0.05% Trypsin (Gibco BRL, USA) , gently mixed and incubated at 37°C in CO_2 incubator for 5 minutes. Observation of detached cells was performed by inverted microscope. After reaching the setting time the 2 ml of fetal bovine serum were added to the culture flask for neutralizing the activity of Trypsin. The trypsinized cells were washed twice with DMEM at 800g for 5 minutes.

5. Passaging of Mesenchymal Stem Cells

The 2 x 10^{5} cells/ml of trypsinized cells were cultured in DMEM supplemented with 10% FBS and antibiotics in new 25 cm^{3} culture flask, this called passage1 cells. The cultured cells were incubated at 37°C 5% CO_2 . Medium changing was performed every 4 days. Upon 90% confluent reaching of cultured cells, trypsinization and passaging would be done for the next expanding.

6. Enrichment of Mesenchymal stem cells by positive selection with CD105 conjugated microbeads.

Prepare single cell suspension from density gradient centrifugation using Ficoll-Paque. To remove clumps, pass cell through nylon mesh. Wash cells, remove supernatant completely and resuspend cell pellet in 80 μl of buffer (PBS pH7.2, supplemented with 0.5% fetal bovine serum and 2mMEDTA) per10^{7}total cells. Add 20 μl of MACS CD105 microbeads per 10^{7}total cells, mix well and incubate for 15 minutes at 6-12°C. After that, wash cells by adding 10-20X the labeling volume of buffer, centrifuge at 200xg for 10 minutes, remove supernatant completely and resuspend cell pellet in appropriate amount of buffer. Place the column in the magnetic field of an appropriate MACS separator. Prepare the column by washing with appropriate buffer. Apply cell suspension in appropriate amount of buffer onto the column. Let the negative cells pass through and rinse with appropriate amount of buffer. Remove column from separator,

place column on a suitable collection tube, pipette appropriate amount of buffer onto the column and firmly flush out positive cells using the plunger supplied with the column. To achieve a higher purity, apply positive fraction to a new, fleshly prepared column. Let the negative cells pass through. Rinse with appropriate amount of buffer. Elute positive fraction as described above.

7. Enrichment of Hematopoietic stem cells by positive selection with CD133 conjugated microbeads

Prepare single cell suspension as described above. Add 100μl FcR blocking reagent to 10^8 total cells resuspended in 300μl of buffer, to inhibit unspecific or Fc-receptor mediated binding of antibodies to non-target cells. Label cells by adding 100μl CD133 microbeads (final volume 500 μl per 10^8 total cells. Mix well and incubate for 30 minutes at 4-8 °C. Wash cells by adding 10-20X the labeling volume of buffer, centrifuge at 300xg for 10 minutes. Pipette off the supernatant , resuspend cell pellet in buffer and proceed to magnetic separation as described in enrichment of mesenchymal stem cells. The morphology of isolated CD105 and CD133 cells were assessed using the Giemsa stained cytospin preparations.

8. Phenotypic Analysis of Mesenchymal Stem Cells by Flow Cytometry

The cells from isolation were adjusted the concentration to 10^5 cell/100μl . The 100μl of adjusted cells were incubated with 10 μl of monoclonal antibody CD105-PE, CD34-FITC, CD133-PE , CD45-TRI color , respectively , for 20 minutes at 4°C in the dark . the 2 ml of PBS were added to each monoclonal antibody-treated cells, centrifuged for 5 minutes at 800xg and removed the supernatant, these steps were repeated once. The cells were fixed with 0.5% paraformaldehyde and stored at 4°C in the dark until acquisition on flow cytometry (FACScan, Becton Dickinson, USA) using CELLQUEST software programme.

9. Studies of Mesenchymal Stem Cells Gene Expression by Quantitative Real-Time PCR

Total RNA was isolated from isolated cells by using AquaPure RNA isolation kit (Bio-Rad) method as described by manufacturer. CDNAs were reverse transcribed from total RNA using Taqman Reverse Transcription System (Applied biosystems) with random hexamer as primer as described by manufacturer. Quantitative real time PCR was performed using ABI Prism 7000 sequence detection system (Applied Biosystems , CA, USA) 3 μl of cDNA was analyzed in 25μl reaction of SYBR Green PCR mastermix (Applied Biosystems) . real time PCR reaction was performed with target and reference genes (18SrRNA) in the separate wells. Each sample was analyzed in triplicate. Primer concentration 100nM for Cbfa1, Osterix , Osteocalcin , 20nM for Alkaline Phosphatase , Bone Morphogenetic protein-2 , 50nM for Type I Collagen and 200nM for 18SrRNA. Relative expression levels of the gene of interest, normalized by the amount of 18SrRNA, were calculated by Sequence detection software version 1.2 (Applied Biosystems) using Relative Quantification Study approach. The average values of ΔCts from each sample were used for statistical analysis.

10. Statistic Analysis

Result are presented as mean ± SEM. Student *t* test was used to compare group means of two samples. The difference was considered significant at *P* value below 0.05.

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CHAPTER IV

RESULTS

1.Patients data

Mesenchymal stem cells and Hematopoietic stem cells were isolated from 10 severe thalassemic children patients. Three males and seven females with ages between 1 to 11 years old were included for this studying. Beta-thal/Hb disease and beta-thalassemia major were the final diagnosis for these patients. The 20-25 ml of bone marrow aspiration were collected form these 10 patients. These bone marrow cells were separated by 1.077 g/ml Ficoll density gradient centrifugation to yield bone marrow mononuclear cells. Mean average of bone marrow mononuclear cells is 2.5×10^8 cells range from 1.4×10^8 to 3.75×10^8 cells. After that, mesenchymal stem cells and hematopoietic stem cells were isolated by using immunomagnetic method. Mean average of mesenchymal stem cells is 3.2×10^5 cells rang from 1.1×10^5 to 6.4×10^5 cells. Mean average of hematopoietic stem cells is 4.4×10^5 cells rang from 1.4×10^5 to 6.1×10^5 cells. Data shown in table 1.

Table 1. Data of Thalassemia Patients

Patients No.	Sex	Ages (Years)	Total mononuclear cells	MSC (CD105)	HSC (CD133)
1	F	---	1.79×10^8	1.5×10^5	---
2	M	1	1.98×10^8	2.1×10^5	3.3×10^5
3	M	10	1.40×10^8	1.6×10^5	1.4×10^5
4	F	4	2.15×10^8	2.2×10^5	3.6×10^5
5	F	11	1.66×10^8	1.1×10^5	2.2×10^5
6	M	5	3.20×10^8	5.4×10^5	6.1×10^5
7	F	11	3.75×10^8	6.4×10^5	8.2×10^5
8	F	7	3.31×10^8	3.9×10^5	4.8×10^5
9	F	4	2.45×10^8	3.3×10^5	3.9×10^5
10	F	10	3.49×10^8	4.4×10^5	5.9×10^5

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In control group, mesenchymal stem cells and hematopoietic stem cells were isolated from 12 healthy donor. Six males and six females with ages between 2 to 17 years old were included for control group. Mean average of bone marrow mononuclear cells is 258.9 x 106 cells range from 1.15×10^8 to 3.66×10^8 cells. After that, mesenchymal stem cells and hematopoietic stem cells were isolated by using immunomagnetic method. Mean average of mesenchymal stem cells is 1.9×10^5 cells range from 5.0×10^4 to 4.1×10^5 cells. Mean average of hematopoietic stem cells is 2.2×10^5 cells range from 6.3×10^4 to 5.5×10^5 cells. Data shown in table 2.

Table 2. Data of Healthy Donor

Donor No.	Sex	Ages (Years)	Total mononuclear cells	MSC (CD105)	HSC (CD133)
1	M	5	2.60×10^8	6.3×10^4	8.4×10^4
2	F	8	2.55×10^8	1.1×10^5	1.3×10^5
3	M	14	2.72×10^8	1.3×10^5	1.1×10^5
4	F	17	3.66×10^8	4.1×10^5	4.4×10^5
5	F	13	2.21×10^8	1.2×10^5	1.1×10^5
6	M	14	2.85×10^8	2.6×10^5	4.2×10^5
7	M	8	1.58×10^8	5.1×10^4	6.3×10^4
8	F	8	1.15×10^8	5.0×10^4	1.2×10^5
9	M	15	2.66×10^8	1.3×10^5	1.8×10^5
10	F	2	2.98×10^8	2.8×10^5	2.0×10^5
11	F	3	3.15×10^8	3.3×10^5	2.9×10^5
12	M	4	2.96×10^8	3.5×10^5	5.5×10^5

2. Culture of Mesenchymal Stem Cells

The isolated mesenchymal stem cells from thalassemia patients were culture in 12-well plate. The 2 x 10^4 cells of mesenchymal stem cells were introduced to the 12-well plate for initial plating. There were 3-5 colonies of adherent fibroblast- like cells observed on days 5 of cultivation. These observed colonies rapidly expanded after days 5 and reached 90 % confluent in 14 days in passage 0 cultivation. The culture of mesenchymal stem cells were expanded and maintained to passage 10. Data shown in figure 2. In addition, the morphology of fresh isolated mesenchymal stem cells were observed by using the Giemsa stained cytospin preparations. Data shown in figure 3-4.

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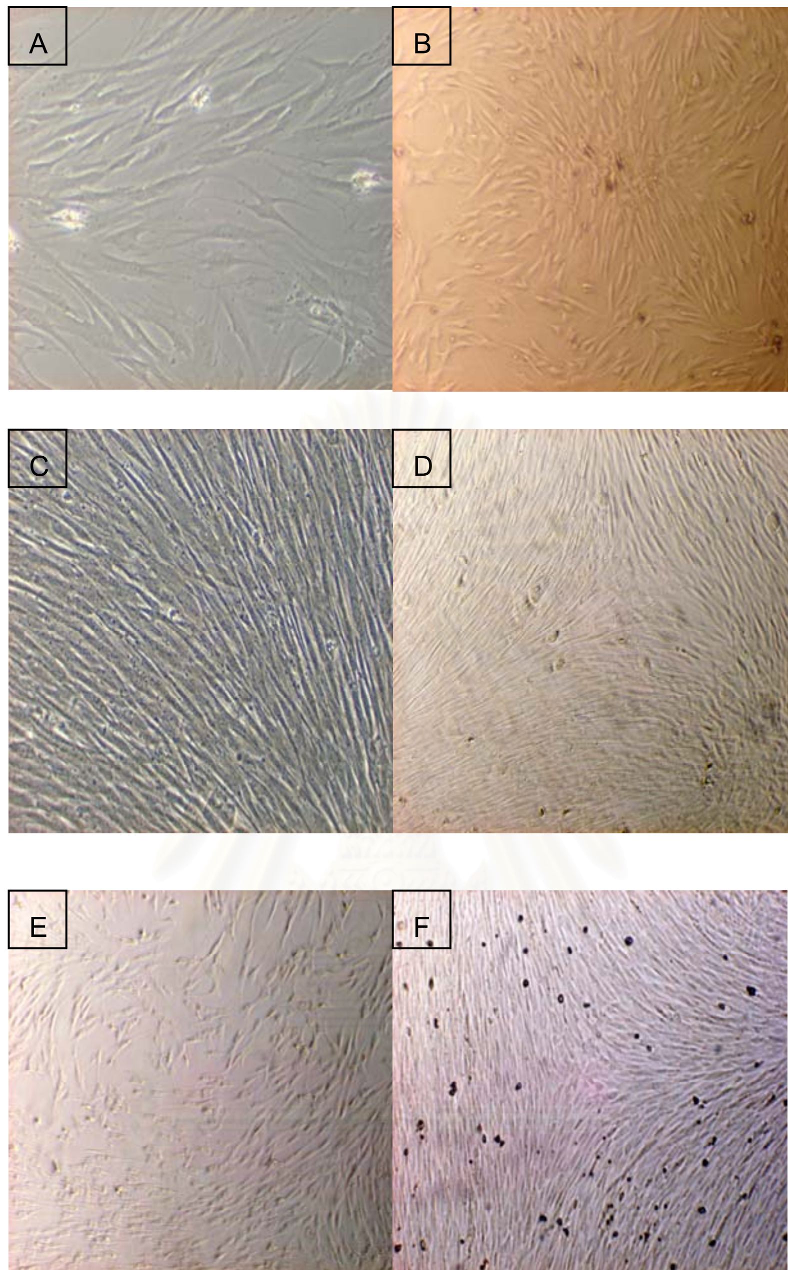

Figure 2. Culture of mesenchymal stem cells. A-B, MSCs from thalassemic patients, day 7. C-D, MSCs from thalassemic patients, day 14. E, MSCs from healthy donor, day 7. F MSCs from healthy donor, day 14.

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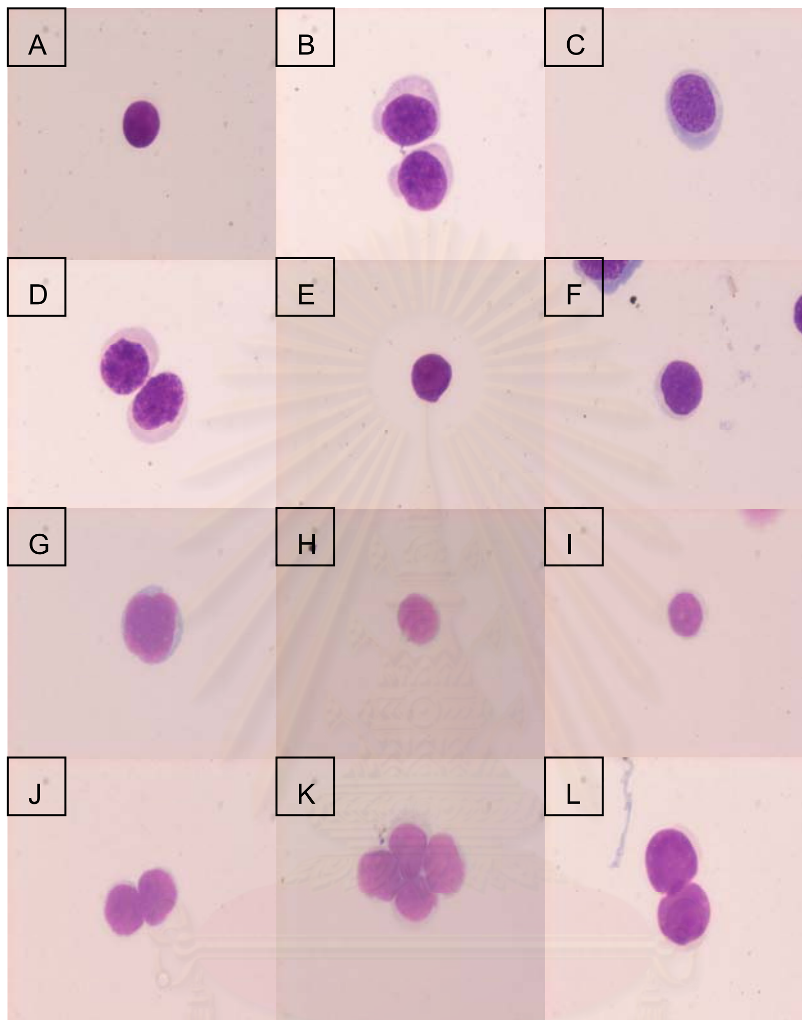

Figure 3. Isolated mesenchymal stem cells (CD105 positive cells), A-F mesenchymal stem cells from thalassemic patients. G-L mesenchymal stem cells from healthy donor.

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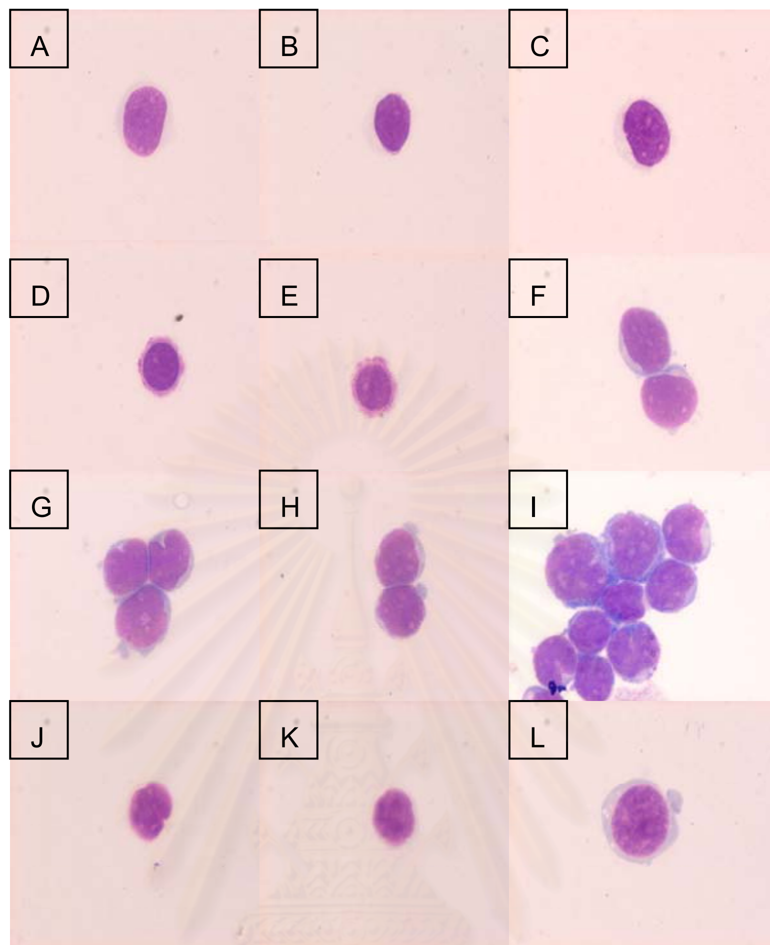

Figure 4. Isolated hematopoietic stem cells (CD133 positive cells). A-F,HSCs from healthy donors. G-L, HSCs from thalassemic patients.

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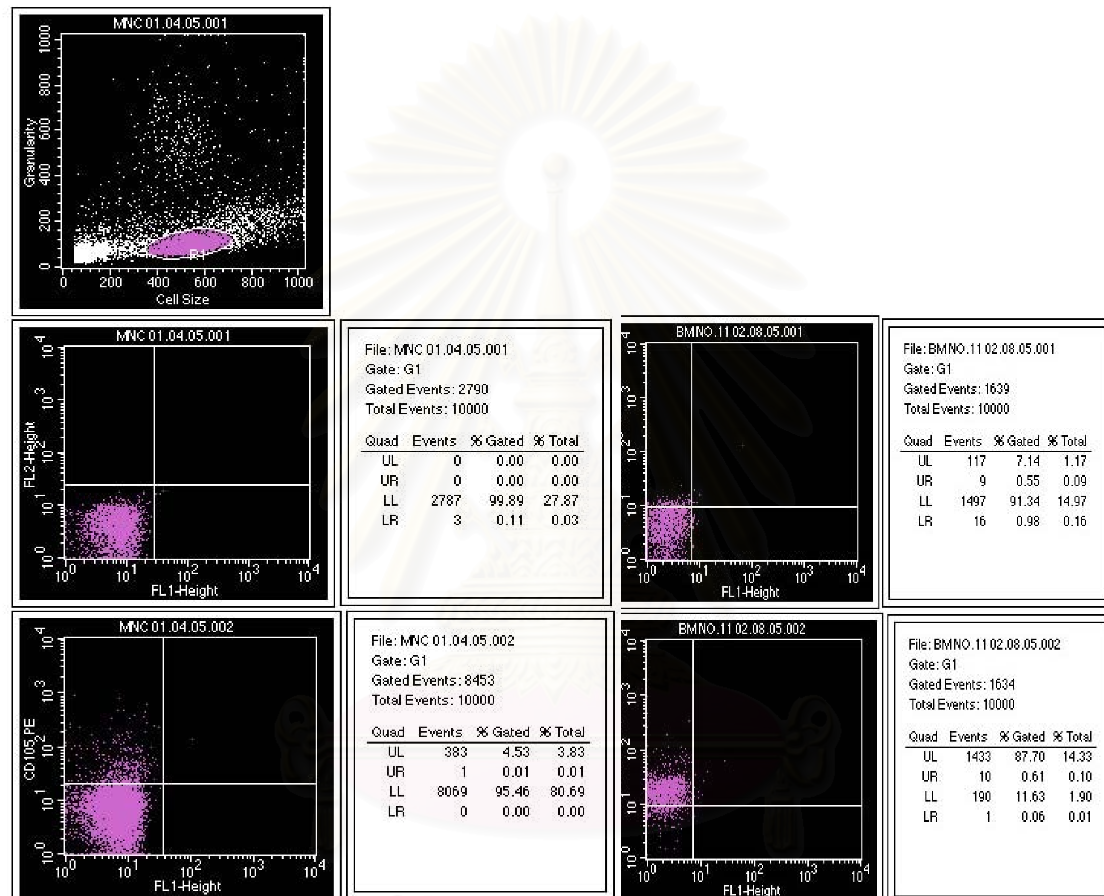
3. Phenotype Analysis by Flow Cytometry

Phenotypic surface markers of mesenchymal stem cells and hematopoietic stem cells were analyzed by FACScan (Becton Dickinson, USA) using the CellQuest software program. Data shown in figure 5-6.



A B

Figure 5. Phenotypic analysis characterization of MSCs. A:mononuclear cells, B: isolated mesenchymal stem cells.

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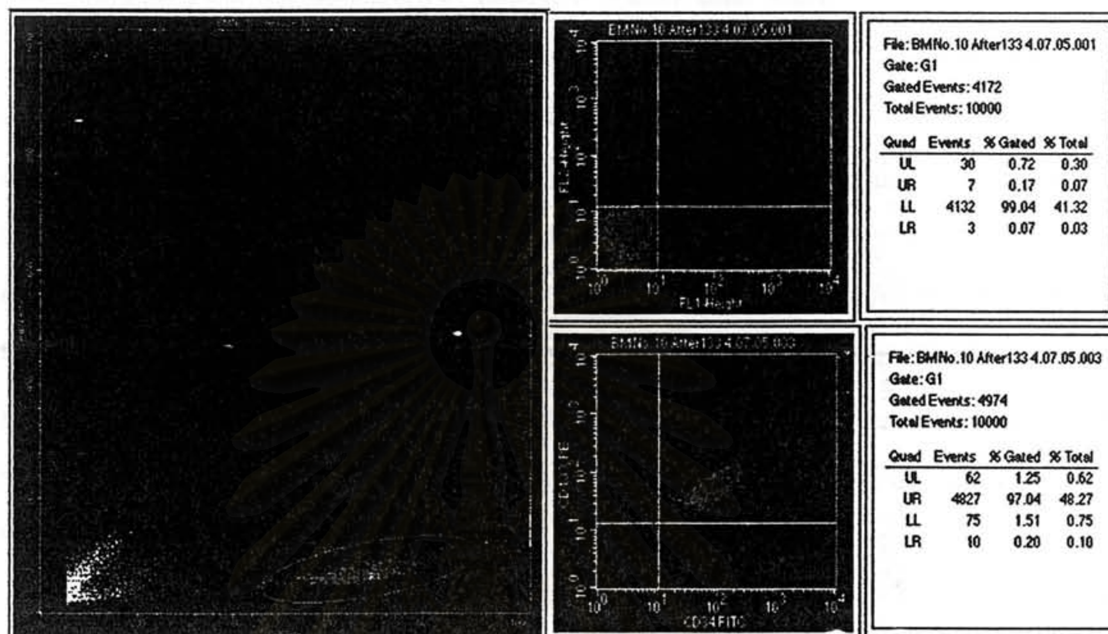

Figure 6. Phenotypic analysis characterization of isolated HSCs after positive selection.

4. Studies of Mesenchymal Stem Cells Gene Expression by Quantitative Real-Time PCR

Quantitative real-time PCR was used to examined mesenchymal stem cells gene expression. In this experiment, we examined the effect of bone marrow expansion on mesenchymal stem cells gene expression. The purified mesenchymal stem cells from 10 thalassemia patients were assessed for the expression of the genes of interest at mesenchymal stem cells differentiation into osteoblast. Relative expression levels of the gene of interest, normalized by the amount of 18SrRNA, were calculated by Sequence detection software version 1.2 (Applied Biosystems) using Relative Quantification Study approach. The average values of ΔCts from each sample were used for statistical analysis. The expression of Cbfa1 gene was decreased, fold difference is 0.228 ± 0.2 ($P < 0.05$). The expression of osterix gene was decreased ,

fold difference is 0.356 ± 0.1 ($P < 0.05$). The expression of Osteocalcin gene was decreased, fold difference is 0.245 ± 0.1 ($P < 0.05$). The expression of Bone Morphogenetic proteins-2 (BMP-2) , Type I Collagen and Alkaline phosphatase gene was decreased but not significant in statistic $P < 0.05$, data shown in figure 7, details of each of genes that interest were shown in figure 9-14. Next , we examined hematopoietic stem cells gene expression. The expression of Osterix gene was decreased, fold difference is 0.397 ± 0.1 ($P < 0.05$). The expression Cbfa1, Osteocalcin gene was decreased but not significant in statistic $P < 0.05$. Unlike the expression of Type I Collagen gene was not difference from control, data shown in figure 8,details of each of genes that interest were shown in figure15-18. Interestingly, the mRNA of PPARγ2 was not detected from both of mesenchymal stem cells and hematopoietic stem cells from healthy donor and thalassemic patients. Moreover, the mRNA of BMP-2, Alkaline Phosphatase was not detected from hematopoietic stem cells.

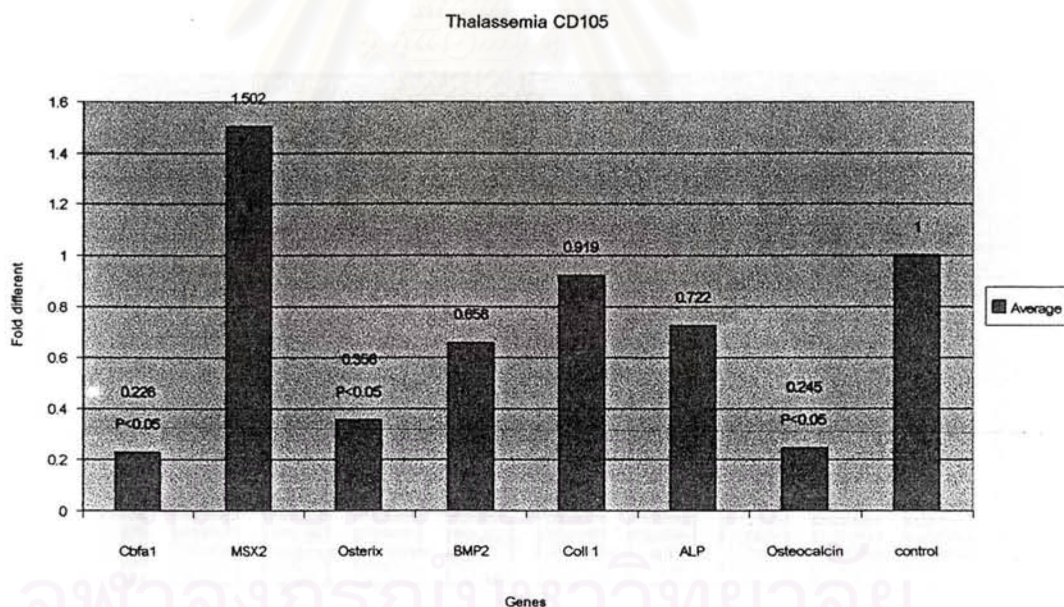

Figure 7. Relative gene expression of mesenchymal stem cells from thalssemic patients.

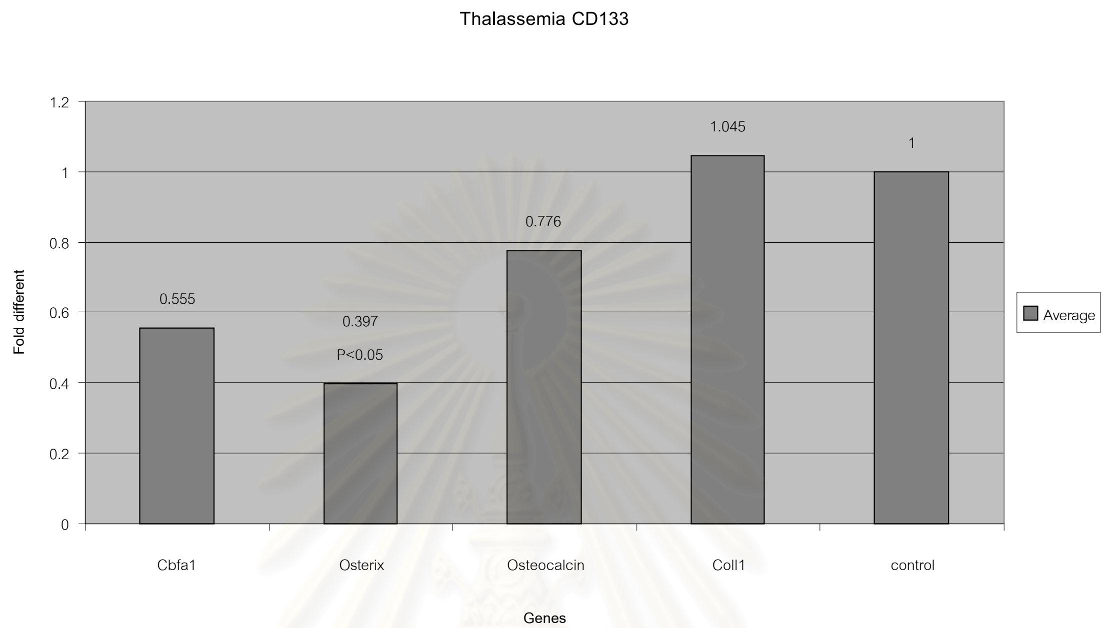

Figure 8. Relative gene expression of hematopoietic stem cells from thalssemic patients.

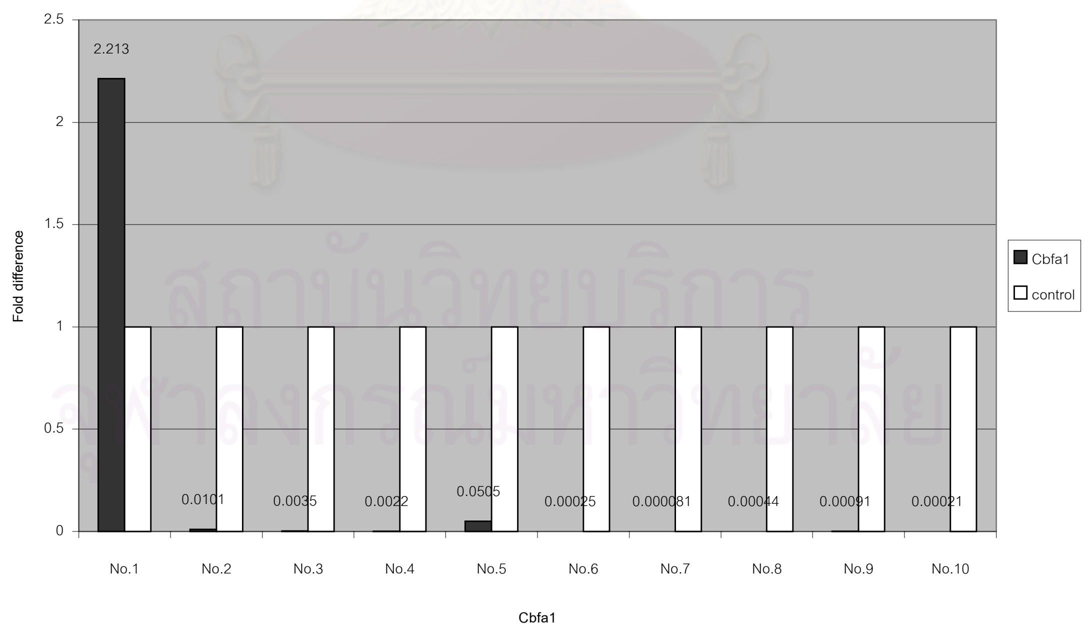

Figure 9. Relative gene expression of Cbfa1 from MSCs.

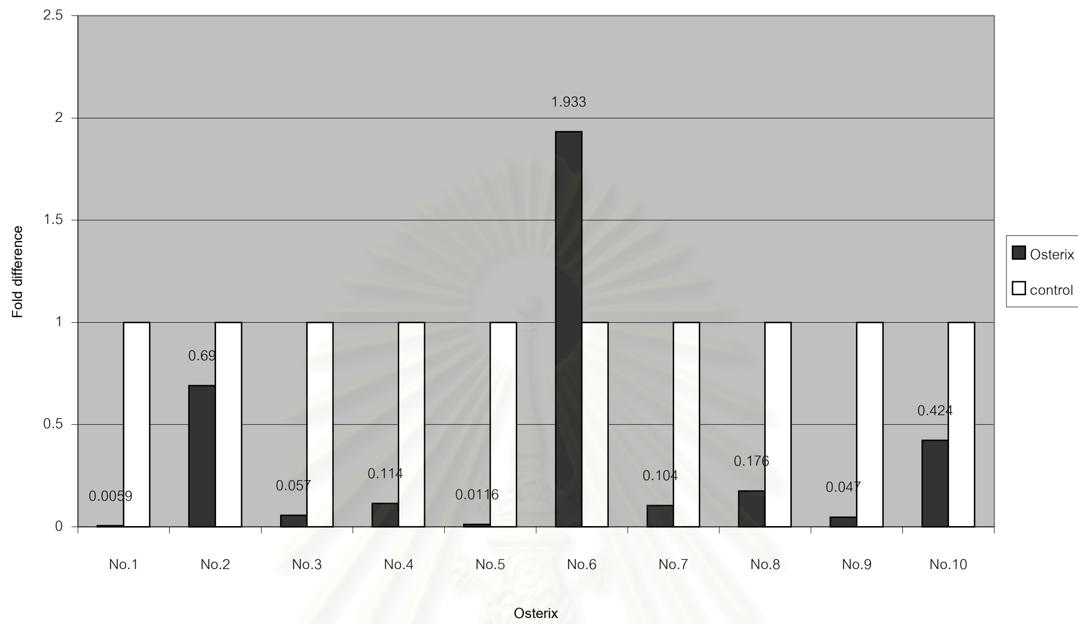

Figure 10. Relative gene expression of Osterix from MSCs.

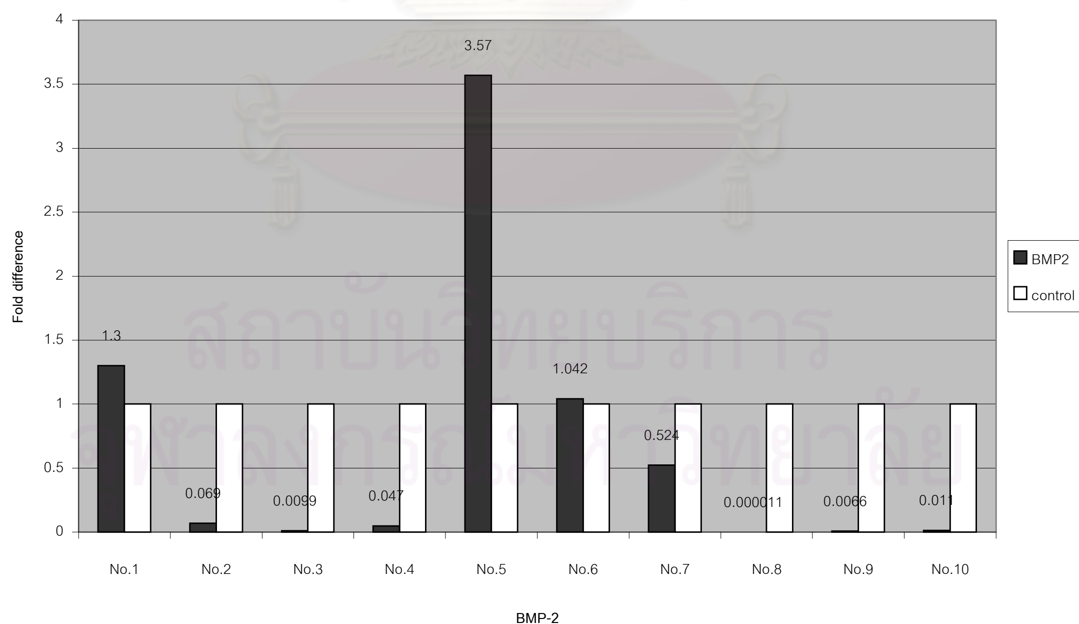

Figure 11. Relative gene expression of Bone Morphogenetic protein-2 (BMP-2) from MSCs.

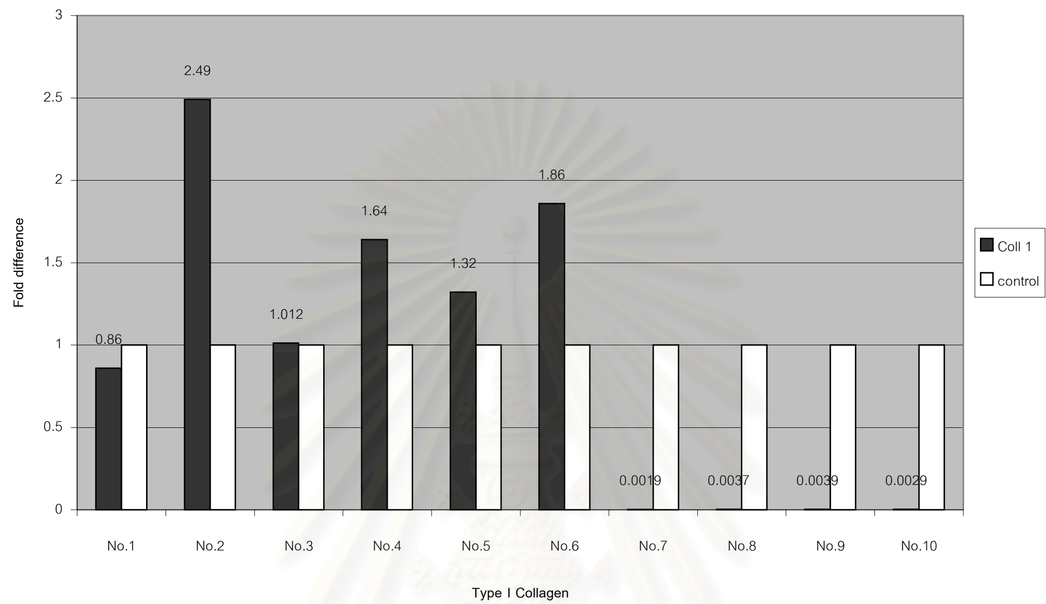

Figure 12. Relative gene expression of Type I Collagen from MSCs.

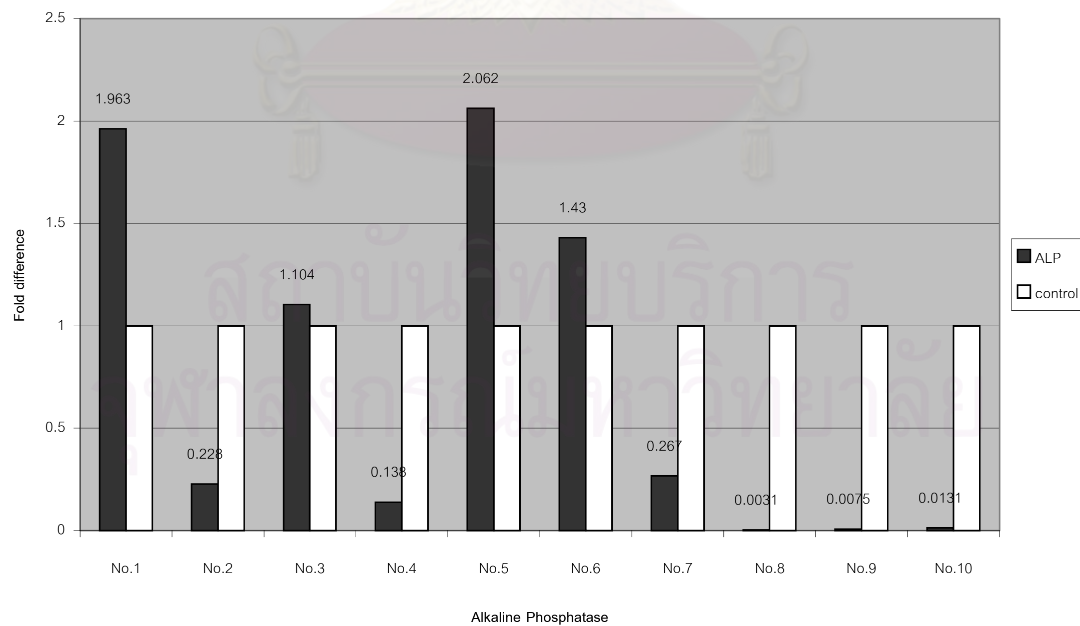

Figure 13. Relative gene expression of Alkaline Phosphatase from MSCs.

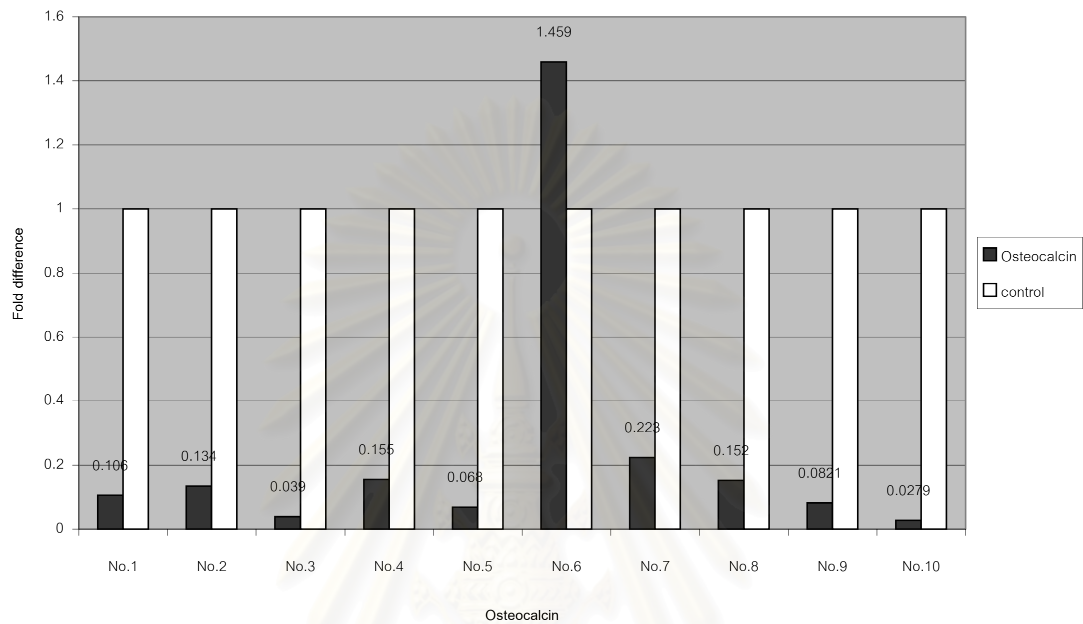

Figure 14. Relative gene expression of Osteocalcin from MSCs.

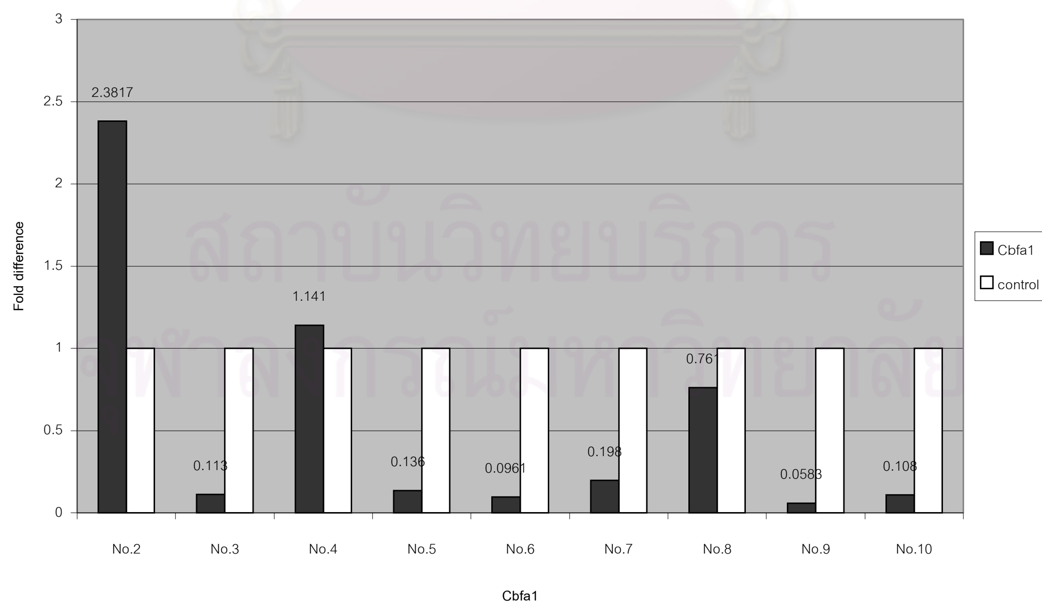

Figure 15. Relative gene expression of Cbfa1 from HSCs.

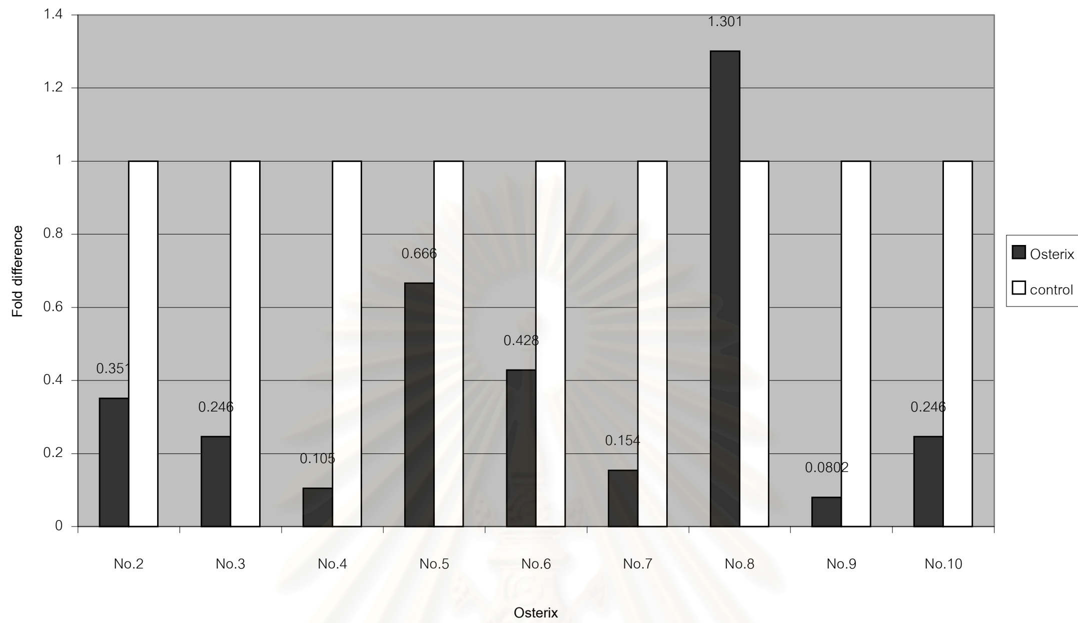

Figure 16. Relative gene expression of Osterix from HSCs.

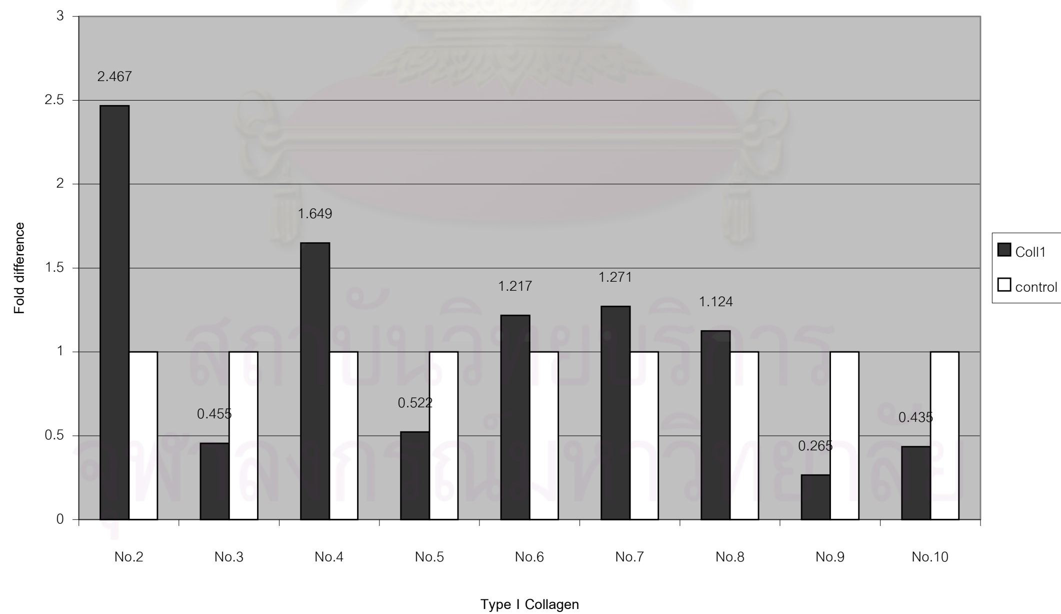

Figure 17. Relative gene expression of Type I collagen from HSCs.

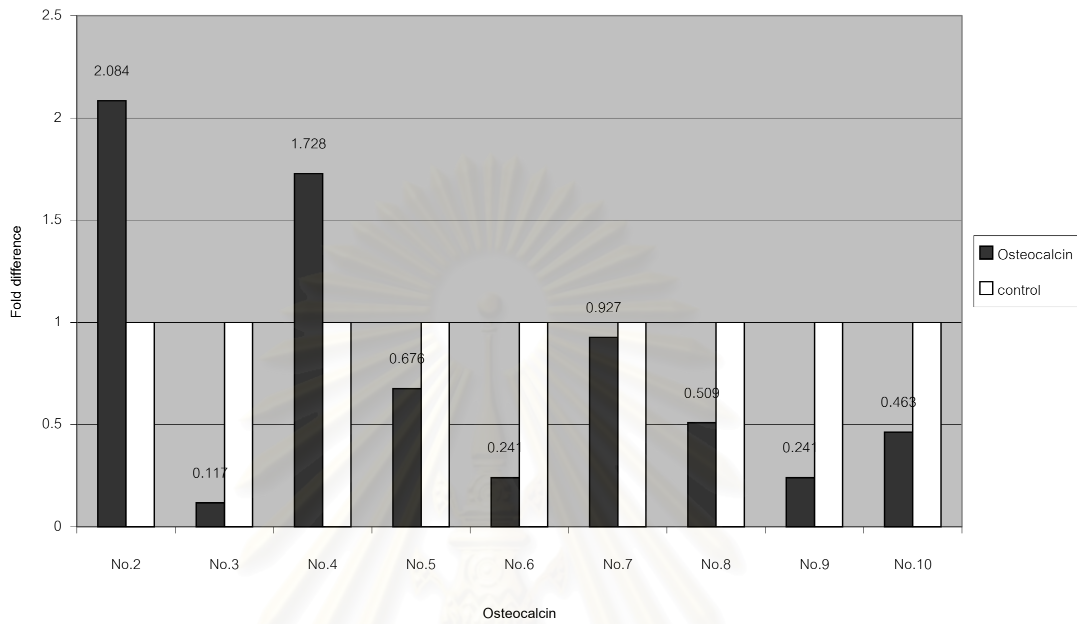

Figure 18. Relative gene expression of Osteocalcin from HSCs.

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CHAPTER V

DISCUSSION

Bone disease is emerging as a major cause of morbidity in severe thalassemia individuals. Patients commonly present with bone deformity , osteoporosis , fractures , and chronic bone pain. Osteoporosis is presented in severe thalassemia due to ineffective in erythropoiesis. More than 15-20 times comparing to normal individual expansion of bone marrow causes bone loss and osteoporosis. The patients may be suffered from bone change depending on the degree of osteoporosis. These defect could be observed in the radiological changing in the skull, facial bone, and other extremities.

Bone marrow are contains at least two types of stem cells: hematopoietic stem cells and mesenchymal stem cells. Mesenchymal stem cells, an initiated cells of mesenchymal tissues origin, have the capability of commitment and differentiation into mesenchymal lineages including osteoblastic, tendogenic, chondrogenic, adipocytic, myogenic, and other lineages. The transition from mesenchymal stem cells to each lineage are controlled by many signaling pathways such as cell-to-cell contact, cytokines and growth factors, and pathologic condition. Mesenchymal stem cells reside in a variety organs including bone marrow, cord blood, muscle,dermal, and other tissues. These could explain the supplied mesenchymal cells during tissue injury or regeneration of new organs.

In our study. First , we isolated mesenchymal stem cells by using magnetic microbeads. The less amount of human mesenchymal stem cells reside in the bone marrow with the estimation rate of 1-10 MSC in 10,000 bone marrow mononuclear cells. From our patients data , Mean average of mesenchymal stem cells is 3.2×10^5 cells from 251.8×10^6 cells of bone marrow mononuclear cells , the frequency of mesenchymal stem cells is 13 in 10,000 cell of bone marrow mononuclear cells and mean average of hematopoietic stem cells is 4.4×10^5 cells , the frequency of HSC is 16 in 10,000 cell of bone marrow mononuclear cells. These data suggest that the isolated cells may have some contamination with unwanted cells such as lymphocyte. In control

group mesenchymal stem cells and hematopoietic stem cells were isolated from healthy donor bone marrow , the frequency of mesenchymal stem cells is 7.3 in 10,000 cells and the frequency of hematopoietic stem cells is 8.5 in 10,000 cells of bone marrow mononuclear cells. These data suggest that the isolated cells may have a high purity than the isolated cells from thalassemic patients, because the pathologic effects of the bone marrow expansion may produced some factors that interrupt on stem cell isolation.

The isolated mesenchymal stem cells from thalassemia patients were culture in 12-well plate. The 2×10^4 cells of mesenchymal stem cells were introduced to the 12-well plate for initial plating. There were 3-5 colonies of adherent fibroblast- like cells observed on days 5 of cultivation. These observed colonies rapidly expanded after days 5 and reached 90 % confluent in 14 days in passage 0 cultivation. The culture of mesenchymal stem cells were expanded and maintained to passage 10. These data suggest that the appropriate environment contributed mesenchymal stem cells growth and expansion.

Quantitative real-time PCR was used to examined mesenchymal stem cells gene expression. In this experiment, we examined the effect of bone marrow expansion on mesenchymal stem cells gene expression. The purified mesenchymal stem cells from 10 thalassemia patients were assessed for the expression of the genes of interest at mesenchymal stem cells differentiation into osteoblast. The results show that the expression of Cbfa1 , Osterix , BMP-2 , Type I Collagen , Alkaline Phosphatase and Osteocalcin was downregulated. In previous study have been shown that human myeloma cells suppress the formation of human osteoblast progenitors in bone marrow cultures. Moreover an inhibitory effect on osteocalcin , alkaline phosphatase , and collagen type I mRNA and protein expression and on Runx/Cbfa1 activity by human pre-osteoblastic cells was observed in co-culture with myeloma cells and moreover they have shown that the presence of high IL-7 levels in the bone marrow plasma of multiple myeloma patients inhibit bone formation. However the role of IL-7 in the inhibition of bone formation in multiple myeloma is not known. Several factors have been identified as potential osteoblast inhibitors in both mouse and human system such as the secreted Frizzled-related proteins (sFRP)-1,-2,-3,-4, noggin , gremlin and interleukin-7 (IL-7).

These data suggest that the pathologic effect of ineffective of erythropoiesis in thalassemic may produce some kind of the secreted osteoblast inhibitors.[135]

Recent data have shown decreased levels of serum osteocalcin, a protein produced by osteoblasts, in patients with thalassemia major. Although osteoblast dysfunction is hitherto thought to be the major pathogenetic mechanism for osteoporosis in thalassemia major, there is also evidence of increased osteoclast activation in these patients. Previous study have shown that patients with thalassemia major and osteoporosis have elevated marker of bone resorption, such as urinary level of N-telopeptides of collagen type I (NTX), which is a specific marker of bone resorption, and increased serum levels of tartrate resistant acid phosphatase isoform 5b (TRACP-5b), an enzyme that is produced only by activated osteoclasts.[136] Furthermore, both NTX and TRACP-5b levels correlated with bone mineral density of the lumbar spine in thalassemia patients. Moreover, pyridinoline and deoxypyridinoline, other markers of bone resorption, are increased in patients with thalassemia major and osteoporosis compared with normal controls.

Receptor activator of nuclear factor-kappa B ligand (RANKL) is a type II transmembrane glycoprotein, produced by bone marrow stromal cells, while a soluble form can be also released its membrane-bound state by metalloproteinases. RANKL binds the RANK receptor on osteoclast precursors and induces the formation of osteoclasts by signaling through the nuclear factor-kappa B and Jun N-terminal kinase pathway. Osteoprotegerin (OPG), which is a member of the tumour necrosis factor receptor family secreted by stromal cells, is the decoy receptor for RANKL. OPG blocks the RANKL-RANK interaction and thus inhibits osteoclast differentiation and activation.[136]

The RANKL/RANK/OPG system seems to be of great importance for the activation and proliferation of osteoclast precursors. Previously shown, in 13 patients with thalassemia major and osteoporosis, the serum levels of sRANKL were slightly increased, compared with controls, while the OPG serum levels were significantly reduced in this cohort of patients, thus the ratio of sRANKL and KOPG was increased in thalassemia patients.[136]

Furthermore, a correlation between the sRANKL and OPG ratio and erythropoietin levels that has also been reported recently, represents a mechanism

through which anaemia, by continuously stimulating the erythropoietin synthesis and determinning bone marrow hyperplasia, may increase bone resorption through enhanced RANKL levels.[136]

Genetic factors seem to play an important role in the development of low bone mass and osteoporotic fractures. These factors have been implicated in the pathogenesis of postmenopausal osteoporosis, as regulator genes of bone mineral density, but have not been studies thoroughly in thalassemia induced osteoporosis.

The polymorphism at the Sp1 site of the collagen type Ia 1 (COLIA 1) gene (collagen type I is the major bone matrix protein) was studied by Wonke et al 1998, whi found, that approximately 30% of the thalassemia major patients were heterozygotes (Ss) and 4% were homozygotes (SS) for the Sp1 polymorphism. Female to male ratio was 2:1. They concluded that male patients with thalassemia major carrying the Sp1 mutation may develop severe osteoporosis of the spine and the hip more frequently than patients who do not carry this mutation. The collagen type 1 polymorphism has been associated with reduced bone mineral density in postmenopausal osteoporosis and predisposes women to osteoporotic fractures. The study of the collagen type 1 polymorphism may help to identify those thalassemia patients who are at a higher risk to develop osteoporosis and pathologic fractures. The sequence variation of the TGF-β1, 713-8delC ,has also been associated with very low bone mass in osteoporotic and normal women. Perrotta et al 2000, studied the association between bone mineral density , collagen type 1 gene polymorphisms and 713-8delC of TGF-β1 in 135 beta-thalassemia patients. A remarkable incidence of osteopenia and osteoporosis (90%) among regularly transfused patients was observed in this study; bone mass was lower in men than in women while mal e patients developed more prevalent osteopenia/osteoporosis of the spine than female patients. TGF-β1 polymorphism failed to demonstrate a statistical correlation with bone mineral density, while subjects who were heterozygous or homozygous for collagen type 1 gene polymorphism showed a lower bone mineral density than subjects without the sequence variation.[136]

In summary, the bone marrow expansion in thalassemic patients may be directly reduce osteogenic commitment of mesenchymal stem cells by downregulated expression of transcription genes and osteoblast differentiation genes.

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สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

APPENDICES

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX

1. Phosphate Buffer Saline pH7.2 (PBS)

Phosphate buffer saline (PBS) solution is prepared following the instruction of Biochrome KG (Germany) , briefly, the PBS powder is dissolved by 1,000 ml of distilled water, and sterilized by steam autoclave. This solution is stored at 4°C.

2. 25X PBS Solution

To prepare 25XPBS solution , follow the formula listed below

NaCl	200	g
KCl	5	g
$Na_2HPO_4.12H_2O$	72.4	g
KH_2PO_4	5	g

All the listed chemical are dissolved in 1,000 ml distilled water , and finally sterilized by steam autoclave. This solution is stored at room temperature.

3. 1X PBS Solution

For preparing 1XPBS solution, the 40 ml of PBS solution are dissolved in 940 ml distilled water, sterilized by steam autoclave. This solution is stored in room temperature.

4. PBS/2% EDTA

25X PBS	40	ml
2%EDTA phenol red	10	ml
Distilled water	950	ml

This solution is sterilized by steam autoclave, and stored at 4°C.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

5. 2% EDTA with Phenol Red (100X Solution :W/V)

EDTA.Na_2	10	g
Phenol red	100	mg
Distilled water	500	ml

Gently mixed for fine dissolving, stores at room temperature.

6. 0.2% Trypan Blue Dye

Trypan blue dye is used for cell counting in order to exclude dead cells, can be prepared by

Physiological saline (85%)

NaCl	0.85	g
Distilled water	100	ml

0.2% trypan blue

Trypan blue	0.2	g
Physiological saline	100	ml

Paper filter before use.

BIOGRAPHY

Chokdee wongborisut was born on december 4, 1980 in Phetchabun, Thailand. He received his bachelor degree of science in biology, Srinakharinwirot university in 2002. He continued his master's degree study at Chulalongkorn university in medical science program, in 2002 and completed the program in 2005.

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