EFFECT OF HUMAN CANCELLOUS BONE GRAFT SOLUBLE MOLECULES ONTO DIFFERENTIATED HUMAN ADIPOSE DERIVED MESENCHYMAL STEM CELLS TOWARDS OSTEOGENIC LINEAGES

by
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This thesis is dedicated to my husband and my cousin Gamze Özcan…

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ABSTRACT

EFFECT OF HUMAN CANCELLOUS BONE GRAFT SOLUBLE MOLECULES ONTO DIFFERENTIATED HUMAN ADIPOSE DERIVED MESENCHYMAL STEM CELLS TOWARDS OSTEOGENIC LINEAGES

The involvement of cellular and molecular mechanisms of bone tissue sometimes fail during bone repair, resulting in either delayed-union or non-union. Numerous signaling molecules near the fracture site have a major role in mesenchymal stem cell adhesion, proliferation and differentiation towards osteogenic lineages. The aim of this study was to determine potential effects of human cancellous bone chip secretion (BCPs) onto differentiated human adipose-derived mesenchymal stem cells (hAD-MSCs) into osteogenic lineage. The cells within the bone chips secrete proteins and soluble molecules that inhibit the process of osteogenesis. In the previous studies, the effect of the BCPs on hAD-MSCs in bone repair has not been discussed before. In this study, hAD-MSCs were cultured in either basal medium (BM) or osteogenic medium (OM) in the presence of the BCPs. Results from this study demonstrated that proliferation and viabilities of hAD-MSCs were decreased by the BCPs. Furthermore, BCPs distinctly led to a reduction of alkaline phosphatase activity in comparison with the control cells. In the same way, BCPs caused an inhibition of mineralized bone nodules and a reduction in calcium concentration of extracellular matrix mineralization during bone formation. Besides, a significant decrease in the level of bone-related genes, including osteocalcin (OCN), collagen type one (Col1A1), osteonectin (ON), and osteopontin (OPN) were observed in hAD-MSCs with BCPs. Expression of osteoprotegerin (OPG) was also significantly decreased during osteoblasts maturation. Nevertheless, expression of Bone Morphogenetic Protein-2 (BMP-2) was constantly induced by BCPs during bone regeneration. Moreover, a significant increase in the level of sclerostin expression was also observed in hAD-MSCs cultured in OM at the fracture site under the influence of BCPs. Nevertheless, BCPs did not have any significant effect on the expression of Dkk-1. Our results have demonstrated that production of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β was stimulated by BCPs. Altogether, our findings demonstrated that the secretions of bone chips inhibit bone formation process during fracture healing.

ÖZET

İNSAN SÜNGERİMSİ KEMİK DOKUDAKİ ÇÖZÜNÜR MOLEKÜLERİN YAĞ DOKU KAYNAKLI MEZENKİMAL KÖK HÜCRELER ÜZERİNDEKİ KEMİĞE FARKLILAŞMA ETKİSİ

Kemik dokunun hücresel ve moleküler mekanizmaları, kemik onarımı sırasında bazen başarısız olarak gecikmiş kaynama veya kemiğin kaynamaması ile sonuçlanır. Kırık civarında bulunan çok sayıda sinyal molekülleri, mezenkimal kök hücrelerin (MKH) adezyon, çoğalma ve kemiğe doğru farklılaşmasında önemli bir role sahiptir. Bu çalışmanın amacı, insan yağ dokusundan elde edilen kemiğe farklılaşmış MKH'lere, insan süngerimsi kemik dokudaki çözünür faktörlerin (KÇF) olası etkilerini belirlemektir. Kemik parçacıkları içindeki MKH'ler, osteogenezis sürecini inhibe eden proteinleri ve çözünür molekülleri salgılarlar. Önceki çalışmalarda, KÇF'lerin kırık onarımında MKH'ler üzerindeki etkisi daha önce tartışılmamıştır. Bu çalışmada, MKH'ler, KÇF'lerin varlığında, bazal besiyeri (BB) ve kemik besiyeri (KB) içerisinde kültüre edilmişlerdir. Bu çalışmadan elde edilen sonuçlar, MKH'lerin çoğalması ve canlılığının, DKÇF'ler tarafından azaltılmış olduğunu göstermiştir. Ayrıca, KÇF'ler, kontrol hücreleriyle karşılaştırıldığında, alkalin fosfataz aktivitesinde belirgin bir düşüşe yol açmıştır. Aynı şekilde, kemik oluşumu sırasında, KÇF'ler mineralize kemik nodullerinin inhibisyonuna ve hücre dışı matriks mineralizasyonunda kalsiyum konsantrasyonun düşmesine neden olmuştur. Ayrıca, KÇF'li MKH'lerde osteokalsin, kollajen tip 1, osteonektin ve osteopontin gibi kemik ile ilişkili genlerin düzeyinde belirgin azalma gözlemlenmiştir. Osteoprotegrin ekspresyonu, osteoblastların olgunlaşması sırasında anlamlı şekilde azalmıştır. Bununla birlikte, kemik yenilenmesi sırasında, kemik morfojenik protein-2'nin ekspresyonu sürekli olarak KÇF'ler tarafından indüklenmiştir. Ayrıca, KÇF'li kemik besiyerinde kültürlenen MKH'lerde sklerostin ekspresyon seviyesinde belirgin bir artış gözlemlenmiştir. Bununla birlikte, KÇF'lerin Dkk-1 ekspresyonu üzerinde anlamlı bir etkisi bulunmamıştır. Sonuçlar, TNF-α, IL-6 ve IL-1β gibi pro-inflamatuar sitokinlerin üretimlerinin, KÇF'ler tarafından uyarıldığını göstermektedir. Sonuçta, elde ettiğimiz bulgular, KÇF'lerin, kırık iyileşmesi sırasında kemik oluşum sürecini olumsuz yönde etkilediğini göstermektedir.

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ADAM	A dis-integrin and metalloproteinase
ADAM/TACE	Metalloproteinase tumor necrosis α conversion enzyme
ALP	Alkaline phosphatase
APC	Adenomatous polyposis coli
BCPs	Bone chip secretions
BM	Basal Media
Bm	Bone marrow
BMPs	Bone morphogenetic proteins
BMPR-I	Bone morphogenetic protein receptor-I
BMPR-II	Bone morphogenetic protein receptor-II
BMU	Basic multicellular units
CK1α	Casein kinase 1 alpha
Col1A1	Collagen type 1 alpha 1 chain
CPCs	Calcium phosphate cements
CS	Calcium sulfate
CSF-1	Colony stimulating factor-1
c-Src	Proto-oncogene tyrosine- protein kinase
C3H10T1/2	Mouse embryo mesenchymal stem cells
DBM	Demineralized bone matrix
DMEM	Dulbecco's modified Eagle's medium
Dkk-1	Dickkopf-1
DLL	Delta like
DPBS	Dulbecco's Phosphate Buffer Saline
Dsh	Disheveled protein
ELISA	Enzyme-linked immune-sorbent assay
ERK	Extracellular signal-regulated kinase
ESI	Electrospray ionization
FBS	Fetal Bovine serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
GDFs	Growth differentiation factors

GFs	Growth factors
GSK3β	Glycogen synthase kinase 3β
hAD-MSCs	Human adipose derived mesenchymal stem cells
HAp	Hydroxyapatite
HCl	Hydrochloric acid
HES	Transcriptional repressors hairy and enhancer of split
HEY	HES-related with YRPW motif
Hh	Hedgehog
HIV	Human immunodeficiency virus
IBM®-SPSS®	Statistical Package for the Social Sciences
IGF-I	Insulin like growth factor I
IGF-II	Insulin like growth factor II
Ihh	Indian Hedgehog
IL-1	Interleukin-1
IL-IR	Interleukin-1 receptor
IL-IIR	Interleukin-II receptor
IL-6	Interleukin-6
ITS	Insulin transferrin selenium
JAG	Jagged
JNK	C-jun N-terminal kinase
Lef/TCF	Lymphocyte enhancer factor/T cell transcription factor
LiCl	Lithium chloride
Lrp5	Low-density lipoprotein receptor related protein-5
Lrp6	Low-density lipoprotein receptor related protein-6
M	Molar
µl	Microliters
mM	Milimolar
mAB	Monoclonal antibody
MAML	Mastermind-like protein
MAPKs	Mitogen activated protein kinase cascade
MCP-1	Monocyte chemoattractant protein-1
MCS-F	Macrophage colony- stimulating factor
MC3T3-E1	Mouse osteoblast precursor cells

MS	Mass spectrometry
MSCs	Mesenchymal stem cells
MMPs	Matrix metalloproteinases
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
NaOH	Sodium hydroxide
NF-ĸB	Receptor activator of nuclear factor kappa-B
NH_4Cl	Ammonium Chloride
NICD	Notch intracellular domain
OCN	Osteocalcin
OM	Osteogenic media
ON	Osteonectin
OPG	Osteoprotegerin
OPN	Osteopontin
PCL	Polycaprolactone
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PGA	Poly-glycolic acid
pH	Negative log of hydrogen ion concentration
PLA	Poly-lactic acid
PLGA	Copolymer of PLA and PGA
Ptch	Patched
PTH	Parathyroid hormone
PMMA	Poly-methyl-methacrylate
PMNs	Poly-morphonuclear leukocytes
PMS	Phenazine methosulfate
QUAD	Quadrupole time-of-flight
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RBPjk	Immunoglobulin Kappa J Region
rhBMP-2	Recombinant human BMP-2
rhBMP-7	Recombinant human BMP-7
hTGF-β	Recombinant Human Transforming Growth Factor

RIPA	Radio immunoprecipitation assay
Runx2	Runt-related transcription factor 2
SD	Standard Deviation
SDS	Sodium dodecyl sulfate
Shh	Sonic Hedgehog
sFRPs	Secreted frizzled related proteins
Smo	Smoothened
ST2	Mouse bone marrow stromal cells
TCP	Tricalcium phosphate
TGF-β	Transforming growth factor-β
TNF	Tumor necrosis factor
TNF-α	Tumor necrosis factor-α
TNFRI	Tumor necrosis factor receptor-I
TNFRII	Tumor necrosis factor receptor-II
VEGF	Vascular endothelial growth factor

1. INTRODUCTION

1.1. CELLULAR BIOLOGY OF BONE

Bone is a complex and highly mineralized tissue that supports framework of the body [1]. It has important functions including facilitation of movement, structural support, preservation of soft tissues [2], reservoirs for minerals and growth factors (GFs) [3]. Bone tissue has four types of cells: osteoclasts, osteoblasts, osteocytes and bone lining cells [4]. Osteoblasts are originated from mesenchymal stem cells (MSCs) and capable of regulation of organic matrix synthesis [5]. The commitment of MSCs into osteogenic lineage needs canonical Wnt/β-catenin pathway that is essential for MSCs differentiation and osteoblasts formation [6].

Osteoblasts are responsible for collagen synthesis. Collagen proteins provide a framework which is essential for calcium phosphate deposition. Extracellular bone matrix is hardened by hydroxide and bicarbonate ions. The new bone that is composed of the osteoblasts is known as an “osteoid”. Finally, several osteoblasts are entrapped in their own bone matrix, giving rise to osteocytes that finish the secretion of osteoid [7]. Osteocytes are plentiful cells in bone tissue and act as mechano-sensors. An interconnected network of osteocytes is responsible for the detection of mechanical pressures and loads. Additionally, they have an important role during bone remodeling, including regulation of osteoclasts and osteoblasts activities [8].

Osteoclasts are originated from the precursors of monocytes in the hematopoietic stem cells and they are terminally differentiated into multinucleated cells [9]. They degrade extracellular matrix in order to start bone remodeling [10]. Osteoclast activity is regulated by many factors such as cytokines, osteoprotegerin (OPG), receptor activator of nuclear factor kappa-B (NF-κB) ligand (RANKL), interleukin-1 (IL-1), interleukin 6 (IL-6), macrophage colony- stimulating factor (MCS-F) and parathyroid hormone [11-12].

Bone lining cells are inactive straight shaped osteoblasts and found on the bone surfaces. Even though their functions are not completely understood, they inhibit the direct communication between osteoclasts and mineralized matrix, as bone resorption does not take

place. Moreover, they play major roles in osteoclast differentiation, OPG and RANKL secretions [13, 9].

1.1.1 Bone Matrix

Bone matrix possesses two substances; an organic matrix and inorganic salts [14]. The organic matrix includes collagenous proteins, mainly collagen type 1 (Col1A1), and noncollagenous proteins such as osteocalcin (OCN), osteonectin (ON) and osteopontin (OP) [15]. Small leucine- rich proteoglycans such as decorin, lumican and biglycan can be found in organic matrix [16]. Calcium and phosphate ions are the major inorganic salts of the tissue. They form hydroxyapatite crystals [Ca_{10} $(PO_4)6(OH)$ $_2$] that are the main inorganic components of the bone. Highly organized collagen protein and the non-collagenous matrix proteins provides a framework for hydroxyapatite deposition and this association is able to rigidity and strength of the bone tissue [7, 9]. Bone matrix forms a well-organized framework which provides a mechanical support for the bone tissue. It has a remarkable role in maintaining the bone homeostasis. Moreover, it releases several molecules which affect bone cells activities [17].

1.1.2. Physiology of Bone Formation

Bone is regularly formed and reabsorbed in reply to changes such as mechanical loading, a wide range of endocrine and paracrine factors [18]. The process of bone remodeling contains highly well-orchestrated actions of osteoblasts, osteoclasts, bone lining cells and osteocytes. Coupling action between bone formation and resorption is described as a basic multicellular units (BMU) and it is necessary to maintain structural integrity [19]. Bone remodeling occurs asynchronously throughout the life. Osteoblasts and osteoclasts act independently within the bone [20]. Ossification is known as the formation of new bone by osteo-progenitor cells. The new bone formation has two important processes; an intramembranous ossification and an endochondral ossification.

1.1.2.1. Intramembranous Ossification

Intramembranous bone formation takes place during flat bone formation of the skull, maxilla and mandible. It can also occur during bone healing. Intramembranous ossification has four steps: ossification center formation, calcification, trabeculae formation and periosteum growth (Figure 1.1) [7].

Intramembranous ossification starts as some of the MSCs begin to differentiate into osteoprogenitor cells, while other MSCs differentiate into capillaries. Osteoprogenitor cells initiate to differentiate into osteoblasts that form osteoid in the middle of cell accumulation [21].

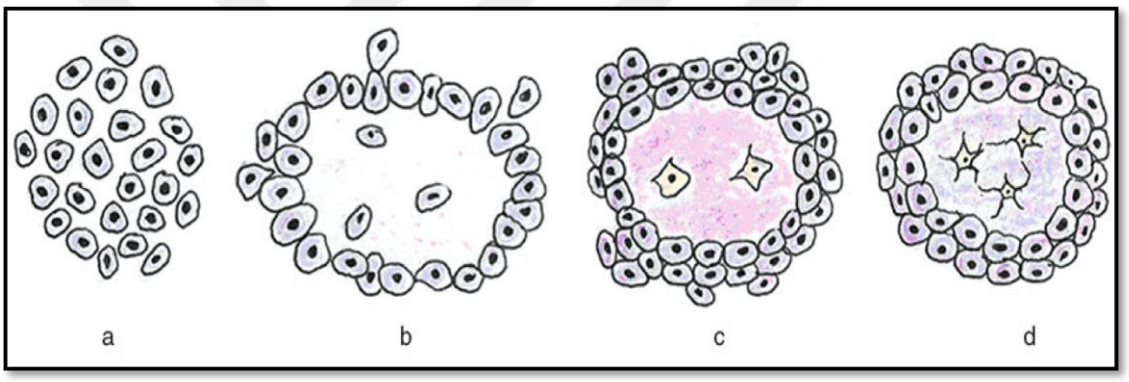

Figure 1.1. Intramembranous ossification displaying; a) accumulation of osteoprogenitor cells, b) collagen network formed in the center, c) differentiation of MSCs into osteoblasts that form osteoid in the middle of the gathering, d) new bone tissue produced by osteoblasts, and numerous osteoblasts begin to become osteocytes within the osteoid [7].

Osteoblasts secrete organic compounds of bone matrix, such as collagens and proteoglycans which are responsible for binding of calcium salts. Osteoid matrix becomes calcified throughout those binding. Deposition of minerals into bone matrix causes entrapping of the osteoblasts. When osteoblasts are entrapped, they start to become osteocytes [22]. By the way, osteogenic cells in the surrounding connective tissue continue to differentiate into new osteoblasts. Osteoid forms a trabecular matrix, whereas osteoblasts on the surface of the spongy bone turn into the periosteum. The periosteum then forms a sheet of the compact bone surface to the trabecular bone. The trabecular bone gathers nearby blood vessels that finally condense into red marrow [23, 21].

1.1.2.2. Endochondral Ossification

Endochondral bone formation is a major embryonic process of bone formation. Cartilage tissue is progressively converted into vascularized bone tissue during endochondral ossification [24]. Endochondral ossification takes place at two sites of a long bone, including diaphyseal (primary ossification center) and epiphyseal (secondary ossification center) sites of the ossification [25]. It is also the primary process of bone repair [26]. Endochondral ossification initiates cartilage growth, continues with primary and secondary ossification center development, ultimately ends with articular cartilage formation and development of epiphyseal plate (Figure 1.2) [27, 22].

Cartilage model growth: Hyaline cartilage is used as a precursor molecule during endochondral ossification. Highly condensed mesenchyme and perichondrium form the cartilaginous tissue [28]. Some of the MSCs begin to differentiate towards chondrocyte lineage which then form the cartilaginous skeletal precursor of the bones. Chondrocytes continue cell division and start to secrete extracellular matrix proteins on the periphery cartilage surface. A new cartilage model which is known as a "growth plate" begins to grow in thickness, because of increasing more extracellular matrix and producing new chondrocytes from the perichondrium [29].

Primary center of ossification: Initial part of the ossification takes place primary center of the ossification in the center of diaphysis [30]. As blood vessels start to invade diaphyseal site, the perichondrium turns into periosteum that has a layer of osteoprogenitor cells. After periosteum formation, osteoprogenitor cells differentiate into osteoblasts. Osteoblasts start to secrete osteoid to form a bone collar. In the meantime, chondrocytes in the middle of ossification initiate to secrete alkaline phosphatase enzyme (ALP) which is responsible for mineral deposition. ALP provides calcification of the matrix [31]. Some of the hypertrophic chondrocytes are then undergone cell death by apoptosis and create a cavity within the bone. Vascular endothelial growth factor (VEGF) is secreted by hypertrophic chondrocytes [32]. It induces spreading out blood vessels throughout the cavity. The blood vessels have osteoprogenitor cells and hematopoietic stem cells that will later create the bone marrow [25]. Meanwhile, osteoblasts begin to enter the cavity via the periosteal bud. Mineralized matrix acts as a scaffold for osteoblasts. Bone trabecula is formed by osteoid. After

osteoclast formation from macrophages, they begin to break down cancellous bone to form bone marrow cavity [33].

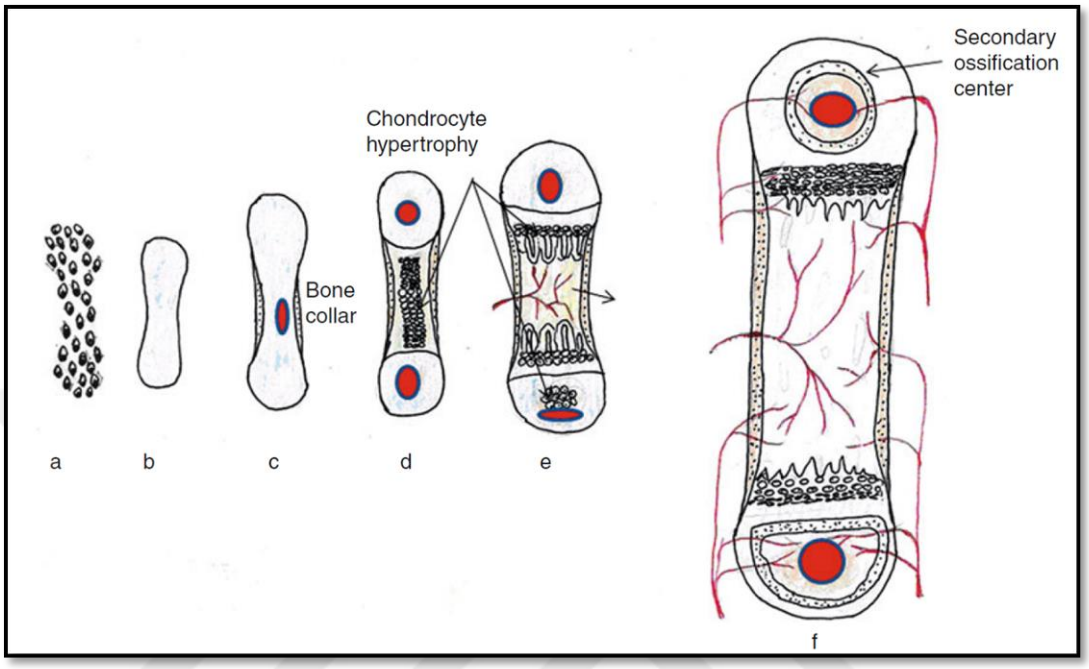

Figure 1.2. Endochondral ossification following; a) recruitment of osteoprogenitor cells, b) cartilage model, c) primary ossification center, d) secondary ossification center, e) medullary cavity, f) blood vessels feeding of new bone [7].

Secondary center of ossification: Secondary ossification occurs in the epiphyseal site of long bones. Secondary ossification is similar to primary ossification formation [34]. Cartilage tissue between primary and the secondary ossification centers is named as an epiphyseal plate. Cartilage tissue is then replaced by bone resulting in long bone growth. Secondary ossification continues until a person reaches in his mid-twenties, when the epiphyseal plate is fully replaced by bone. When the whole bone has been matured, epiphyseal ossification gradually overlaps the growth plate, finally, no further growth happens [35, 7]. Ossification plays a major role in the remodeling of the bone. It appears throughout a person's lifetime. Bone formation and resorption take place together systematically to reshape the skeleton, repair microfractures and protect calcium levels in the human body.

1.1.3. Bone Remodeling

Bone remodeling occurs continuously in a balanced manner and involves mainly two sub-processes; bone formation and bone resorption which take place in a coordinated manner. Bone remodeling has five stages: activation, resorption, reversal, formation and quiescence stages (Figure 1.3) [36].

Activation Stage: Activation phase initiates recruitment and activation of mononuclear monocytes which are formed from osteoclast precursor cells [37, 38]. Osteoclast precursors are attached to the bone surface and become mature. Mature osteoclasts show their resorptive activities by secreting osteoclastic enzymes, such as cathepsin K, and hydrogen ions resulting in degradation of all elements in extracellular matrix.

Resorption Stage: When osteoclasts start to decompose whole bone matrix including collagen, osteoid and mineral matrix, macrophages induce release of GFs, such as transforming growth factor-β (TGF-β) [39], platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) [40] and insulin-like growth factor I-II (IGF-I and II) [41]. The cavity on the trabecular bone surface which is named as a "Haversian canal" is formed as a consequence of osteoclastic activity [7]. Haversian canal formation continues 2-4 weeks during each bone remodeling period.

Reversal Stage: Following osteoclast-mediated resorption, monocytes remove the collagen remnants in Haversian canal and make arrangements on the bone surface [42]. Macrophages produce matrix metalloproteinases (MMPs) that are responsible for matrix degradation [43]. Although coupling signals associating with bone resorption and bone formation have still been unknown, it has been proposed that the cells in reversal stage such as monocytes, pre-osteoblasts and osteocytes create coupling signals which permit the progression from resorption to formation within BMU [40]. Coupling signal molecules such as TGF-β, PDGF, FGF, IGF-I –II and bone morphogenetic proteins (BMPs) can be stored in the extracellular matrix during bone resorption.

Formation Stage: When osteoclasts are resorbed, osteoblasts are then replaced on the bone surface to initiate bone formation. GFs stimulate osteoprogenitor cell proliferation.

Particularly, BMPs are considered as osteoinductive agents and responsible for the enhancement of osteoblast differentiation [44].

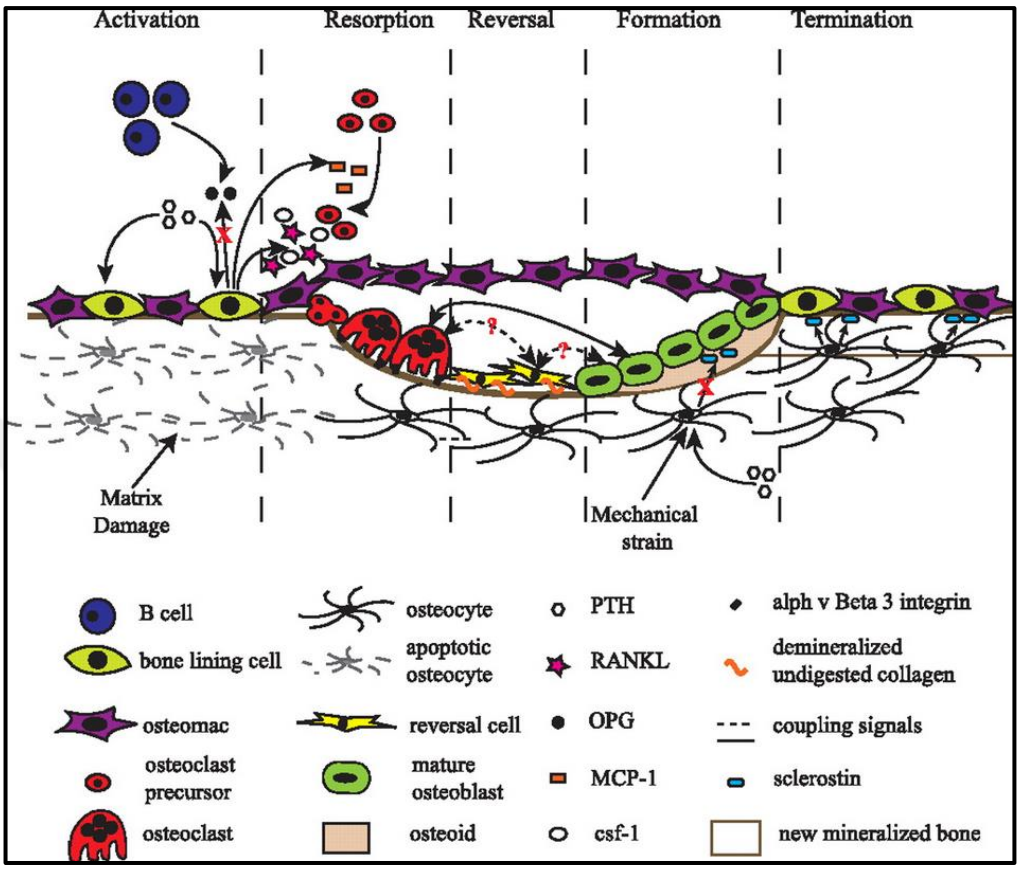

Figure 1.3. Bone remodeling stages. Quiescent stage: bone lining cells are lined on the bone surface. Activation stage: mature osteoclasts display their resorptive activities. Osteoclasts reabsorb bone matrix components during resorption stage. Formation stage: osteoclasts are rechanged by osteoblasts inside osteoid. Extracellular matrix mineralization is initiated by osteoblasts. Reconstruction of new bone in a structure unit progresses to quiescent stage [46].

Osteoblasts start to express osteoid matrix proteins which fill resorption cavity. Furthermore, hydroxyapatite minerals are incorporated into this newly deposited matrix [45]. Osteoblasts continue to secrete those matrix components until they ultimately turn into bone lining cells. Then the bone surface is entirely covered by bone lining cells. Eventually, bone lining cells are attached to osteocytes in the extracellular matrix by a framework of canaliculi. As an equal amount of bone has been reabsorbed, remodeling period terminates. Following mineralization, some of the mature osteoblasts are subjected to cell death by apoptosis;

whereas the others continue to differentiate into osteocytes. The bone surface is reconstructed until remodeling period starts again [46].

Quiescent Stage: It is the stage of the bone during rest. All lining cells are inactive and maintained themselves attached to the bone surface. Factors in quiescent stage starting to bone remodeling are still not known.

1.2. BONE SIGNALING PATHWAYS

Bone remodeling and growth provide an appropriate bone tissue structure and function. Bone tissue is regulated by systemic and local paracrine signals [47].

1.2.1. Canonical Wnt/β-Catenin Signaling Pathway

Wnt proteins are a group of secreted proteins which play significant roles in the activation of cell proliferation, differentiation and function [48, 49]. Wnt/β-catenin cascade is responsible for osteoblastogenesis, including stimulation of osteoprogenitor cells, suppression of osteoblast and osteocyte apoptosis [50]. Some of Wnt molecules, such as Wnt1, 3a, 4, 5, 10b and 13 play critical roles in bone formation [51]. Canonical Wnt/β-Catenin signaling cascade contains binding of Wnts through Frizzled family receptors (Fzd) and their co-receptors comprising of low-density lipoprotein receptor-related proteins (Lrp) [52, 53]. This binding leads to activation of Wnt/β-catenin pathway that causes an intracellular accumulation of β-catenin [54, 55].

In the absence of the Wnts, cytoplasmic β-catenin is controlled by 'β-catenin destruction complex' consisting of Axin, adenomatous polyposis coli (APC), and glycogen synthase kinase 3β (GSK3β) and casein kinase 1 alpha (CK1α) [56]. Axin protein serves as a scaffolding protein to facilitate binding of all other members. Cytoplasmic β-catenin is phosphorylated by CK1α and GSK3β. As a consequence of phosphorylation, polyubiquitination and proteasome-mediated degradation of β-catenin occurs [57]. Conversely, in the presence of the Wnts, they bind to their receptors and co-receptors, (Fzd and Lrp), following phosphorylation of the intracellular protein, which is called Disheveled (Dsh). Phosphorylated Dsh suppresses GSK3β. So, β-catenin destruction complex is not

formed as a consequence of GSK3β inhibition. As a result, cytosolic gathering of β-catenin is stabilized, translocate into the nuclei and binds to lymphocyte enhancer factor/T cell transcription factor (Lef/TCF) to regulate upregulation of the target genes (Figure 1.4) [58-60].

Nowadays, studies on the important role of Wnt signaling pathway in bone remodeling have been increased, since various human diseases such as osteoporosis, sclerosteosis, pseudo glioma syndrome and van Buchem's disorder have been related to abnormal canonical Wnt signaling cascade. Improvement of therapeutic targets for the cure of bone disorders has been focused by considering the significant role of canonical Wnt signaling pathway in osteogenic pathologies [61, 57].

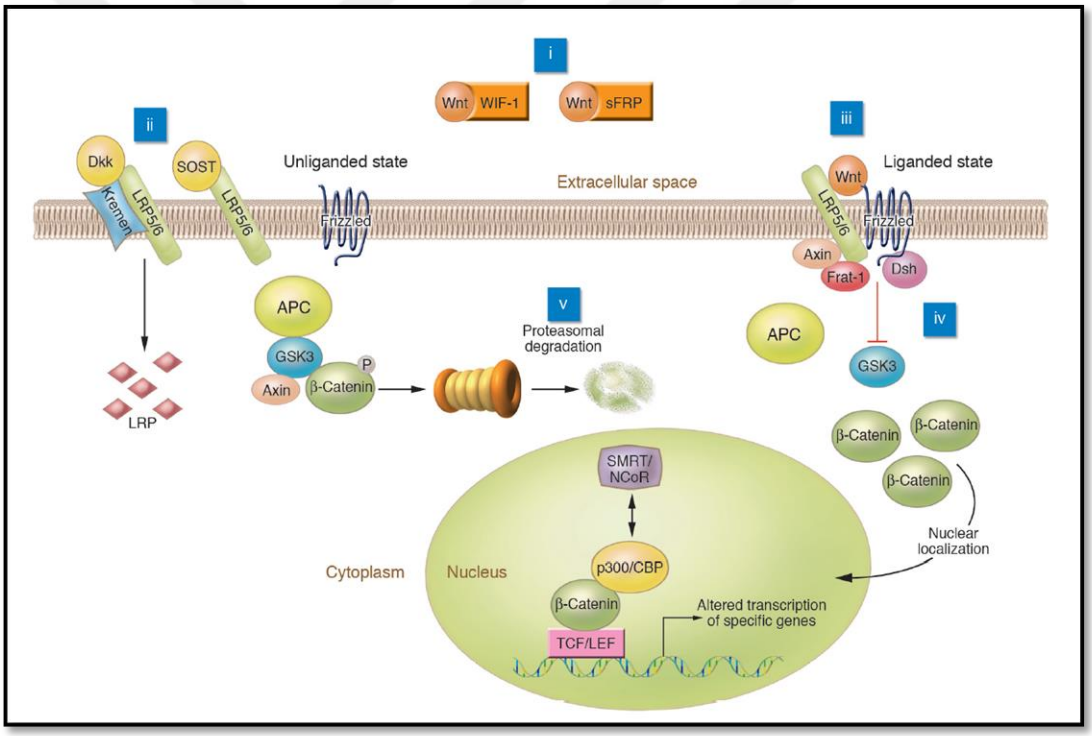

Figure 1.4. Canonical Wnt/β-Catenin Signaling Pathway. GSK3β is inhibited by binding to Frizzled and their co-receptor complex Lrp5/6 through mechanisms, including axin, Dsh and Frat-1. β-catenin gathers in the cytoplasm and is translocated into the nucleus, in which it binds to Lef/TCF resulting in upregulation of the target genes [50].

Several extracellular proteins associated with bone biology regulate the activity of Wnt/β-catenin signaling pathway. They interact with either Wnt molecules or their receptors/co-receptors, Lrp5/Lrp6, Fzd. These secreted proteins such as the Dickkopf family of proteins

(Dkk-1) [62-64], the secreted frizzled-related proteins (sFRPs) [65, 66], and sclerostin [67, 68] are capable of regulation of the Wnt/β-catenin signaling cascade (Table 1.1). Dkk-1 is a soluble secreted protein and expressed by MSCs. It binds to Lrp5/6, and then inhibits Wnt-Lrp5 proteins interaction [69]. Dkk-1 also creates a complex with Wnt co-receptor, Lrp6 and other transmembrane protein, named Kremen. This formation complex starts to internalization and degradation of Lrp resulting in decreased Lrp for Wnt ligands. Dkk-$1^{-/-}$ mice have displayed an increase in bone formation [70]. It has been suggested that bone mineral density can increase in several models of bone disorders in mice by preventing Dkk-1 activity [71].

The sFRPs are competitively responsible for inhibition of Wnt/β-catenin signaling pathway [72]. sFRPs bind to Wnt molecules to inhibit their binding to Fzd and Lrp5/6 [73]. Particularly, sFRP-1 plays a important role in osteoblast formation and regulation of osteoblast/osteocyte apoptosis [74]. Deletion of the sFRP-1 gene (sFRP-$1^{-/-}$) in mice results enhancement in trabecular bone mass. Moreover, sFRP-1 also binds to RANKL and thereby, osteoclast formation is suppressed [75].

Sclerostin is encoded by the SOST gene and produced by osteocytes. It serves as an antagonist of canonical Wnt/β-catenin signaling cascade by inhibiting bone formation [76]. Sclerostin binds to co-receptor Lrp5/6 to avoid binding of other Wnt ligands to Lrp5/6 [77]. Mutations in SOST gene in humans result in sclerosteosis. Clinical studies have displayed that anti-sclerostin treatment in several rats enhanced bone mass and bone strength [78]. Moreover, studies have suggested that sclerostin can be an anabolic therapy for the treatment of bone mass diseases [79].

GSK3β is also another significant signal regulator for the Wnt/β-catenin signaling cascade. In the absence of the Wnt signal, 'β-catenin destruction complex' including GSK3β, initiates degradation of β-catenin. However, the activity of GSK3β molecule is modulated by its inhibitor or small molecules, so that target genes are expressed for the enhancement of bone density [80, 81, 58]. Lithium chloride (LiCl) is used as an inhibitor of GSK3β to improve bone mass and reduce fracture risk. It has been shown that treatment with LiCl of MSCs stimulates differentiation of the MSCs into osteoblasts. Studies in rat models displayed that bone mass is increased by inhibition of GSK3β using small molecule, called GSK3α/β dual-inhibitor [82-84].

Table 1.1. Potential therapeutic targets and their physiological roles in canonical Wnt/β-catenin signaling pathway [60].

Therapeutic Targets	Roles	Agents	Physiological Mechanisms	Potential Effects
Sclerostin	Suppresses canonical Wnt/β-catenin signaling by binding LRP5/6	Anti-sclerostin antibody (AMG 785)	Neutralizes sclerostin	Enhanced bone mass and strength
Dickkoppf (Dkk-1)	Inhibits β-catenin signaling pathway by binding to low-density lipoprotein receptor-related protein (LRP)	Anti-Dkk-1 antibody (BHQ 880)	Neutralizes Dkk-1	Decreased bone loss in rheumatoid arthritis in mice Increased fracture healing
Secreted frizzled-related protein (SFRP)	Suppresses β-catenin signaling by binding toWnt molecules	Diphenylsulfonyl sulfonamide	Suppresses SFRPs	Suppresses SFRP-1 *in vitro* models and stimulates bone formation *ex vivo*
Glycogen synthase kinase β (GSK3β)	Proteasomal degradation of β-catenin	Lithium GSK3β inhibitors	Inhibits GSK3β	Increases fracture repair and avoids tumor growth in multiple myeloma mouse. Enhances bone mass in normal mice.
β-catenin	Binds to T-cell factor/ lymphoid enhancer-binding factor (Tcf/Lef-1) and activates expression of target gene	Deoxycholic acid	Regulates activation of β catenin	Activates of target gene expressions

Understanding the function of canonical Wnt/β-catenin signaling pathway is essential for the development of new pharmaceutical approaches for the treatment of bone diseases and fracture healing. Antibodies against Wnt antagonists, particularly anti-sclerostin, anti-Dkk-1 and anti-sFRP-1 proteins have been candidates as therapeutic targets to increase bone formation and decrease bone loss [51]. Modulation of Wnt/β-catenin signaling cascade has served as a promising strategy to enhance bone density through using of GSK3β inhibitors. Although these novel therapeutic targets offer great approaches, systemic regulation of Wnt signaling pathway may have carried several potential risks. Long-term safety of these therapies should be determined [79].

1.2.2. TGF-β/BMP Signaling

TGF-β superfamily includes over forty secreted members, including TGF-βs, activin and BMPs which have significant roles in regulating cells proliferation, differentiation, adult tissue homeostasis and embryonic development [85]. TGF-β/BMP signaling is a key element in bone formation by providing homeostasis of bone function. It increases proliferation of osteoprogenitor cells and differentiation of MSCs towards osteogenic lineage via the Smad-independent pathway, p38 mitogen-activated protein kinase cascade (MAPKs) and Smad-dependent pathway [86, 87].

BMPs are expressed by osteoblasts and they are potent inducers of bone formation [87-90], BMP-2, -4 -7 and -9 stimulate both endochondral and intramembranous ossification. Meanwhile, BMP-2 and 6 have considerable roles on induction of MSCs differentiation into osteoblasts [91, 92]. BMPs have two different subtypes of receptors, which are named as BMPR-I and BMPR-II that are serine-threonine kinase receptors. When BMPs bind to their receptors, BMPR-II, quaternary complex is formed. This complex is then trans-phosphorylated to BMPR-I. After that, it activates intracellular Smad proteins which then translocate into the nuclei to mediate as transcription factors (Figure 1.5) [93, 51]. One of the BMP-Smad target genes is Runt-related transcription factor 2 (Runx2) that is responsible for bone formation. Mutations in Runx2 cause degradation of Smad-Runx2 interactions. Consequently, early osteoblasts differentiation is inhibited [94]. *In vitro* experiment in human marrow stroma-derived cell line has showed that BMP-2 treatment enhances expression of Runx2 gene and ALP protein [95].

Smad-independent pathway requires the interaction of BMP with its receptor, BR1a. Activation of BR1a initiates other downstream signaling pathways, including extracellular signal-regulated kinase (ERK), map kinase p38, and C-jun N-terminal kinase (JNK). It has been suggested that protein-protein interactions of BMP receptors with downstream signaling molecules activate Smad-independent pathway, resulting in expression of the target gene. Although Smad-independent pathway initiation is not very well known, BMPs activate ERK, JNK and map kinase p38. Thus, they are able to show their effects on cell proliferation, migration, and MSCs differentiation [96-98].

TGF-β/BMP signaling pathway has remarkable roles in bone tissue homeostasis. It is regulated by several ligand-receptor complexes and transduced by MAPK cascade and Smad proteins. Deterioration of this signaling pathway results in several bone diseases. Manipulating of TGF-β/BMP signaling pathway might be therapeutic implications for the cure of various bone diseases such as osteoarthritis, osteoporosis and fracture healing [99, 100].

1.2.3. OPG/RANKL/RANK Signaling

RANK [101], its ligand (RANKL) [102], the decoy receptor for RANKL, OPG [103] are the members of tumor necrosis factor (TNF) family. They have major roles in controlled of bone resorption and balanced bone remodeling [36]. RANK binds to its ligand, RANKL, which provides a significant signal to develop osteoclasts from hematopoietic progenitor cells. RANK/RANKL complex is responsible for the stimulation of mature osteoclasts. OPG adversely regulates RANK activity by binding to RANKL. Consequently, OPG inhibits bone resorption. However, the relative ratio of RANKL/OPG detects the extent of the bone resorption [104]. Therefore, the ratio of RANKL to OPG expression is a key element of resorption stage. Loss of functional OPG in mouse causes brittle bones due to excessive bone resorption [103]. RANKL is a major element for formation of mature osteoclasts. It interacts with various downstream signaling molecules, such as NF-κB, TNF receptor associated factor-6 and MAPK [105, 106]. It also regulates expression of αvβ3 integrin that is a part of osteoclast precursor cells. Proto-oncogene tyrosine- protein kinase (c-Src) is stimulated to activate GTPase that is necessary for the formation of actin. As a result, osteoclast proliferation and maturation occur [107-109]. Several studies exhibited the importance of

the RANKL gene. RANKL knockout mice have displayed lacking osteoclast cells resulting in osteoporosis [110, 111].

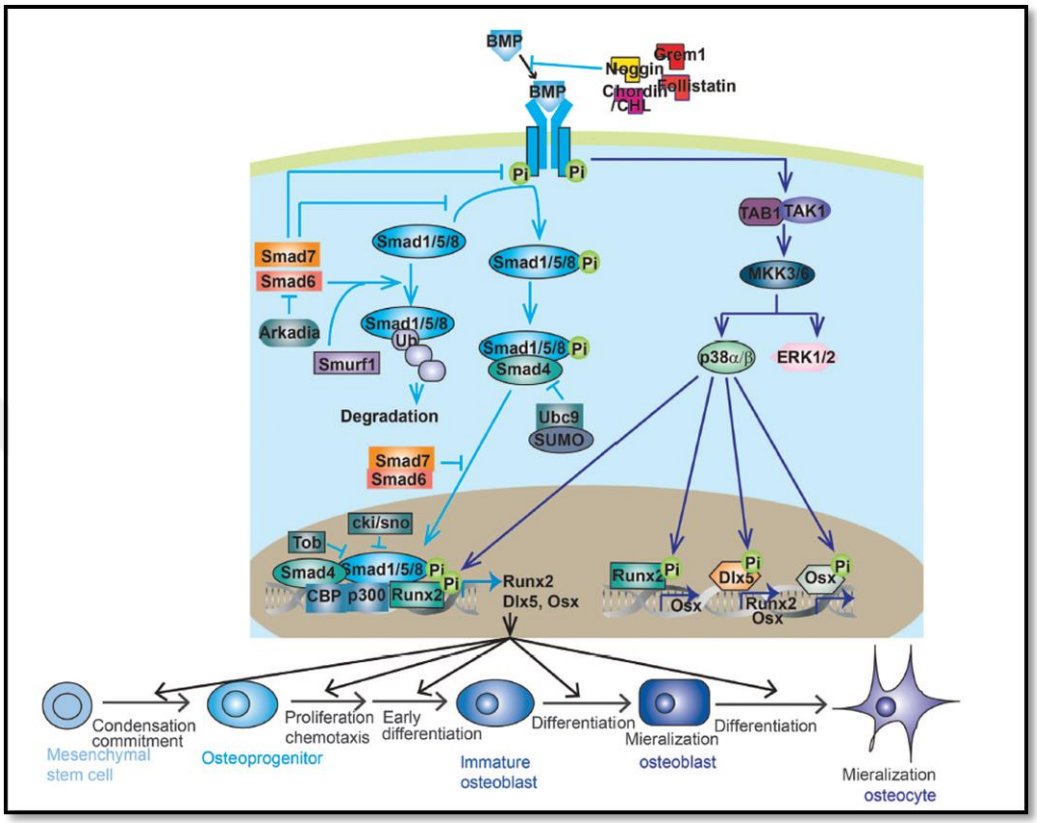

Figure 1.5. BMP signaling. BMP binds to its receptor BMPII, that transphosphorylases BMPI to stimulate Smad-dependent and independent pathways. In Smad-dependent pathway, Intracellular Smad proteins (Smad -1, -5 and -8) translocate into the nuclei, in which they gather co-factors and Runx2 to control the target gene upregulation. In Smad-independent signaling, phosphorylated TAK1 gather TAB1 to begin the p38, Erk or JNK signaling pathway, resulting in expression of Runx2. BMP signaling increases during osteoblast differentiation and maturation [101].

Several systemic osteotropic factors, such as 1α,25 dihydroxyvitamin D3 [1α,25$(OH)_2D_3$], prostaglandin E_2 and Parathyroid hormone (PTH) also regulate osteoclastogenesis by inducing expression of RANKL [112, 113]. Expression of RANKL is increased by PTH. Besides, PTH regulates calcium concentration of serum, whereas, 1α,25 dihydroxyvitamin D3 is responsible for the absorption of calcium in the intestinal system and stimulation of osteoclasts. Macrophage-colony stimulating factor (M-CSF) and prostaglandin are secreted by osteoblasts. They regulate the formation of osteoclasts [114, 36].

Various studies have displayed that PTH enhances both bone formation (anabolic effect) and bone resorption (catabolic effect) in human, depending on the length of the treatment [115, 104]. RANKL-mediated osteoclastogenesis is stimulated during increasing level of PTH [116]. Osteoblasts secrete monocyte chemoattractant protein-1 (MCP-1) that acts as a chemoattractant for osteoclast precursor cells. Meanwhile, they also express colony stimulating factor-1 (CSF-1) by responding increased level of PTH and hereby, RANKL/OPG modulates. CSF-1 and RANKL work together in a harmony; RANKL enhances osteoclast precursor cells proliferation and differentiation of osteoclast precursor cells into multinucleated osteoclasts (Figure 1.6). Thus, resorption activity of the mature cells is increased by RANKL [117]. At the same time, CSF-1 is also responsible for survival and proliferation of osteoclast precursors. It mediates cytoskeletal organization and spreading of mature cells [118, 46]. However, when expression of OPG is increased, the levels of RANKL and CSF-1 expressions are decreased resulting in a reduction of osteoclast formation [119].

RANKL/RANK/OPG signaling cascade are essential for osteoclast maturation and discovery of this pathway has opened a new avenue for the cure of bone related diseases including osteoporosis and rheumatoid arthritis [120, 121]. Consequently, controlling of RANK and RANKL expressions at the receptor level could be a remarkable therapeutic therapy in order to improve novel drugs for the treatment of systemic or local bone loss [122, 123]. Antibodies such as blocking RANKL can affect bone resorption. It was shown that parathyroid hormone-related peptide (PTHrP)-mediated bone loss resulted in a tremendous inhibition of bone resorption using OPG in mouse models. A monoclonal antibody, named Denosumab (AMG 162), binds to RANKL and thus mimics the effect of OPG. It has been applied as a novel therapeutic strategy for the treatment of osteoporosis in human [124, 125].

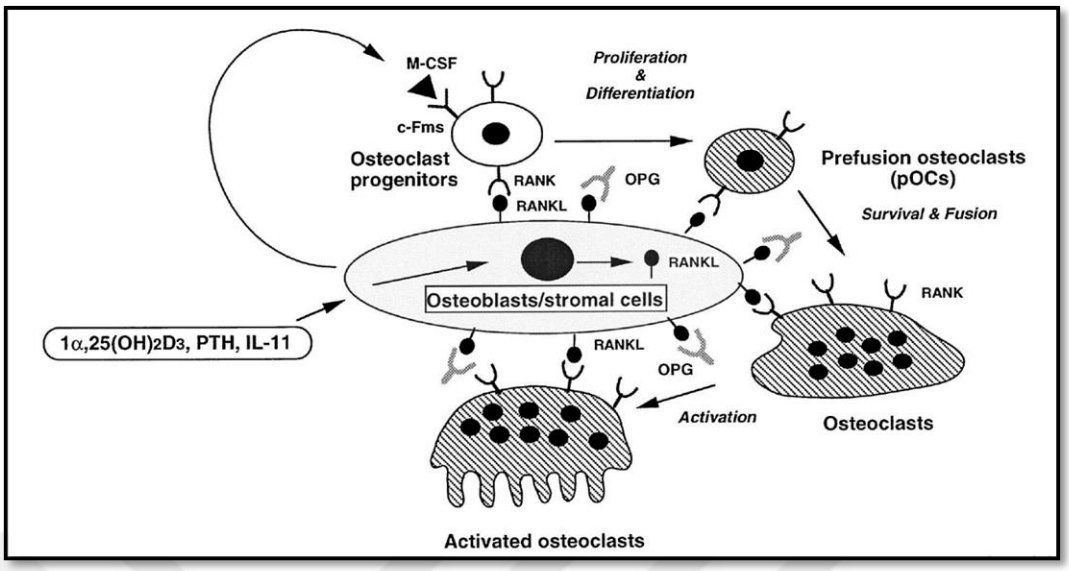

Figure 1.6. A diagrammatic representation of OPG/RANKL/RANK signaling in osteoclast differentiation. RANK interacting with RANKL stimulates differentiation of osteoclast progenitors. The decoy receptor for RANKL, OPG, regulates RANK activation. PTH induces RANKL-mediated osteoclastogenesis [120].

1.2.4. Hedgehog Signaling Pathway

Hedgehog (Hh) signaling pathway [126] plays significant roles in bone development and homeostasis. Sonic Hedgehog (Shh) has the main function for the early stage of the embryonic limb development, whereas Indian Hedgehog (Ihh) possesses a vital role for endochondral ossification [127, 128]. Ihh, which is a secreted molecule of Hedgehog family, is expressed by early hypertrophic chondrocytes. Ihh signaling acts as a transition molecule within inner perichondral mesenchyme to start bone formation. It provides differentiation of osteoprogenitor cells into osteoblast precursors [129-131].

Basically, Runx2 expression [132, 51] is modulated by Hh signaling pathway (Figure 1.7). Hh binds to cell surface receptor, Patched (Ptch) and Hh/Ptch is activated by patched-mediated suppression of a transmembrane protein, named Smoothened (Smo). After that, Smo is stimulated in an intracellular signaling cascade following by stabilization of transcription factor Gli2. Furthermore, Gli2 induces other transcription factors, including Gli1 and another Hh target genes [133, 134]. Several reports suggested that degradation of Hh signaling cascade results in bone disorders. Deletion of Shh gene in mice leads to

formation of skeletal malformations [135]. Moreover, it has been showed that Ihh$^{-/-}$ mouse embryos were lacking multiple early osteogenic markers such as Runx2, type I collagen and ALP [136, 137].

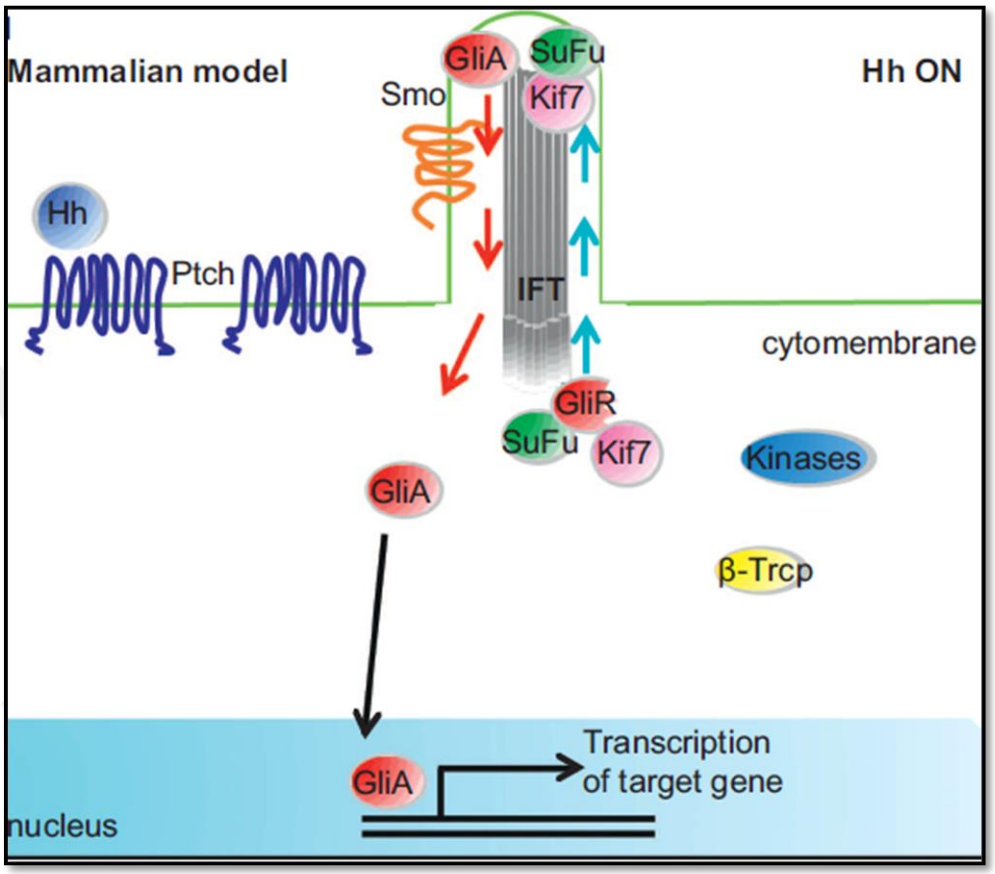

Figure 1.7. Hedgehog signaling in bone remodeling. As Hh binds to Ptch, Smo is activated by Hh/Ptch complex, resulting in stabilization of transcription factor, Gli that translocates into the nuclei to transcript Hh target gene [128].

1.2.5. Notch Signaling Pathway

In bone tissue, Notch signaling pathway affects both osteoblasts and osteoclasts. The Notch signaling depends on differentiation stages of the cells [138]. The Notch signaling pathway is a cell-to-cell signaling cascade that regulates cell proliferation, differentiation and apoptosis [139]. It is initiated when Notch ligands such as Jagged 1, 2 (JAG) and Delta-like 1, 4 (DLL) [140] interact with Notch receptors, Notch 1-4, which are expressed on the surface of neighboring cells [141]. Ligand binding causes to proteolytic cleavage [142] of

the Notch receptors via two mechanisms: i) a disintegrin and metalloproteinase family metalloproteinase tumor necrosis α conversion enzyme (ADAM/TACE) [143], ii) γ-secretase complex. Consequently, Notch intracellular domain (NICD) is released. After that, NICD translocates into the nuclei in which it binds to recombination signal binding protein for immunoglobulin kappa J region (RBPjκ), transforming it from a repressor into an activator. At the same time, Mastermind-like protein (MAML) also binds to NICD-RBPjκ to create a complex. Afterward, NICD-RBPjκ-MAML complex initiates upregulation of Notch target genes including the transcriptional repressors hairy and enhancer of split (HES) and HES-related with YRPW motif (HEY) (Figure 1.8) [144, 145].

Notch signaling can either suppress or induce osteoblast differentiation. It inhibits terminal differentiation of osteoprogenitor cells. Nonetheless, mature osteoblasts are not affected [146, 147]. Activation of the Notch signaling in osteogenic lineage at various stages of differentiation causes Notch arrested differentiation of the pre-osteoblasts resulting in osteopenia [148] because of the enhancement of osteoclasts activities. Osteoprogenitor cells in an undifferentiated stage are maintained by activation of the Notch signaling [149].

Although, Notch signaling causes suppression of osteoblasts differentiation by inhibition of osteogenic markers including Runx2, ALP, Col1A1 and OCN, activation of Notch signaling in mouse osteoblast precursor cells shows induction of osteoblasts differentiation [150]. Loss of functions of Notch 1 and 3 promotes a number of osteoclasts due to stimulation of cell proliferation. Studies suggested that Notch-1 receptor causes a reduction of M-CSF expression and an increase in RANKL/OPG expression. As a result, osteoclastogenesis in stromal cells is decreased. Likewise, as Notch 1 is inactivated in osteogenic lineage, expression of RANKL is enhanced and OPG is reduced resulting in increased osteoclastic activity [151, 152].

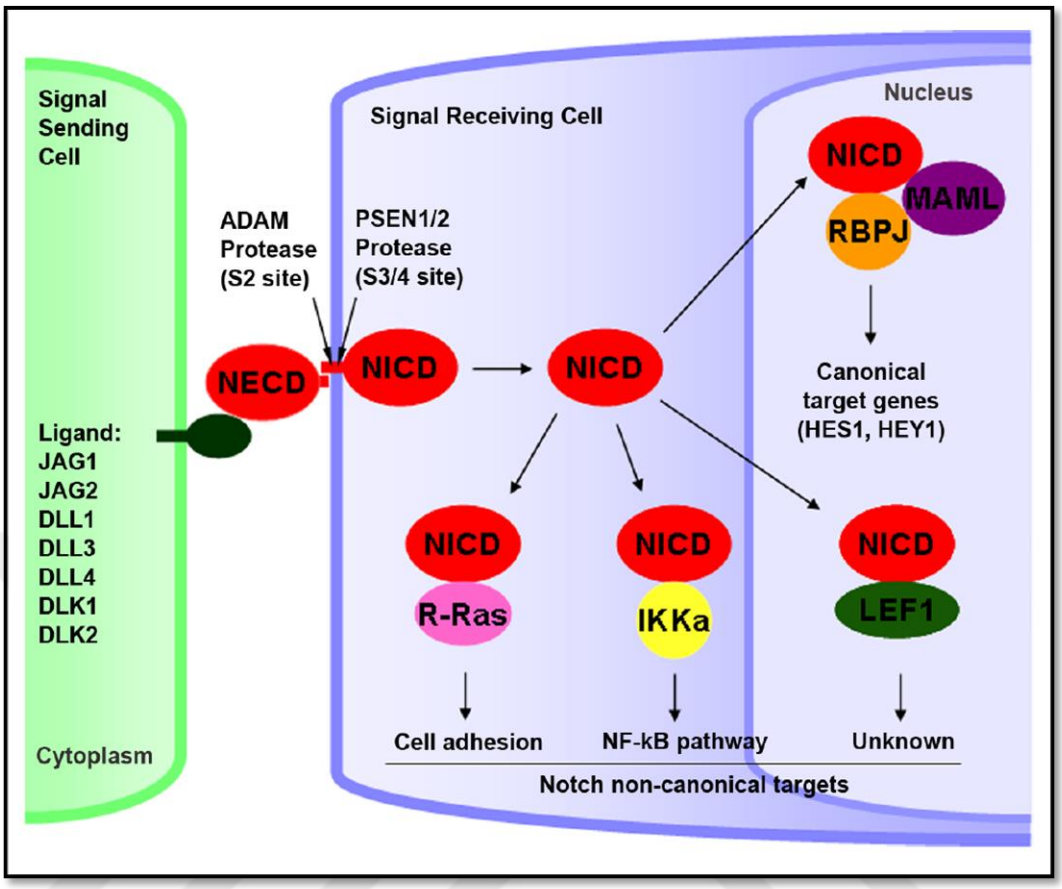

Figure 1.8. Notch signaling. Binding between Notch receptors and their ligands, JAG, DLL, causes a proteolytic cleavage resulting in the release of NICD to translocate into the nuclei, in which NICD binds to RBPjκ and co-activator MAML. NICD- RBPjκ-MAML transcriptional complex regulates transcription of the target genes, including HES1 and HEY1 [139].

1.2.6. Signaling cross-talk in bone development

Wnt, TGF-β/BMP signaling Pathway: BMP signaling has dual effects in regulating Wnt signaling pathway. Cross communicate between canonical Wnt/β-catenin signaling pathway and TGF-β/BMP signaling has a reverse effect on osteoprogenitor cells and cooperative effect in osteoblasts [129]. Activation of Wnt/β-catenin signaling pathway regulates differentiation of mesenchymal stem cell precursors into osteoprogenitor cells, whereas TGF-β/BMP signaling indirectly increases chondrogenesis because of blocking Wnt/β-catenin cascade [153, 127]. Viability and proliferation of osteoprogenitor cells are provided

by Wnt/β-catenin pathway, while TGF-β/BMP signaling pathway stimulates those cells to turn into mature osteoblasts [154, 155]. On the other hand, BMP signaling is responsible for regulation of osteoblast differentiation synergistically with canonical Wnt/β-catenin signaling. Particularly, BMP-2 increases Wnt/β-catenin signaling pathway by stimulating expression of several Wnt proteins and Lrp5 [156]. In the presence of the BMP signaling together with Wnt signaling, osteoblastogenesis is maximized, since Smad and LEF/TCF/β-catenin cooperatively affect each other. Consequently, the highest expression of osteoblast gene is observed [157]. However, various studies demonstrated that BMPR-1 mediated BMP signaling increases expressions of sclerostin and Dkk-1 that cause an inhibition of canonical Wnt/β-catenin signaling. It indirectly regulates RANKL/RANK/OPG signaling resulting in osteoclastogenesis. Consequently, BMP signaling pathway regulates bone mass in a negative way by the stimulation of sclerostin expression and inhibition of canonical Wnt/β-catenin signaling.

Cross-talk between BMP and Wnt signaling pathways have numerous different molecular steps such as Runx2, Dsh, β-catenin and GSK3β [51]. BMP signaling also regulates Wnt/β-catenin signaling via Smad molecules. Previous studies have exhibited that Smad1 inhibits activation of Dsh in the absence of Wnt molecules. As Wnt3a is found devoid of BMP-2 in mouse bone marrow stromal cells (ST2 cell line), Dsh is activated resulting in suppression of GSK3-mediated degradation of β-catenin. A reduction of Wnt3a is observed with the addition of BMP-2 since BMP might induce the formation of Smad-Dsh complex [158]. Otherwise, it has been suggested that BMP-2 increases Wnt signaling by expression of various Wnt receptors and ligands [159].

Hedgehog, BMP and Wnt Signaling Pathway: BMP signaling pathway and Hh signaling pathway seem to have a cooperative effect on osteogenesis in MSCs. Shh/Ihh treatment of either mouse embryo mesenchymal stem cells (C3H10T1/2 cells) or mouse osteoblast precursor cells (MC3T3-E1 cells) stimulate ALP activity synergistically with BMP-2 [160]. Integration of Hh signaling with Wnt signaling in osteoblast differentiation occurs in a cooperative way. $Ihh^{-/-}$ mouse embryos showed a lack of β-catenin accumulation since Wnt/β-catenin signaling was broken down in the absence of Ihh signaling [137]. Even though *in vitro* and *in vivo* findings have suggested that Hh signaling induces expression of Runx2 and differentiation of MSCs towards osteoprogenitor cells, further studies are need to understand the downstream of the canonical Wnt/β-catenin signaling pathway [161, 162].

Notch, Wnt and BMP signaling: Notch and BMP signaling pathways work together synergistically to enhance osteogenic differentiation [51]. Several studies proposed that Notch signaling in osteoblastogenesis increases BMP-induced signaling. Activation of Notch signaling in MC3T3-E1 cells with the addition of adenovirally-overexpressed Notch intracellular domain resulted in stimulation of BMP-2-stimulated osteoblastogenesis. An increase in the level of ALP activity and extracellulae matrix mineralization were demonstrated [150]. However, supprresion of Notch signaling resulted in a reduction of promoter activity of BMP target genes followed by a decrease in ALP activity [163]. Conversely, the integrated effects of those signaling pathway have been created dichotomous results. Another study displayed that activation of Notch targets, Hey 1, inhibited BMP-2-stimulated osteoblast differentiation in MC3T3-E1 cells [160]. In the same way, BMP-2 treatment of MC3T3-E1 cells regulates the induction of Hey1 expression [164, 51].

1.3. BONE GRAFTING

Bone repair is a complicated, well-organized physiological mechanism of bone remodeling that has occurred during continuous bone remodeling throughout adult life and normal fracture healing in response to damage [165]. It has series of biological events involved in coordinated actions of a lot of cell types and complex molecular signaling cascades to reconstruct skeletal function [166]. Nevertheless, there are several clinical circumstances where bone regeneration is required on a large-scale including skeletal reconstruction of massive bone disorders, skeletal abnormalities, trauma and tumor resection [167]. Although standard approaches have been broadly used in clinical practice for augmentation of bone regeneration, there has been the usage of different strategies for enhancement of inadequate bone regeneration including autologous bone grafts, allogeneic bone grafts, bone graft substitutes and growth factors. A bone graft is described as an implanted material which enhances bone repair [168-171].

Bone graft materials are the second most commonly used transplantation tissue after blood in the human body [172] and approximately 2.2 million surgeries [173, 174] have been carried out each year worldwide in the field of orthopedics, dentistry and neurosurgery, including traumatology, maxillofacial and plastic surgery [175, 176]. Particularly, bone grafts are mainly used for major indications in orthopedics following; i) reconstruction of

skeletal defects, including tumors, osteotomies and trauma; ii) enhancement of fracture repair, for the cure of non-union and delayed-union; iii) fusing joints; augmentation of joint reconstruction, such as large bone loss in revision arthroplasties [177]. Beyond how often bone grafts are used, it is noted that the use of appropriate and effective bone grafts requires not only proper technical surgical skills, including harvesting procedure and delivering graft to host site, but also a profound theoretical knowledge of its biological and mechanical mechanism during host-graft integration [178, 167].

1.3.1. Properties of bone grafts

Bone grafts show biological and mechanical features. The biological properties can be divided as follows: osteoconduction, osteoinduction and osteogenesis. The primary function of bone grafts is to enhance bone healing by supporting new bone formation. Bone grafts ensure a framework so that new bone can be constituted due to osteoconduction, osteoinduction and osteogenesis [179]. They should have at least two of these biological features [180]. Osteoconduction is the process that provides a framework for ingrowth of new bone. Bone grafts have a porous three-dimensional structure which supports migration of host cells including MSCs, osteoblasts, and osteoclasts [181].

Grafts are able to incorporate the graft with host bone for accomplished osteogenic activity. When a new bone starts to be formed, the graft initiates partial or entire resorption throughout bone formation [182]. Osteoinduction is described as an improvement of new bone formation by induction of host osteoprogenitor cells and differentiation of them into osteoblasts. MSCs around the host site are gathered and differentiated towards osteogenic lineage. This process is modulated by GFs including BMPs -2, -4 and -7, FGF, IGF and VEGF [183]. New bone growth can arise from live cells in the graft. Osteointegration refers to the capacity of chemical bonding to the host surface without aside of a layer of fibrous tissue [184].

Osteopromotion is defined as the capability of a substance to promote osteoinduction without having osteoinductive features [185]. Understanding of the mechanical and biological features of each graft material is essential to determine which bone graft is more suitable for a given situation (Table 1.2). Besides, the choice of an ideal bone graft depends

on other factors such as graft shape, size, volume, graft handling, cost and ethical issues [186].

1.4. TYPES OF BONE GRAFTS

Bone grafts can be classified into various main categories such as autograft, allograft and xenograft. Autograft refers to a graft harvested from one site and transferred to another site within the same person, whereas tissue moved between two different donors of the same species is known as an allograft [187, 182]. Xenograft is described as a graft of tissue removed from a individual of one species and transferred into the recipient of other species [177, 179].

1.4.1. Autografts

Autogenous bone grafts are the most frequently used graft material in musculoskeletal reconstruction. They still remain as a "*gold standard*" [188] for bone regeneration since they show the best osteogenic, osteoinductive and osteoconductive properties. Autografts, including osteogenic cells and matrix proteins, enhance bone ingrowth. Moreover, they are able to complete major histocompatibility and they don't have any risk of disease transmission [180]. Nevertheless, there are several disadvantages for using autografts: i) additional surgical process, ii) inadequate amounts of graft material, especially in children and in revision restoration, iii) critical donor site morbidity, iv) postoperative complications such as muscle weakness, nerve injury, pain and infection, v) enhanced operation time, and vi) additional cost [189-191].

They can be cancellous, cortical (vascularized/non-vascularized) and bone marrow aspirate [192]. Sources of the autograft are the iliac crest, fibula, femoral head and the other long bones [193]. Cancellous bone autografts have more osteogenic capacity than cortical autografts since areas in cancellous structure permit wide distribution of nutrients and revascularization [194, 195]. Cancellous autografts are already re-vascularized and integrated rapidly with the host site because low pH and oxygen tension of the recipient site act as attractants to host undifferentiated MSCs to the graft site. Eventhough they possess

more osteogenic and osteoinductive abilities than other bone grafts, they lack important mechanical features [196-198].

Cortical autologous bone grafts have osteoconductive properties and their solid, highly organized structure cause a decrease in cellular activity, osteoinductive and osteogenic potentials [199]. Several studies suggested that after transplantation of cortical autografts, osteocytes started to die resulted in a reduction of osteogenesis; on the other hand surviving osteoblasts can still display osteogenic features [200]. Consequently, cortical autografts are incorporated much slower than cancellous autografts [201].

The bone marrow is used to induce bone formation by regulation of GFs and cytokines that are secreted from transplanted cells [202]. It was previously thought that bone marrow includes a high amount of MSCs and promotes osteogenic capacity. Nevertheless, recent research has exhibited that the existing amount of MSCs in bone marrow aspirate is importantly much lower than as earlier thought [183]. The efficiency of bone marrow in fracture repair actually comes from GFs and endothelial progenitor cells which are able to induce angiogenesis and re-build up blood flow at the host site [203]. In spite of possible indirect osteoinductive feature of bone marrow aspirate, it does not give any structural support. Moreover, its viscous form causes leakage from the host site. The mixture of bone marrow with demineralized bone matrix (DBM) is a great carrier due to osteoinductive and osteoconductive properties of the carrier. It is usually used to fill bone defects [204, 205]. Proliferation and differentiation of MSCs in bone marrow can also be enhanced by combining bone marrow aspirate in collagen [206].

1.4.2. Allografts

Allogeneic bone grafts are obtained from human cadavers, then processed for sterilization before transplanting into a patient [207]. They display osteoconductive and occasionally osteoinductive capacities which are determined according to harvesting and preparation processes. They don’t contain viable osteogenic cells [199, 200]. Allogeneic bone grafts are usually used when there is a massive bone loss that requires structural support or when insufficient autograft volume is available [207]. Limitations of the use of autografts can also be accomplished by the use of allografts. Main advantages of allogeneic bone grafts have

their availability in different sizes and shapes, prevention of the requirement to sacrifice host bone tissue and avoidance of the morbidity [206]. However, the most important concern with the use of allografts is the probability of viral disease transmissions such as human immunodeficiency virus (HIV), hepatitis B and C. Furthermore, toxins and autoimmune diseases have been reported [208, 209].

A bone banking system is essential to provide safety issues of allografts. American Association of Tissue Banks outlined a standard including sterile processing, rapid procurement, and donor screening [210]. The USA Food and Drug Administration (FDA) requires HIV and hepatitis tests. Preparation procedure of allografts is another limiting factor [211]. The irradiation causes a reduction of graft-host osseo-integration [212]. Effective vascular infiltration is not provided in the presence of immune responses. Consequently, osseo-integration of the graft to host site is decreased and fibrous tissue is occurred rather than new bone formation [213-215].

Allografts are available in many forms including cortical grafts, cancellous bone chips, cortico-cancellous grafts, osteochondral segments and DBM [206]. Cancellous allograft is used to fill osseous defects, including total hip arthroplasty associated with retro-acetabular osteolysis [179]. Although its mechanical strength is similar to autogenous cancellous grafts, it shows relatively poor enhancement of bone healing because of preparation process that eliminates growth factors [199]. Consequently, it is only used as an osteoconductive graft. On the other hand, cortical allografts are used to fill massive defects and give rigid structural support [216]. Even though they have both osteoinductive and osteoconductive properties, they undergo slow vascular invasion and gradual integration, due to their solid structure [47]. Osteochondral allografts possess articular cartilage, diaphyseal cortical bone and metaphyseal spongy bone and they are generally used in joint reconstruction [183].

DBM is mainly used in a structurally stable environment since it does not provide any structural strength. DBM has an osteoconductive (type I collagen and non-collagenous protein) and osteoinductive potentials (FGF, PDGF, IGF, BMPs and TGF-β) [217]. The mineral substances of the allogenous bone are usually eliminated by demineralization method to get demineralized bone matrix. Moreover, DBM acts as an appropriate carrier for bone marrow aspirate. Moreover, it re-vascularizes rapidly and several proteins and GFs existing in the host bone matrix are attracted by DBM [218, 207]. However, DBM has a

number of limitations including potential infectious disease transmission, particularly HIV. Besides, different batches can have different features due to a number of donors used in the harvesting of grafts [206].

1.4.3. Bone Graft Substitutes

Synthetic ceramic-based bone graft substitutes are an alternative to both allogeneic and autologous bone grafts. They have osteoconductive capacity and can be found in various forms, such as blocks, pellets, granules, powders, coatings and cement [219]. They permit migration and proliferation of osteogenic cells. Reabsorption of the bone graft substitutes are related to the host site and type of the materials used [217]. The porous structure of bone graft substitutes is essential for supporting new bone formation. Migration of MSCs and infiltration of blood cells are provided by their 3D structure [220].

1.4.3.1. Calcium Phosphate Based Bone Graft Substitutes

Among various ceramic-based bone graft substitutes, synthetic forms of calcium phosphate based substitutes, including β-tricalcium phosphate (β-TCP), hydroxyapatite (HAp) and calcium phosphate cement (CPCs) have been used in orthopedics and dentistry for long years [221, 222]. Osteoconductive capacity, biodegradability, the absence of toxicity, bioactivity and lack of disease transmission are several advantages of their usage. Nonetheless, they have several limitations regarding the lack of osteogenic and osteoinductive potential and minimal mechanical support [223, 173].

β-TCP [Ca_3 $(PO_4)_2$] is a biodegradable and biocompatible calcium salt-based bone graft substitutes. It is used as void fillers in orthopedic and dental operations [224, 206]. The chemical composition of β-TCP is similar to mineral phase of bone. The small particle size and micro-porous structure of β-TCP provide an enhancement of its osteoconductive feature and improvement of timely resorption during bone remodeling [225]. Several studies have exhibited that β-TCP together with osteoinductive proteins show osteogenicity and effective healing for large bone defects [226, 227]. Nevertheless, β-TCP sometimes processes reabsorption more quickly than new bone formation following an imbalance between β-TCP and new bone replacement [180, 208].

Synthetic HAp [C_{10} $(PO_4)_6$ (OH) $_2$] which is the major mineral substance of the bone possesses osteoconductive capacity and biocompatibility. It was shown that although HAp has no osteoinductive role, MSCs can still differentiate into osteoblasts and so a new bone is formed [228]. Additionally, HAp has been usually used to cover metal implants to improve their graft-host osseo-integration. The porous structure of HAp allows migration of osteogenic cells and invasion of blood cells. HAp supports to begin bone remodeling. Even though HAp provides a great compressive strength, it shows an insufficient tensile strength [208]. It can be fragile and weak particularly under shearing forces. In spite of its biocompatibility, it shows a low solubility [47].

CPCs are the combination of calcium carbonate, monocalcium phosphate and β-TCP. They are essentially used as filling materials [173]. They can be pasted or injectable and react in the body temperature. Deposition of calcium phosphate results in improvement of host-graft matrix interface strength [229]. Although degradation rate of the CPCs is associated with physio-chemical properties of the host site, CPCs are degraded in a few months [230]. Moreover, CPCs have the highest compressive strength when compared to other bone substitutes [231].

1.4.3.2. Bioactive Glass & Glass Ionomers

Bioactive glass (commercial name Bioglass®) ceramics mainly consist of sodium oxide, silica, calcium phosphates and oxides [206]. They have osteoconductive capacities and are able to bind directly to bone. They can be available as fibers, beads and porous structures. They have been broadly used for filling bone defects alone [232, 233]. They have been also used as a graft expander to enhance their osseo-integration in a mixture of either autografts or allografts [234, 235].

Bioactive glass-ceramics can be adhered to the host site without forming intermediary fibrous tissue [236, 237] resulting in a series of reactions on the surface of the bioactive glass [238]. They directly bind to collagen, GFs and fibrin to create a microporous extracellular matrix which permits migration of osteoblasts. They can be either completely absorbed by osteoclasts or can partially form little amount of fibrous tissue [239, 207].

Bioactive glass ionomer is a glass powder of aluminum, calcium and flora-silicate combined with a polyacrylic acid, polycarboxylic acid, citric acid and poly maleic acid. Its biocompatibility and osteo-integrative properties are similar to Bioglass®. Its porous structure permits osteoconduction. Moreover, it has high compressive strength like cortical bone [208]. Glass ionomer is not replaced by new bone due to lack of reabsorption. Consequently, it has been used as an alternative to poly-methyl-methacrylate (PMMA) which is a calcium phosphate-based cement. Moreover, glass ionomer releases GFs more slowly and effective than the PMMA [240].

Glass ionomers and bioactive glasses provide various advantages for bone formation in fracture healing. They provide an actual stability and strength for the surrounding bone tissue and immunogenic reactions are not occurred [241]. Nevertheless, the risk of fibrous tissue formation is the limitation of bioactive glass and glass ionomer [47]. Moreover, the metal ions found in glass ionomers can be toxic to bone cells resulting in inhibition of bone tissue formation [242].

1.4.3.3. Calcium Sulphate

Calcium sulfate (CS) bone graft substitute is defined as an alpha-hemihydrate and has primarily osteoconductive role. It provides efficient gap filler and allows vascular ingrowth and quick reabsorption [243]. It has been commonly used for filling of bone cysts, segmental bone defects and bone lesions [244]. Recently, several adverse effects were noted that CS is degraded too fast (approximately 12 weeks) and can display immune reactions without bone formation [245, 246].

1.4.3.4. Aluminum Oxides

Aluminum oxide [Al_20_3] is an element of various bioactive materials. It has been used as a graft substitute on its own. It has no osteointegration with host site due to lack of ionic exchange between implant and bone. It has been used as graft expanders for wedge osteotomies. However, its application in the orthopedic field has been restricted due to insufficiency in osteointegration. It has been widely used in dentistry, particularly in orbital implants and ossicular replacements [247, 208].

1.4.3.5. Polymer-based bone graft substitutes

Polymers are covalently bound repeating monomers. They can be either linear chains or cross-linked with other chains [248]. Polymers are promising bone graft substitutes. They exhibit different chemical, physical and mechanical properties. They can be classified as natural and synthetic polymers. They are used either alone or combination with other grafts [206].

Table 1.2. Properties of bone grafts and their substitutes [206].

Graft Types	Definition	Examples	Features of action
Autologous bone grafts	Used alone		Osteoconductive Osteoinductive Osteogenic
Allogeneic bone grafts	Allogeneic bone graft used alone or in mixture with other bone grafts	Allegro, Orthoblast,	Osteoconductive Osteoinductive
Growth Factors	Recombinant growth factors used alone or in mixture with other bone grafts	b-FGF, rhBMP-2,-7, TGF-β, PDGF,	Both osteoinductive and osteoconductive with carrier materials
Usage of Cells	Cells used to form new bone alone or harvested onto a scaffold	Mesenchymal stem cells	Osteogenic, Both osteogenic and osteoconductive with carrier materials
Ceramic based bone graft substitutes	Contains calcium sulfate, bioactive glass and glass ionomers used alone or in mixture with other grafts	Osteograf, Osteoset, NovaBone	Osteoconductive
Polymer based bone graft substitutes	Contains biodegradable and nondegradable natural and synthetic polymers	Cortoss, OPLA, Immix	Osteoconductive biodegradable polymers
Miscellaneous	Coral HA composites	ProOsteon	Osteoconductive

Natural polymers such as hyaluronic acid (HA), collagen act as a natural biological guidance to bone cells. Although they support cell proliferation and enhancement of chemotactic responses, they have several limitations including the risk of disease transmission,

immunogenicity and weak mechanical properties (Stevens 2008). Besides, synthetic polymers such as polyglycolic acid (PGA), polylactic acid (PLA), a copolymer of PLA and PGA (PLGA), poly-fumarates and polycaprolactone (PCL) provide great alternatives. They can be processed using different methods to create series of 3D scaffolds [249].

Many of bone graft materials have been only showed classical scaffolding properties: osteo-integrative and osteoconductive. Conversely, osteoinduction and osteogenesis are essential for differentiation of MSCs into osteogenic lineage resulting in an enhancement of new bone formation. Nowadays, various methods have been improved to provide those two properties in order to increase an effectiveness of new bone regeneration [47]. New strategies, including gene therapy, novel biological approaches, bone tissue engineering by using stem cells, scaffolds and GFs have been evaluated for bone healing. Furthermore, three-dimensional printing would be in a preliminary stage of new inspiration in the near future of the bone regeneration [207].

1.5. FRACTURE HEALING MECHANISMS

Fracture repair is regulated by molecular and cellular mechanisms of bone tissue involved in various changes of the expression of numerous genes [250]. Even though several pathways of bone regeneration are entirely not comprehended, all pathways of biochemical events have been thoroughly considered to give a general point of view about how fracture heals. Fracture healing is a well-organized process, including bone cells, GFs, cytokines and inflammatory cells [1]. The coordinated manner of these molecules results in reconstruction of normal bone tissue. In spite of regenerative capacity of bone tissue, biological process of bone regeneration is sometimes unsuccessful during fracture healing following by non-unions, pseudo-arthritis and malignant tumors. A better understanding of the biology of the fracture healing leads to the development of new strategies [251].

Fracture repair can be divided into two types: primary healing by internal modeling and secondary healing by callus formation [252]. Primary healing includes a direct interference into the cortex to rebuild itself without callus formation. It requires a rigid stabilization [253-255]. Primary healing is rare since it needs an anatomical reduction and firmly stable

conditions. Open reduction and internal fixation are usually required [256, 166]. Both intramembranous and endochondral ossification takes place during secondary healing.

Secondary healing is the most common type of bone repair and it follows four stages; i) inflammatory phase, ii) soft callus formation, iii) hard callus formation, and iv) bone remodeling (Figure 1.9) [257]. Fracture healing starts an acute inflammatory response resulting in recruitment of MSCs and follows differentiation of MSCs into chondrocytes. After formation of cartilage matrix, a transition from a calcified cartilage to bone tissue is occurred by resorption of calcified cartilage. At the same time, primary soft callus is formed by resorption to reconstruct the anatomical structure. The cellular and molecular mechanisms of those stages are firmly regulated by signaling molecules such as GFs and cytokines (Table 1.3) [258, 259].

Inflammation: When the fracture occurs, an inflammation stage begins quickly and ends until bone formation starts. The inflammatory response is essential for fracture healing [260]. Vascular endothelial injury leads to an activation of a cascade including recruitment of platelet cells and release of their α-granule contents. Monocytes, macrophages and polymorphonuclear leukocytes (PMNs) are attracted to the injury site and then they are stimulated to release cytokines which regulate angiogenesis [261, 259]. Inflammatory response causes a hematoma formation in medullary canal and around the fracture ends. Hematoma formation acts as a hemostatic filling for inhibition of further hemorrhage. Additionally, it serves as a fibrin network which is required for cellular migration [254]. It also aids as a source of signaling molecules that start molecular and cellular events of fracture healing [257]. The acute inflammatory response reaches at highest level within 24h and it is almost completed after 1 week of post-fracture [258]. Although pro- inflammatory cytokines have negative effects both on bone formation and transplanted grafts, secretion of cytokines following an acute injury is essential and critical for bone regeneration [253].

Soft callus formation: After hematoma formation and development of fibrin-rich granulation network, endochondral ossification occurs in the fracture sites and outside of the periosteal sites resulting in soft callus formation [262]. Soft callus formation is defined as the development of a callus tissue near the fracture site that gradually is turned into bone. Meanwhile, an intramembranous ossification takes place adjacent proximal and distal sites of the fracture, following by a hard callus formation. Bridging of the central hard callus

provides semi-solid structure to fracture that permits weight bearing [263, 253]. Role of the soft callus formation is to increase the mechanical stability of the fracture site by supporting fracture [211].

Damaged bone marrow, cortex, periosteum and other surrounding tissue cause a necrotic tissue near the fracture site. When the necrotic tissue starts to be reabsorbed, MSCs initiate formation of other cell types such as osteoblasts, chondrocytes and fibroblasts [260, 254].

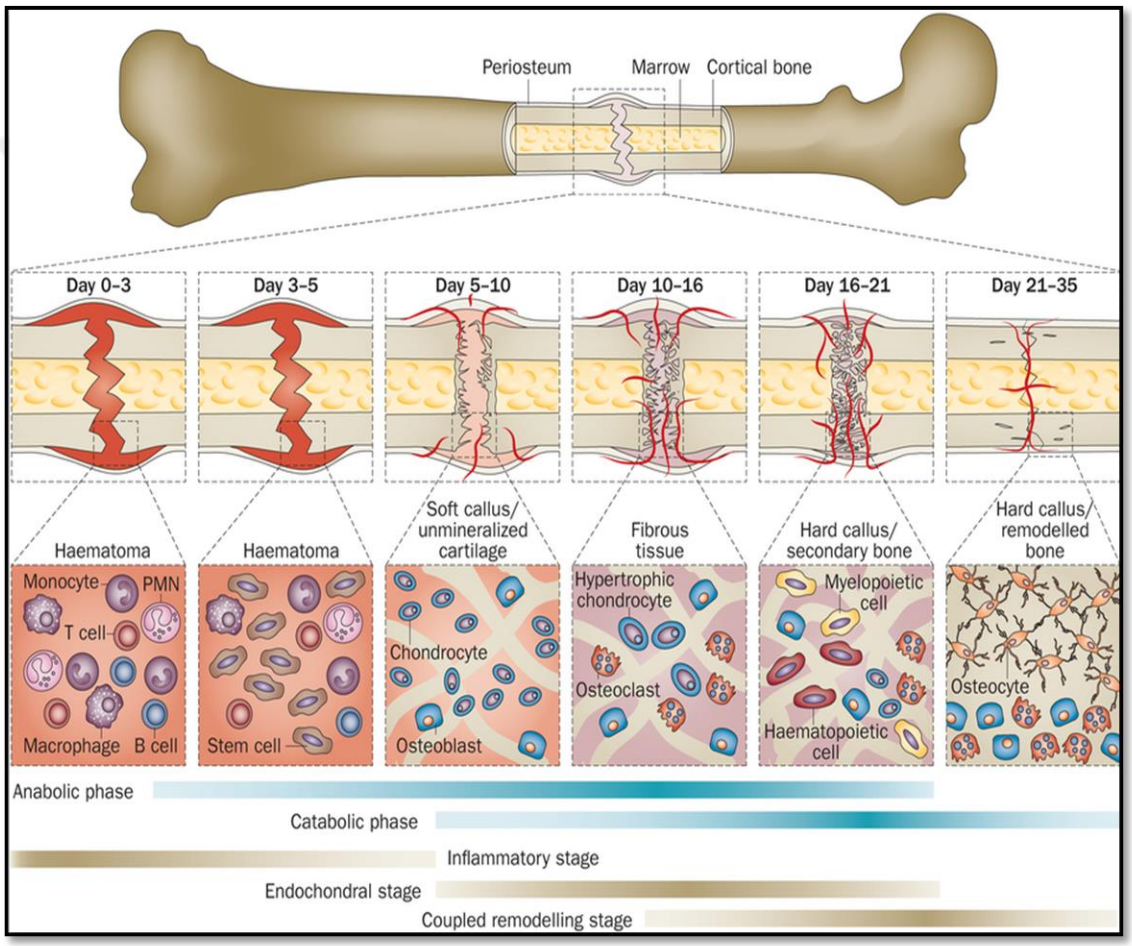

Figure 1.9. Progression of bone healing. After the injury, inflammatory response immediately leads to secretion of cytokines and GFs that begin the process of fracture repair. MSCs proliferation and differentiation towards osteogenic lineage enhance the production of existing capillaries' from pre-existing blood vessels between days 1-7. New bone formation takes place through endochondral and intramembranous ossification which is ultimately mineralized. Mature bone is formed which is constantly remodeled rest of life [270].

Table 1.3. Phases of bone repair and functions of signaling molecules during fracture healing [259].

Phases of Fracture Healing	Molecular Processes of Bone Repair	Functions of Signaling Molecules
Inflammatory Stage	Hematoma	Pro-inflammatory cytokines play critical roles in starting the repair cascade.
	Inflammation	TGF-β and BMP-2 expressions increase to begin callus formation.
	Gathering of mesenchymal stem cells	GDF-8 is responsible for controlling cellular proliferation.
Cartilage Formation	Chondrogenesis and endochondral bone formation initiates	Expressions of TGF-β2, -β3 reach at high levels because of their involvement in chondrogenesis and endochondral ossification.
	Cell proliferation in intramembranous bone formation	BMP-5 and -6 increase
	Vascularization	Angiopoietins and VEGFs are stimulated to activate vascularization from cappillaries in the periosteum.
	Neo-angiogenesis	
Cartilage Resorption and Primary Bone Formation	Stage of osteogenesis	TNF-α increases in related to cartilage resorption. This enhances the gathering of MSCs and stimulates apoptosis of hypertrophic chondrocytes.
	Recruitment of osteoblasts and woven bone formation	RANKL and MCSF increase.
	Chondrocyte apoptosis and matrix proteolysis	
	Osteoclast gathering and cartilage resorption	BMP-3, -4, -7, and -8 increase differentiation of MSCs towards osteogenic lineage.
	Neo-vascularization	BMP-5 and -6 reach high levels during this phase. They are responsible for regulation both endochondral and intramembranous bone formation.
Secondary Bone Formation and Remodeling	Bone remodeling	IL-1 and IL-6 increase again. However, RANKL and MCSF show decreased levels.

Although several data has indicated that MSCs are originated from bone marrow, systemic circulation of MSCs into the peripheral blood can be considerably important in bone healing [264, 253]. However, which molecular pathways regulate recruitment of MSCs from peripheral blood is still not known. It has been proposed that BMP-2 has a critical role in the recruitment of MSCs [265]. Other studies have proposed that BMP-7 might play more significant role than BMP-2 in the MSCs gathering around the fracture sites [266]. BMP-2 is actually necessary for post-natal osteogenesis in bone healing but not for the gathering of MSCs.

Hard callus formation: When the fracture sites are fused to soft callus, hard callus formation continuous until the fragments are tightly linked together by new bone. Both hard and soft callus formation occur at the same time near the fracture site. Soft callus is transformed into rigid mineralized tissue, named woven bone [253]. When chondrocytes turn into hypertrophic cells, extracellular matrix starts to be calcified and soft callus becomes more rigid. Ingrowth of capillaries and enhancement of vascularization occur. Vascularization is primarily regulated by VEGF-dependent pathway and angiopoietin-dependent pathway [267]. Angiopoietins, mainly angiopoetin-1 and-2 which are vascular morphogenetic proteins are stimulated for enhancement of vascularization from existent capillaries in the periosteum. On the other hand, VEGF- dependent pathway plays a major role in the arrangement of vascular regeneration rather than vascular in-growth [268]. VEGF is expressed by hypertrophic chondrocytes. It increases invasion of blood vessels. It is responsible for converting the cartilaginous extracellular matrix into a vascularized bone tissue [269, 270]. Vascularization at the fracture site is followed by migration of osteoblasts and osteoclasts [257]. Well-organized actions of M-CSF, RANKL, OPG and TNF-α begin to resorb calcified cartilage [271]. It is thought that these molecules also regulate osteoblast and osteoclast formation. Furthermore, TNF-α induces recruitment of the MSCs. It might also start chondrocyte apoptosis [258]. Several studies showed that hard callus formation is generally completed at day 14 in animal models according to the results of histomorphometry of calcified tissue and measurement of levels of ALP, OCN, ON and Col1A1 [166].

Remodeling: Although hard callus formation provides a rigid structure to the fracture site, it does not completely reconstruct the biomechanical features of normal bone. A second resorptive stage of fracture healing occurs in order to provide more strong bone structure

than hard callus. For this reason, woven callus initiates to remodel slowly for turning into a lamellar bone [253, 258]. Resorption of excess callus proceeds and it can take years to be completed [272, 273]. Bone remodeling is regulated by cytokines, including TNF-α and IL-1. High levels TNF-α and IL-1 expressions occur during bone remodeling. Furthermore, BMPs particularly BMP-2, are also expressed with high levels [274, 253]. Bone remodeling is accomplished by a balance of resorption of hard callus and formation of lamellar bone. The success of bone remodeling is associated with the progressive modification of fracture sites under the effect of optimal mechanical loads until bone cortex becomes similar to the original bone [254, 260].

1.6. MOLECULAR MECHANISM OF INCORPORATION OF BONE GRAFTS-OSSEO-INTEGRATION

Fracture healing around the grafts includes biochemical and cellular events that occur at the host-graft site interface until the graft surface eventually covered with a recently formed bone [275]. Although the biological mechanism of bone graft incorporation is fully not understood, that process is similar to the bone fracture healing mechanism. Stages of incorporation primarily initiate with the formation of hematoma and specific immune response, continues with vascular in-growth, focal osteoclastic resorption of the graft and it lasts with intramembranous/endochondral ossification on graft surface [276-279].

Autograft incorporation: After transplantation of autogenous cancellous bone graft, hemorrhage and inflammation rapidly occur. The graft is encircled by blood cells including lymphocytes, monocytes, leukocytes and macrophages. They are invaded from the recipient site into the graft site. The blood cells are activated and secrete pro-inflammatory cytokines, GFs and other soluble molecules [280]. Meanwhile, as macrophages are infiltrated, they remove necrotic tissue within the Haversian canals of the graft resulting in secretion of various cytokines and GFs which act as a chemo-attractant for MSCs. Neovascularization takes place within the graft at first 2 days, after graft transplantation. MSCs begin to differentiate under osteoinductive effects.

This stage of bone repair with cellular regeneration is defined as a primary stage of bone regeneration in which inflammation, revascularization, and osteoinduction continuously take

place with bone formation and resorption [183]. The secondary stage of bone regeneration is initiated by osteoclasts which reach at the graft site through the newly formed vessels. Osteoclasts remove the necrotic tissue, whereas osteoblasts initiate to lay down osteoid throughout the dead trabecula of the graft [182]. New bone formation is developed into mature Haversian canals and lamellar bone which tolerates normal shear and compressive forces. At the same time, autograft is gradually reabsorbed and gradually replaced by new host cells. MSCs start to form new bone marrow inside the transplanted graft [281, 282].

Bone remodeling can take several months to complete progress. Cortical autograft incorporation is similar to the cancellous autograft. However, the most apparent differences between cancellous and cortical autografts are in the rate of revascularization and amount of new bone formation. Cancellous autograft is re-vascularized more quickly and more completely than cortical autograft [283].

Allograft incorporation: Cancellous allografts show poor properties in bone healing as compared to cancellous autografts. After transplantation of cancellous allograft in the first 2 weeks, it invokes a comprehensive host response. The inflammatory response against the graft-derived cellular antigens leads to inhibition of GFs-mediated bone formation which is essential for host-graft incorporation. Furthermore, neovascularization is also delayed. New capillaries surrounded by inflammatory cells cause an occlusion and formation of necrotic tissue. After 8 weeks, the fibrous tissue starts to encapsulate the allograft. This stage takes 8 months or longer depending on an inequality in histocompatibility between the graft and the recipient site [284].

Cancellous allografts are incorporated much faster than cortical allografts. They serve as a scaffold onto which host bone is laid. They are completely not reabsorbed and so that they can stay entrapped inside the host bone [282]. Inflammatory response of cortical allografts is similar to that of cortical autografts. Nevertheless, an aggressive inflammatory response leads to occlusion of invading host capillaries and causes graft necrosis.

The new appositional bone formation occurs during the integration of cortical allograft. As a consequence of the lack of vascularization, the cortical allograft is less stable as compared to cortical autograft. Its incorporation can take up to 1 year, after its transplantation [285]. The duration of each stage during host-graft integration can change depending on the types

of graft. Incorporation of the cancellous autograft is the fastest and a complete one. It is followed by cortical autograft, cancellous and cortical allografts, respectively [183, 207].

1.7. BIOLOGICAL FACTORS IN FRACTURE HEALING

There are various clinical circumstances which require promotion of bone regeneration either systemically or locally. A comprehensive understanding of the molecular mechanism during bone repair is fundamental for patient benefit. Further understanding of the role of cytokines and GFs is expected to facilitate the improvement of new anabolic and catabolic therapies for bone healing [252].

1.7.1. The Role of Proinflammatory Cytokines in Fracture Healing

Immune systems are necessary during anabolic and catabolic stages of bone repair. In the primary inflammatory phase of injury, immune functions clear away necrotic tissue, enhance angiogenesis and start repair [286, 287]. During inflammatory stage, pro-inflammatory cytokines are released by macrophages and other inflammatory cells including PMNs and cells of mesenchymal origin that are found in the periosteum [288]. Some of the pro-inflammatory cytokines are produced at a mid-stage in repair. Thereby, osteoclastogenic activity is induced to remove calcified cartilage and other cytokines are stimulated at a later phase during bone regeneration [259].

IL-1, IL-6 and TNF-α play significant regulatory roles in initiating fracture repair cascade [289, 258]. These pro-inflammatory cytokines have main functions during stimulation of downstream responses by acting as chemotactic stimulators on other inflammatory cells and gathering of MSCs resulting in enhancement of matrix synthesis, induction of angiogenesis and accumulation of endogenous fibrogenic cells to the site of the injury [289, 267]. Even though IL-1 is expressed at a low level throughout fracture healing, it shows high activities in the early inflammatory phase [288]. Besides, it induces the release of IL-6 and M-CSF. It is thought that early expression of IL-1 may demonstrate a triggering mechanism which starts a cascade of events that regulate bone remodeling in fracture healing [290].

Expression of IL-1 also regulates angiogenesis. Furthermore, IL-1 might induce the activity of neutral proteases to degrade cartilaginous callus tissue [291, 254]. Various functions of IL-1 during bone repair may be associated with different expression levels of its receptors, IL-1RI and IL-1RII. In a mice model of bone repair, the expression of IL-1RII shows the same pattern as IL-1. On the other hand, IL-1RI displays very low level during the early inflammatory stage [289]. Moreover, several studies have suggested that IL-1RI$^{-/-}$ mice have reduced bone mass density and increased osteoclastic activity. Altogether, the significance of IL-1 molecular signal in bone hemostasis is emphasized [292, 293].

IL-6 is secreted by osteoblasts in response to secretion of IL-1 during fracture healing [294, 295]. IL-6 is only secreted during the initial phase of inflammation. It increases angiogenesis in regulating the release of VEGF. It also regulates differentiation of osteoblasts and osteoclasts resulting in enhancement of the production of cartilaginous callus at the injured site. The expression of IL-6 remains basic levels in the remodeling phase. Two weeks after injury, IL-6 knockout mice had decreased extracellular matrix mineralization and enhanced cartilage tissue in the fracture site. However, 4 weeks after fracture, bone repair is more comparable to that of IL-6 knockout mice [296]. In human patients, IL-6 stays high levels for a few months, after the fracture occurs. High level of its expression is connected with the reduced load-bearing capacity at the fracture site [297, 293].

TNF-α enhances recruitment of MSCs and stimulates apoptosis of hypertrophic chondrocytes. It also regulates the osteoclastic activity of osteoclasts. In the absence of TNF-α, resorption of calcified cartilage is delayed. Thus, its absence causes prevention of new bone formation. TNF-α has been displayed in the induction of differentiation of MSCs into osteoblasts [298, 258, 267].

TNF-α receptors, TNFRI and TNFRII that are secreted by both osteoblasts and osteoclasts, follow a biphasic pattern. TNFRI is always presented in bone, while TNFRII is only secreted following a bone fracture. So, TNFRII has more specific role than that of TNFRI during fracture healing [293]. It was showed that TNF-α signaling increased bone formation both in normal and TNFRI$^{-/-}$ mice. Nevertheless, TNFRII-deficient mice showed TNF-α-signaling induced osteoclasts differentiation and bone resorption [299].

The expression level of TNF-α peaks 24h after injury in mice models and returns to its normal level in 72h [258]. During this period, it is believed that TNF-α signaling induces the

secrete of secondary signaling molecules and shows a chemotactic stimulator, gathering cells which are essential for bone remodeling. Besides, the expression level of TNF-α increases again 2 weeks later, during endochondral ossification [293].

OPG and RANKL are the two members of the TNF-α superfamily and play key regulatory roles in osteoclastogenesis in control of bone mass. The levels of their expression increase after the initial injury and in the course of the period of cartilage resorption. However, the expression levels of RANKL, OPG and M-CSF begin to reduce during the stage of secondary bone formation and bone remodeling. On the other hand, IL-1 and IL-6 expressions rise during late stage of bone remodeling [258, 259].

1.7.2. The role of GFs in Fracture Healing

The process of fracture healing can be divided into distinct stages and a large number of cytokines, GFs, receptors, and intermediate signaling molecules have major roles during each stage of bone repair [254]. GFs are the most important molecules during fracture healing (Table 1.4).

TGF-β superfamily includes numerous growth and differentiation factors such as BMPs, TGF-β, growth differentiation factors (GDFs), activins, inhibins and Mullerian inhibiting factors. Particular members of that family such as BMPs and TGF-β enhance both endochondral and intramembranous ossification during fracture repair [300, 259]. TGF-β is secreted by platelets, inflammatory cells including monocytes, macrophages, as well as by osteoblasts, osteoclasts and chondrocytes at the later stages [257]. It is weakly present in hematoma during the early phase of the inflammatory response. However, it is highly expressed during intramembranous ossification and endochondral ossification [301].

TGF-β has several roles including MSCs proliferation and differentiation [302]. It regulates mineralization of cartilage matrix and stimulates osteoblastic activity. It also increases the production of extracellular matrix proteins including collagen, ALP, ON, OP and proteoglycans [303]. However, TGF-β is thought to have significant roles during chondrogenesis. Moreover, it might also begin a cascade for BMPs synthesis. It may inhibit the formation of osteoclasts and enhance apoptosis of osteoclasts. Even though, previous

studies have suggested that TGF-β induces cellular proliferation, osteoinductive potential of TGF-β seems to be limited in terms of unexpected side effects [304-306].

BMPs are secreted by MSCs, osteoprogenitor cells, osteoblasts and chondrocytes. They stimulate a consecutive cascade of events for chondro-osteogenesis. They have major roles such as chemotaxis, differentiation of MSCs, the proliferation of osteoprogenitor cells, angiogenesis and regulation of synthesis matrix proteins. Recent studies have showed that particularly, BMP-2, -4 and -7 play a key role during fracture healing. In studies of mice fracture healing, the expression of BMP-2 displays a maximum level in 24h of fracture. Thus, it was proposed that BMP-2 has a key role especially in the beginning of the repair cascade [307-309, 265]. In a rat model, MSCs which have migrated into the injury site exhibited an increased level of BMP-2 and -4 expression [254, 304]. Further studies have proposed that BMP-2 is necessary for the late phase of bone repair and genetically associated with the protection of normal bone mass [310, 265].

Table 1.4. Effects of growth factors during fracture healing.

GFs	Sources	Functions
TGF-β	Bone matrix, platelets and chondrocytes	Induces undifferentiated MSCs into osteoblast lineage; mitogenesis to regulate collagen and proteoglycan synthesis
FGF	Osteoblasts, chondrocytes Macrophages, and MSCs	Enhances osteoblasts and chondrocytes proliferation and promotes collagen formation
PDGF	Endothelial cells, platelets, osteoblasts	Stimulates collagen secretion; mitogenic activity for MSCs and osteoblasts
IGFs	Osteoblasts and Chondrocytes	Increases bone remodeling and regulates osteoblast proliferation and chondrocyte formation
VEGF	Vascular endothelial cells, osteoblasts	Enhances angiogenesis mitogenesis for endothelial cells
BMPs (-2, -4, -7)	MSCs, osteoprogenitor cells, osteoblasts	Enhances induction of osteo-progenitor cells to turn into osteoblasts

PDGF is secreted by monocytes, platelets, macrophages and osteoblasts. It acts as a potent mitogen activator for cells of mesenchymal origin. It is synthesized by platelets after immediate injury and serves as a chemotactic stimulator for other inflammatory cells including macrophages and monocytes [259]. PDGF possesses several roles including stimulation of MSCs proliferation and initiation of callus formation. It was shown that PDGF treatment in rabbits promoted bone formation related with healing in tibial osteotomies [311].

FGF is also synthesized by inflammatory cells, osteoblasts, MSCs, and chondrocytes. It induces cell proliferation and differentiation of various cell types including osteoblasts, chondrocytes, fibroblasts and myocytes. FGF has a critical role in angiogenesis resulting in blood vessel formation [312]. There are two forms of FGFs, named α-FGF and β-FGF. α-FGF regulates chondrocyte proliferation and it is necessary for maturation of those cells. However, β-FGF is produced by only osteoblasts during endochondral ossification and responsible for bone formation [267, 306].

IGFs are defined as somatomedins (IGF-I) and skeletal growth factor (IGF-II). Their sources are an extracellular bone matrix, osteoblasts, chondrocytes and MSCs. IGF-I increases extracellular matrix formation in regulating secretion of type I collagen and non-collagenous proteins [267]. On the other hand, IGF-II is a key regulator at a later stage of endochondral ossification and it induces type I collagen synthesis and cartilage matrix production [149, 307]. In addition to osteoblastic activity, it can also modulate osteoclastic activity during fracture repair [301]. VEGF is considered as a key factor in the first stage of fracture healing and bone regeneration. It is a primary regulator of angiogenesis and vascular endothelial proliferation [7]. It also plays a main role in neoangiogenesis and endochondral ossification [313]. Besides, fracture healing is enhanced by exogenous VEGF [33]. Various studies have displayed that BMPs induce the expression of VEGF and their receptors proposing a cross-talk between those two families, which increase new bone formation [314].

1.8. CLINICAL PROBLEMS IN FRACTURE HEALING

Orthopedic injuries have been very common worldwide. Although bone tissue can regenerate spontaneously, it can fail resulting in delayed union or non-union during bone restoration. Fracture healing is normally cured by operations including osteotomies, arthrodesis and bone grafting for uniting fractures. However, the amount of osteoclasts and osteoblasts which make intracellular components of bone remodeling can't be enough to heal the bone tissue during fracture healing. Errors of mechanisms of local and systemic mediators' lead to abnormal bone healing resulting in numerous clinical problems [315].

Current options for the treatment of fracture healing start to turn into biological approaches. Several clinical approaches such as cell-based therapies, recombinant GFs and anabolic agents have been under investigation for bone repair. Even though strategies for using these components have been evolved, the transition from biological process to clinical practice has several obstacles that range from gaps in scientific knowledge to insufficient animal models [316, 317]. For that reason, viable regeneration of bone at the molecular level is the principal component of success in the point of view in clinical trials.

Accomplished fracture healing needs biologically effective micro-environment. Studies are required to optimize these therapies for the improvement of biologic potential at the fracture site in the treatment of delayed unions and non-unions [318].

1.9. AIM OF THE STUDY

Given the proposed roles of numerous local molecular mediators during fracture healing, this study aims to investigate the potential influence of human BCPs onto hAD-MSCs. In the previous studies, the effects of the BCPs on hAD-MSCs in bone repair has not been discussed. Consequently, this study seeks to understand how differentiated mesenchymal stem cells towards osteogenic lineages behave at the fracture site by focusing on the interplay between bone chip secretions and the cells.

We hypothesize that the cells within bone chips secrete soluble molecules and proteins which repress the process of osteogenesis. The second aim of this study is to understand the effects

of BCPs on mesenchymal stem cells growth in basal media. Finally, due to the influences of BCPs in bone repair, the production of pro-inflammatory cytokines are aimed to be investigated.

2. MATERIALS

2.1. CHEMICALS

- Dulbecco's Phosphate Buffer Saline (DPBS) (Gibco, Germany)
- Ham's F12 Dulbecco's Modified Eagle Medium (1.0 g/l) (Gibco, Germany)
- High glucose Dulbecco's Modified Eagle Medium (Gibco, Germany)
- Low Glucose Dulbecco's Modified Eagle Medium (1.0 g/l) (Gibco, Germany)
- Primocin (100 µg/ml) (InvivoGen, USA)
- Fetal Bovine Serum (FBS) (Gibco, Germany)
- Trypsin-EDTA (Sigma-Aldrich Corporation, Germany)
- Basic fibroblast growth factor (bFGF) (Sigma-Aldrich Corporation, Germany)
- Recombinant Human Transforming Growth Factor (rhTGFβ) (R&D Systems, USA)
- Collagenase (Sigma-Aldrich Corporation, Germany)

2.2. CD MARKER ANTIBODIES

- CD90-PE monoclonal antibody (mAb), (BD Pharmingen, USA)
- CD44-FITC mAb, (Invitrogen, USA)
- CD73-PE (BD Pharmingen, USA)
- CD81-PE (BD Pharmingen, USA)
- HLA-ABC-PECy.5 (BD Pharmingen, USA)
- CD117-PE mAb (BD Pharmingen, USA)
- CD34-PECy.5, mAb (BD Pharmingen, USA)
- CD45-PECy5, mAb (BD Pharmingen, USA)
- CD14-PECy.5, (BD Pharmingen, USA)
- HLA-DR-PECy.5 (BD Pharmingen, USA)
- IgG_1 (BD Pharmingen, USA)
- IgG_{2a} (BD Pharmingen, USA)

2.3. KITS

- CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS, Promega, USA)
- Alkaline Phosphatase (ALP) Assay kit (Randox, UK)
- QuantiChrom™ Calcium Assay kit (Bioassays Systems, USA)
- BCA protein assay kit (Intron Biotechnology, South Korea).
- RNA extraction kit (GeneJET RNA purification kit, Thermo Scientific, USA)
- iScript cDNA synthesis (Bio-Rad, USA)
- Maxima SYBR Green/ROX qPCR master mix (Thermo Scientific, USA)
- IL-6, IL-1β and TNF-α AssayMax™ human Elisa kits (AssayPro, USA)

2.4. OTHER REAGENTS

- Ammonium Chloride (NH_4Cl) (Sigma-Aldrich Corporation, Germany)
- Alizarin Red S (ScienceCell, USA)
- Dexamethasone (Sigma-Aldrich Corporation, Germany)
- β-glycerol phosphate (Sigma-Aldrich Corporation, Germany)
- l-ascorbic acid 2-phosphate (Gibco, Germany)
- Paraformaldehyde (Sigma-Aldrich Corporation, Germany)
- Isobutylmethylxanthine (Sigma-Aldrich Corporation, Germany)
- Indomethacin (Sigma-Aldrich Corporation, Germany)
- Oil Red O (ScienceCell, USA)
- l-proline (Sigma-Aldrich Corporation, Germany)
- Sodium pyruvate (Sigma-Aldrich Corporation, Germany)
- ITS (Culture Supplement that consists of 6.25 µg/mL insulin, 6.25 µg/mL transferrin, 6.25 ng/mL selenous acid, 1.25 mg/mL bovine serum albumin, 5.35 mg/mL linoleic acid (B&D Biosciences, USA)
- Alcian blue (American MasterTech, USA)
- von Kossa staining (Sigma-Aldrich Corporation, Germany)
- Silver nitrate solution (500 µl) (Sigma-Aldrich Corporation, Germany)

- Sodium thiosulfate (Sigma-Aldrich Corporation, Germany)
- Hydrochloric acid (HCl) (Sigma-Aldrich Corporation, Germany)
- Sodium hydroxide (NaOH) (Sigma-Aldrich Corporation, Germany)
- TritonX-100 (Thermo Scientific, USA)
- RIPA Lysis Buffer (Santa Cruz, USA)
- Protease inhibitor (Sigma-Aldrich Corporation, Germany)
- Tris base (Merck, Germany)
- SDS (Sigma-Aldrich Corporation, Germany)

2.5. INSTRUMENTS

- Inverted microscopy (Nikon Eclipse TC 100, USA)
- Laminar flow cabinet (ESCO Labculture Class II Biohazard Safety Cabinet 2A, Singapore)
- CO_2 incubator (Thermo Scientific, USA)
- Centrifuge (Hettich micro 22R and Sigma 2-5 centrifuge, Germany)
- Vortex (Stuart, UK)
- pH meter (Hanna, Germany)
- ELISA plate reader (Bio-Tek EL x 800, USA)
- FACS Calibur (BD Biosciences, USA)
- Nanodrop Spectrophotometer (Thermo Scientific, USA)
- CFX96 Touch™ sequence detection system (Bio-Rad, USA)

3. METHODS

3.1. ISOLATION AND CULTURE OF hAD-MSCs

Human adipose tissue was obtained from patients who had liposuction procedure. Lipo-aspirate sample (approximately 200 ml) was transferred to the Yeditepe University Tissue Engineering Laboratory under aseptic conditions (Figure 3.1). Sterile physiological saline solution was added to sample and processed according to established procedure to get a stromal vascular fraction [319]. This method defines the preparation of hAD-MSCs from human lipoaspirate.

Figure 3.1. Liposuction aspirate sample. Human Adipose tissue was taken from patients under liposuction surgery. An equal volume of sterile physiological saline solution was added to lipoaspirate sample.

Briefly, the sample was digested at 37 °C for 1h with 0.075 % collagenase in phosphate buffer saline with gentle agitation at 60 cycles/min. The enzyme was then inactivated with an equal volume of Ham's F12 Dulbecco's Modified Eagle Medium (1.0 g/l) including 10 % fetal bovine serum (FBS). After centrifugation, the stromal vascular fraction was collected and resuspended in erythrocyte lysis buffer (160 mM NH_4Cl) to remove red blood cells.

After centrifugation, stromal vascular fraction pellet containing hAD-MSCs were cultured in complete medium containing Ham's F12 Dulbecco's Modified Eagle Medium (1.0 g/l) supplemented with 10% (v/v) FBS, primocin (100 µg/ml) and basic fibroblast growth factor (1 ng/ml) in an incubator at 37°C in 5% CO_2 and 90% humidity. Following initial culture period, the non-adherent cells were thrown away by aspirating the medium. The medium was refreshed every other day. The cells were cultured and expanded at 90 % confluency through 2 to 4 passages before *in vitro* osteogenesis assay.

3.2. CHARACTERIZATION OF hAD-MSCs

3.2.1. Flow cytometry analysis

hAD-MSCs were characterized according to the definition of the International Society for Cellular Therapy (ISCT). After second passage, phenotypic characteristics of hAD-MSCs ($3x10^5$ cells) were confirmed with the monoclonal antibodies (mAb) for CD90-PE, CD44-FITC, CD73-PE, CD81-PE, HLA-ABC-PECy.5, as a positive surface markers, CD117-PE, CD34-PECy.5, CD45-PECy5, CD14-PECy.5, HLA-DR-PECy.5, as a negative markers. Flow cytometry was used on a FACSCalibur. The data was analyzed by Cell Quest Software. Appropriate isotype controls of conjugated antibodies IgG_1 and IgG_{2a} were used to eliminate non-specific characteristics of the antibodies.

3.2.2. *In vitro* multilineage differentiation of hAD-MSCs

Differentiation potential of hAD-MSCs was assessed by adipogenesis, osteogenesis and chondrogenesis protocols as described previously [320].

3.2.2.1. Osteogenic differentiation of hAD-MSCs

Alizarin Red S staining was performed after osteogenic induction of the cells to determine the osteogenic differentiation potential of hAD-MSCs. Alizarin Red S was used to stain free calcium compounds in cell culture. For osteogenesis studies, hAD-MSCs were cultured into osteogenic induction medium containing 20 mM β-glycerol phosphate; 10 nM

dexamethasone; 50 µM L-ascorbic acid 2-phosphate for 10 and 21 days. The media were changed every other days. Extracellular calcium minerals were confirmed by staining with Alizarin Red S solution. After 10 and 21 days of the osteogenic induction, the cells were fixed with 5 % paraformaldehyde and then incubated for 10 min with 0.5 % Alizarin Red S. The cells were washed 5 times with dH_2O and then the stained cells were photographed under an inverted microscope.

3.2.2.2. Adipogenic differentiation of hAD-MSCs

For adipogenic induction, hAD-MSCs were seeded in 24-well plates at a density of 5,000 cells/well. After the cells reach to confluency, they were treated with adipogenic induction medium including 0.5 µM dexamethasone, 0.5 µM iso-butyl-methyl-xanthine and 50 µM Indomethacin. The formation of adipocytes was proved by staining with Oil Red O method after 10 and 21 days of incubation with adipogenic induction medium. Oil Red O stains intracellular triglyceride droplets. Briefly, after the adipogenic differentiation period, 5 % paraformaldehyde was used to fix the cells. After fixation, the cells were incubated for 20 min with Oil Red O solution and then washed 5 times with dH_2O. Dye was removed from the cells using isopropanol. The appearance of lipid droplets was observed under an inverted microscope.

3.2.2.3. Chondrogenic differentiation of hAD-MSCs

hAD-MSCs were induced to differentiate into chondrocytes under specific culture conditions. Chondrogenic media contained high-glucose DMEM supplemented with: rhTGFβ (10 ng/mL), 50 µg/mL l-ascorbic-2-phosphate, 40 µg/mL l-proline, 100 µg/mL sodium pyruvate, 5 mL ITS (6.25 µg/mL insulin, 6.25 µg/mL transferrin, 6.25 ng/mL selenous acid, 1.25 mg/mL bovine serum albumin, 5.35 mg/mL linoleic acid). The media were refreshed every 3 days for up to 3 weeks. After chondrogenic differentiation process, Alcian blue was used to stain sulfated proteoglycan deposits which are the indications of chondrocytes. Briefly, the pellets were fixed with 5 % paraformaldehyde and stained with 1 % Alcian blue reagent (in 0,1N HCl, pH 1,0) for 30 min. Pellets were washed with 0,1 N

HCl to elute excess stain. Proteoglycan deposits were visualized under an inverted microscope.

3.3. ESTABLISHMENT OF CANCELLOUS BONE CHIP SECRETIONS (BCPs)

Cancellous bone chips were harvested from lateral portions of anterior and posterior chamfer cuts of total knee arthroplasties under aseptic conditions, approved by Yeditepe University Hospital Local Ethics Committee (Figure 3.2). Patients with secondary gonarthrosis, including rheumatoid diseases, avascular necrosis, and post-traumatic arthritis were excluded from the harvesting procedure.

An equal amount of basal medium, including Low Glucose Dulbecco's Modified Eagle Medium (1.0 g/l) supplemented with 10% (v/v) FBS, primocin (100 µg/ml) were added onto cancellous bone chips. After 72h incubation at 37°C, in 5% CO_2 and 90% humidity, these molecules released into the basal medium (BM) which was then filtered through the 70 µm cell strainer. For the preparation of osteogenic media, 10 nM dexamethasone, 50 µM L-ascorbic acid 2-phosphate and 20 mM β-glycerol phosphate were added into BM. Basal and osteogenic media with bone chip secretion (BCPs-BM and BCPs-OM) were used for culturing hAD-MSCs in *in vitro* osteogenic assay.

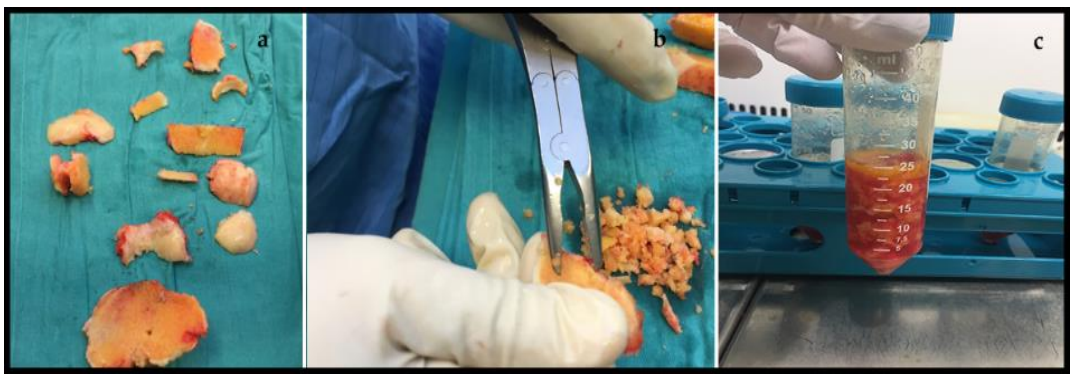

Figure 3.2. a) Lateral portions of anterior and posterior chamfer cuts of total knee arthroplasty, b) cancellous bone chips, c) addition of the basal medium onto cancellous bone chips.

3.3.1. Co-culture of hAD-MSCs with BCPs

hAD-MSCs and BCPs were co-cultured in a permeable transwell system to establish *in vitro* model for understanding the role of soluble molecules that were secreted by bone chips into both basal medium and osteogenic medium (Figure 3.3). Co-culture experiment was carried out by using Transwell® cell culture inserts having 0.4 μm pores which allowed the BCPs transportation across the membrane. By means of this membrane, BCPs were physically separated from hAD-MSCs to prevent direct cell to cell contact. Osteogenic studies were conducted by using third passage cells plated at a density of $2x10^4$ cells/well in 12-well plates cultured in either osteogenic medium (OM) or basal medium (BM). hAD-MSCs were seeded on the lower side of the insert, in the meantime, BCPs in either osteogenic medium (BCPs-OM) 1:1 (v/v) or basal medium (BCPs-BM) 1:1 (v/v) were added in an upper side of the insert. For their negative controls, hAD-MSCs were cultured in osteogenic medium (OM) and basal medium (BM). hAD-MSCs cultured in either OM or BM with/without BCPs were harvested and the medium in the bottom chamber was collected at 1, 3, 5, 7, 14 and 21 days for a subsequent analysis.

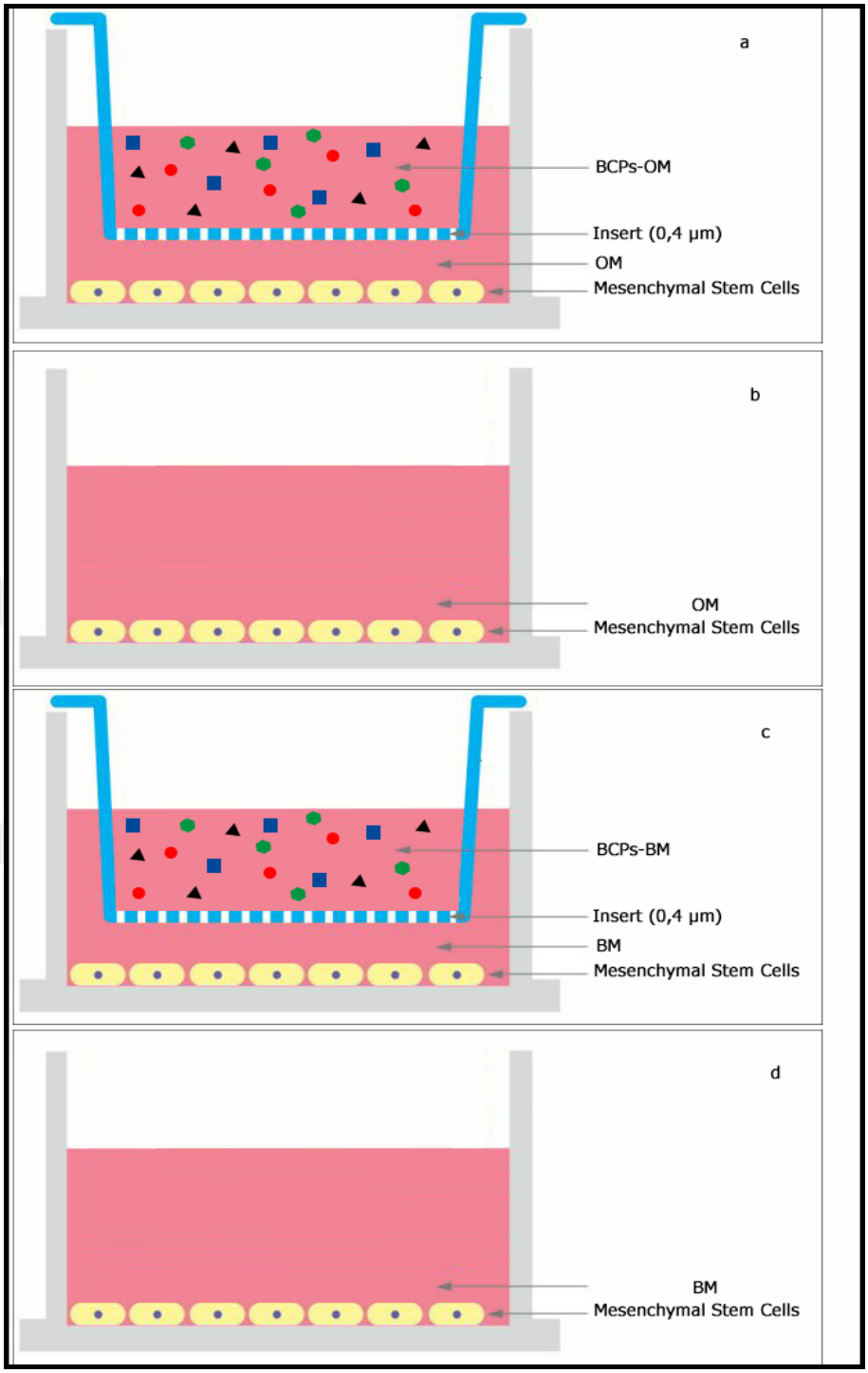

Figure 3.3. Experimental set-up of hAD-MSCs with/without harvested bone chips in transwell plate. a) hAD-MSCs with bone chips in osteogenic medium (BCPs-OM), b) hAD-MSCs without bone chips in osteogenic medium (Only OM), c) hAD-MSCs with bone chips in basal medium (BCPs-BM), d) hAD-MSCs without bone chips in basal medium (Only BM).

3.4. DETECTION OF CELL VIABILITY BY MTS ASSAY

Viability of cells for each experimental group was determined by a chromogenic assay which requires the biochemical reduction of the tetrazolium compound (MTS) by viable cells. When phenazine methosulfate (PMS) is presented, MTS creates a water-soluble formazan salts with an absorbance at 490 nm.

Cell viability of each group was detected using CellTiter 96® AQueous One Solution Cell Proliferation Assay for each time point of examination throughout 21 days of incubation. In the growth curve experiment, 500 µL MTS working solution (a mixture of MTS: LG-DMEM at a ratio of 1:5) was added to each well and the 24 well plate was incubated at 37°C, 5% CO_2 and 90% humidity for 3 hours. The absorbance of each sample was determined at 490 nm by using an Elisa Plate Reader and the viability of cells was calculated using the slope of the calibration curve plotted as absorbance versus cell number.

3.5. ALKALINE PHOSPHATASE ASSAY

Intracellular ALP activity was measured colorimetrically using ALP kit for each group after 1, 7, 14 and 21 days of incubation in culture. The cells were washed 3 times with phosphate buffer saline and lysed with Tris buffer (500 µl, 0.1 M with 0.1% TritonX-100, pH: 9). The samples were frozen at -20 ^{o}C for 10 min and thawed at 37^{o}C for another 10 min, repeating freeze-thaw step 3 times. Then, each sample was sonicated on ice with 30 sec. of sonication and 30 sec. of breaks. After centrifugation steps, ALP activity was determined using p-nitrophenol (6.2 mg/ml) as a substrate. Measurements were performed at 405 nm for the time points of 0, 2, 4, 6, 8, 10, 12 and 14 min by Elisa Plate Reader. A graph of absorbance versus time was plotted and the alkaline phosphatase activity per sample was detected. The total protein content was used to normalize the ALP values.

3.6. DETERMINATION OF MINERALIZATION

3.6.1. Quantitative calcium assay

Calcium ions were measured by QuantiChrom™ Calcium Assay kit for each group at day 7, 14, 21. Calcium deposits were solubilized incubating the cells overnight with 0.5 N HCL and quantified using the kit according to the manufacturer's instructions. The amount of calcium produced was evaluated by measuring the absorbance at 612 nm using microplate reader. Calcium concentration of the cells was normalized with total protein concentration and expressed as µg/ml protein. For this purpose, after decalcification process, the cells were solubilized with a mixture of NaOH 0.1 mol/L and 0.1 % of SDS. The protein content of each sample was detected with a BCA protein assay kit.

3.6.2. von Kossa staining

von Kossa staining protocol was carried out to detect the mineralization of each sample. Silver nitrate solution (500 µl) was added to each sample and then incubated at room temperature under ultraviolet light for 30 min. Silver ions reacted with phosphates in calcium deposits and then silver phosphate was degraded to silver under UV light. After each sample was washed with a physiological saline solution, 500 µl of 5 % sodium thiosulfate was added to stop the reaction, followed by counterstaining with nuclear fast red. Finally, images were obtained by inverted microscope.

3.7. DETECTION OF GENE EXPRESSION LEVELS

3.7.1. RNA isolation

Total RNA was purified using RNA extraction kit as recommended by the manufacturer after 7, 14 and 21 days of incubation period. Briefly, the cells were re-suspended in 600 µl lysis buffer and the cell lysate was transferred up to the GeneJET RNA purification column. The sample was centrifuged for 1 min at 12,000 x g. Each column was washed with kit's

wash buffers and centrifuged for 1 min at 12,000 x g. Nuclease-free water was added onto column membrane to collect total RNA. The RNA concentration was detected using Nanodrop Spectrophotometer. "Concentration of RNA sample (ng/µl) = dilution factor x 40 x A260" formula was used to calculate the RNA concentration.

3.7.2. Reverse transcriptase polymerase chain reaction

cDNA was synthesized using iScript cDNA synthesis. PCR reaction was performed for 20 min at 46 ºC according to manufacturer's protocol (Table 3.1). cDNA concentration was measured using Nanodrop Spectrophotometer.

Table 3.1. Real-time PCR reaction mix.

Components	**Volume per reaction, µl**
5x iScript Reaction Mix	4
iScript Reverse Transcriptase	1
RNA template (100 fg–1 µg total RNA)*	variable
Nuclease-free water	variable
Total Volume	**20**

3.7.3. Real-time PCR analysis

Real-time PCR analysis was performed using Maxima SYBR Green/ROX qPCR master mix. Reaction mixture includes 12,5 µl Maxima SYBR Green/ROX qPCR Master Mix (2X), 0,3 µM primers and 500 ng for each sample.

The real-time PCR assay was carried out using CFX96 Touch™ sequence detection system, according to the reaction conditions (Table 3.2). β-Actin was used as an internal control for normalization of each target gene expression.

Table 3.2. Real-time PCR conditions.

Step	Temperature, ºC	Time	Number of cycles
Initial denaturation	95	10 min	1
Denaturation	95	15 s	40
Annealing	60	30 s	
Extension	72	30 s	

Relative gene expression values were calculated by Pfaffl's method [321]. Primer pairs are listed in Table 3.3. Each sample was studied in triplicate.

Table 3.3. Primer pairs used for real-time PCR.

Primers		Sequence (5'→3')	Product Length
β-actin	F	GACAGGATGCAGAAGGAGATCACT	142
	R	TGATCCACATCTGCTGGAAGGT	
Sclerostin	F	AAATCACATCCGCCCCAACT	173
	R	GGCGGTGTCTCAAAAGGGAT	
Dickkopf-1	F	GTGCAAATCTGTCTCGCCTG	284
	R	GCACAACACAATCCTGAGGC	
Osteocalcin	F	CAGATTCCCCCTAGACCCGC	299
	R	GCCTGGGTCTCTTCACTACC	
Osteonectin	F	AACCCTCCCCTTCGTGTTTC	274
	R	TTTAAGGCAGAGCCCAGCAG	
Osteopontin	F	TCCTAGCCCCACAGACCCTT	259
	R	TCTACTGTGGGGACAACTGGA	
BMP-2	F	TGGCTGGGGACTTCTTGAAC	364
	R	CAGCAACGCTAGAAGACAGC	
Collagen Type I	F	ATGGGGAAGCTGGAAAACCT	286
	R	GCACCATCATTTCCACGAGC	
Osteoprotegerin	F	AAATGGCGACCAAGACACCT	290
	R	CACTGAAAGCCTCAAGTGCC	

3.8. ENZYME-LINKED IMMUNO-SORBENT (ELISA) ASSAY

AssayMax™ human Elisa kit was used to determine the concentrations of IL-6, IL-1β and TNF-α for each group of culture media that was collected at days 7, 14 and 21. The assay contains a quantitative sandwich enzyme immunoassay method which measures each cytokine levels. A murine monoclonal antibody, specific for each human cytokines, was pre-

coated onto a 96- well microplate. Each sample was sandwiched by the immobilized antibody and biotinylated polyclonal antibody that was recognized by a streptavidin-peroxidase conjugate. Briefly, 50 µl sample was added per well and incubated for 2h. After washing steps, 50 µl biotinylated human IL-6 antibody was added into each well and then incubated for 2h. Thereafter, 50 µl streptavidin-peroxidase conjugate was added into each well followed 30 min incubation. Chromogen substrate solution (50 µl) was added to develop color density. Stop solution (50 µl) was added to each well to observe the color change from blue to yellow.

The amount of IL-6 produced was evaluated by measuring the absorbance at 450 nm using microplate reader. The Same procedure was applied to detect the concentrations of IL-1β and TNF-α. Protein contents were used to normalize the amount of IL-6, IL-1β and TNF-α production. Total protein was measured by SMART™ micro BCA protein assay kit. Each sample concentration was performed by the log-log standard curve.

3.9. STATISTICAL ANALYSIS

Statistical analyses were carried out by IBM®-SPSS® software (Statistical Package for the Social Sciences, Version 23.0). Normality was identified by the Shapiro-Wilk and Kolmogorov-Smirnov tests. Paired sample t-test and two related samples followed by Wilcoxon test were applied by comparing any significant changes between groups.

Heterogeneity of group variances of different time points was employed using one-way ANOVA followed by Tukey post multi-comparison tests for parametric tests; while two independent samples followed by Mann-Whitney-U tests were performed for non-parametric tests. Statistically significant levels were provided $^{**}p < 0,01$and $^{*}p < 0,05$. Data were reproduced from at least two independent experiments in duplicated and displayed as the mean ± standard deviation (SD).

4. RESULTS

4.1. MORPHOLOGICAL STRUCTURE ANALYSIS

Morphological structures of hAD-MSCs grown in BCPs cultured with either OM or BM after 1, 7, 14, and 21 days of incubation were analyzed by inverted microscopy (Figure 4.1).

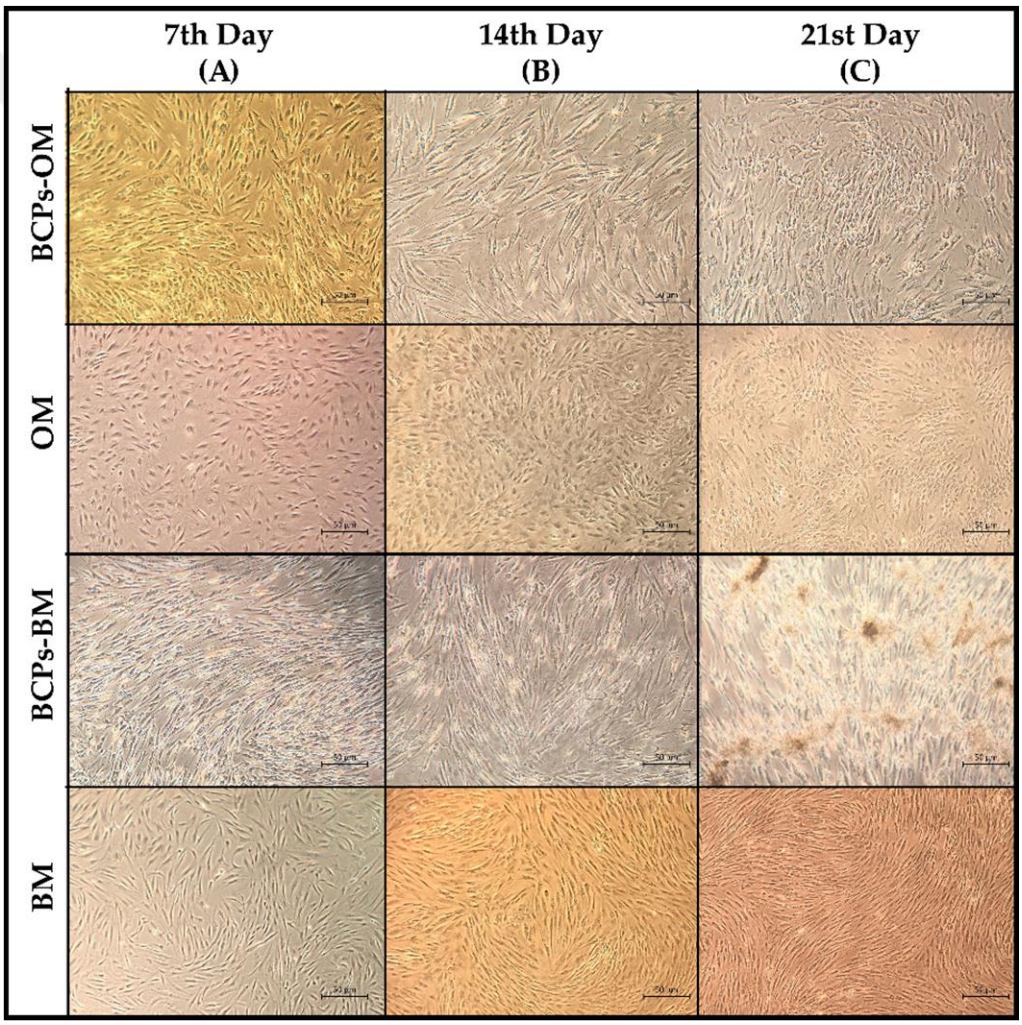

Figure 4.1.Morphological changes of hAD-MSCs cultured in either OM or BM in the presence and absence of BCPs at the end of (A) 7 (B) 14 and (C) 21 days. Magnification is 10X. Scale bars are 50 μM.

Undifferentiated hAD-MSCs in BM exhibited typical adherent spindles and flat polygonal morphologies. Fibroblast-like cells were observed throughout 21 days. According to the results, differentiated hAD-MSCs in OM showed cuboidal appearances at the end of day 7; while the cells became large, round and had multiple nuclei after 21 days of culture. Moreover, hAD-MSCs in OM with BCPs exhibited numerous cell types from day 7 to day 21. In the same way, morphologies of hAD-MSCs in BM with BCPs varied in ranging from flattened-shaped to cuboidal-shaped throughout 21 days of incubation period. Besides, dead cells were observed at day 21.

4.2. CHARACTERIZATION OF hAD-MSCs

4.2.1. Flow Cytometry Analysis

Phenotypic characteristics of hAD-MSCs were determined by flow cytometry. Cell Quest Software was used to analyze the data. $CD90^+$, $CD44^+$, $CD73^+$, $CD81^+$, $HLA\text{-}ABC^+$ were expressed highly and homogenously by hAD-MSCs; whereas $CD117^-$, $CD34^-$, $CD45^-$, $CD14^-$ and $HLA\text{-}DR^-$ displayed low level expressions (Figure 4.2). Isotype controls (IgG_1, IgG_{2a}) were used to determine the positiveness of the cells. Expressions of cell surface markers showed that isolated cells from human adipose tissue have properties of mesenchymal stem cells.

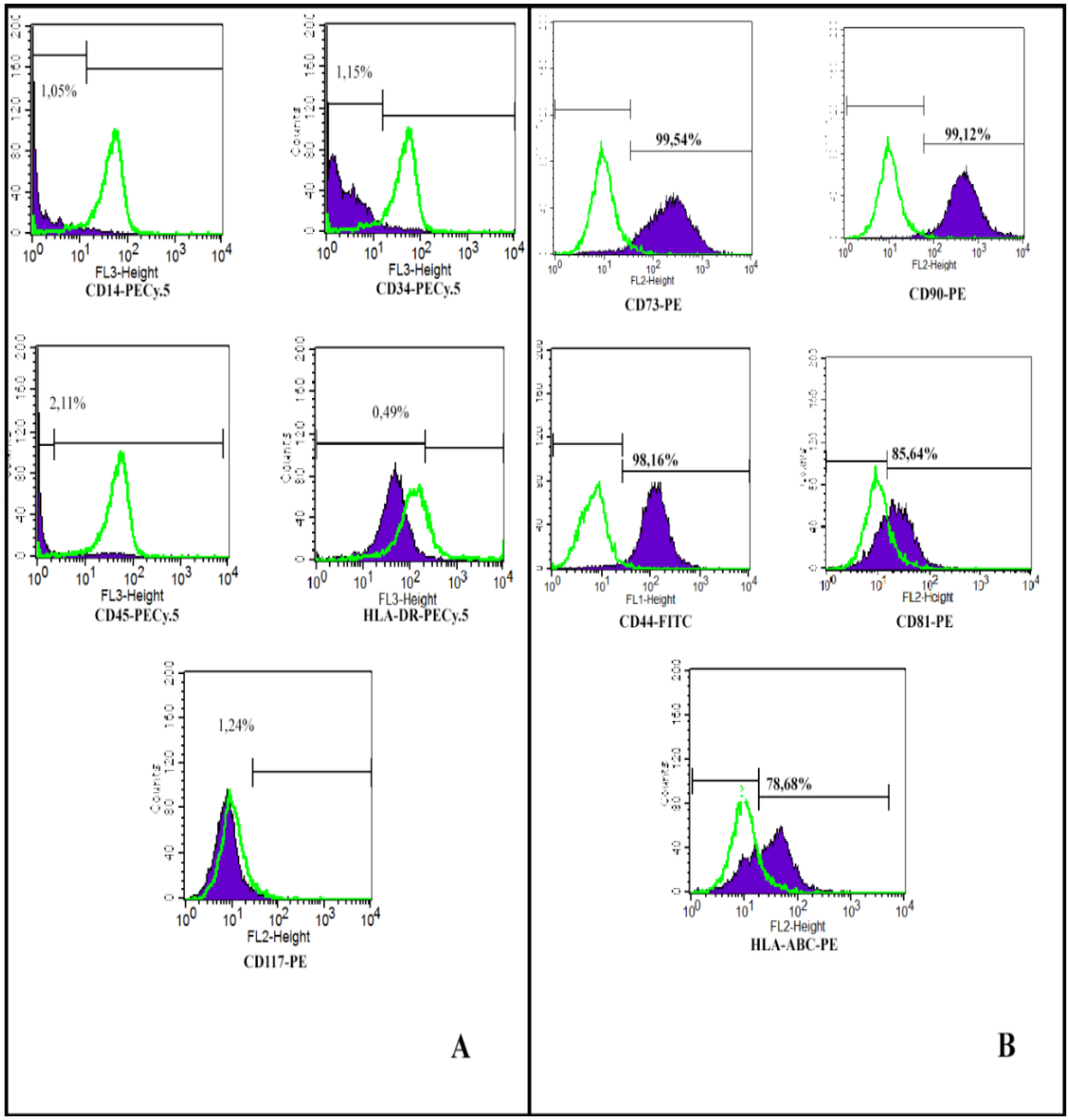

Figure 4.2. Immuno-phenotypic analysis of hAD-MSCs which exhibited less than 1 % (A) CD14-PECy.5, CD34-PECy.5, CD45-PECy.5, CD117-PE, and HLA-DR- PECy.5 markers; on the other hand, they were positive (93 %) for (B) CD73-PE, CD90-PE, CD44-FITC, CD81-PE, and HLA-ABC-PE surface markers. Represented percentages are the mean values of three independent experiments. Isotype controls of each antibody, IgG_1 and IgG_{2a} were displayed as green histograms.

4.2.2. *In vitro* multilineage differentiation of hAD-MSCs

Differentiation potential of hAD-MSCs towards osteogenic, chondrogenic and adipogenic lineages was explored after 10 and 21 days of incubation. Morphological structures of hAD-MSCs were visualized by inverted microscopy (Figure 4.3). hAD-MSCs exhibited a fibroblast-like appearance, while hAD-MSCs cultured in osteogenic medium showed cuboidal shapes. The cells under adipogenic stimulation formed lipid droplets; whereas the cells cultured in chondrogenic medium displayed typical round morphology.

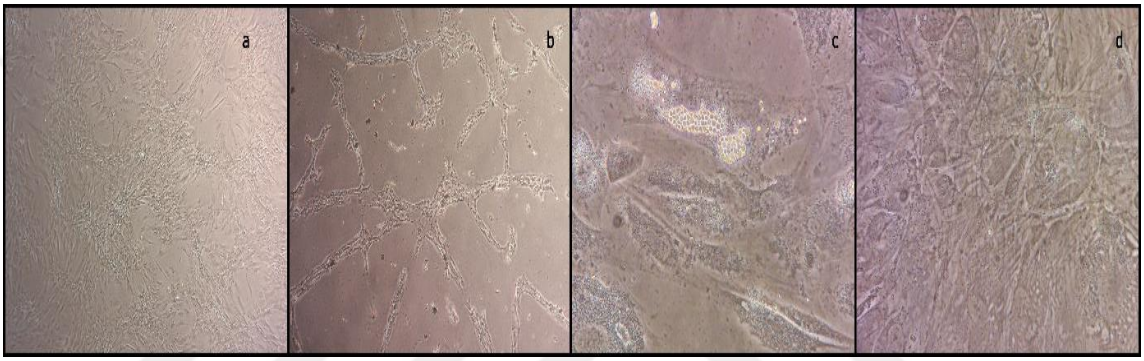

Figure 4.3. Morphological structure analysis of hAD-MSCs under specific stimulation conditions. (a) hAD-MSCs displayed fibroblast- like shapes when cultured in growth medium; (b) hAD-MSCs tended to align themselves and became cuboidal appearance when cultured in osteogenic medium; (c) fatty vacuole deposits were observed after adipogenic induction; (d) hAD-MSCs exhibited the round morphology of chondrocytes when cultured in chondrogenic medium. Scale bars are represented 50 µM; Magnification is 10X.

4.2.2.1. Osteogenic Differentiation of hAD-MSCs

The ability of hAD-MSCs to differentiate into osteogenic lineage was exhibited by using osteogenic induction medium after 10 and 21 days of incubation. Calcium nodules were stained by Alizarin Red S and observed by inverted microscopy (Figure 4.4). hAD-MSCs showed fibroblast-like appearances. However, after differentiation of hAD-MSCs towards osteogenic lineage, they formed cuboidal shapes. Mineralization occurred during osteogenesis of hAD-MSCs. hAD-MSCs cultured in growth medium did not show any

mineralization (Negative controls). Free calcium compounds of differentiated hAD-MSCs at the end of the 21 days was much higher than that of at day10.

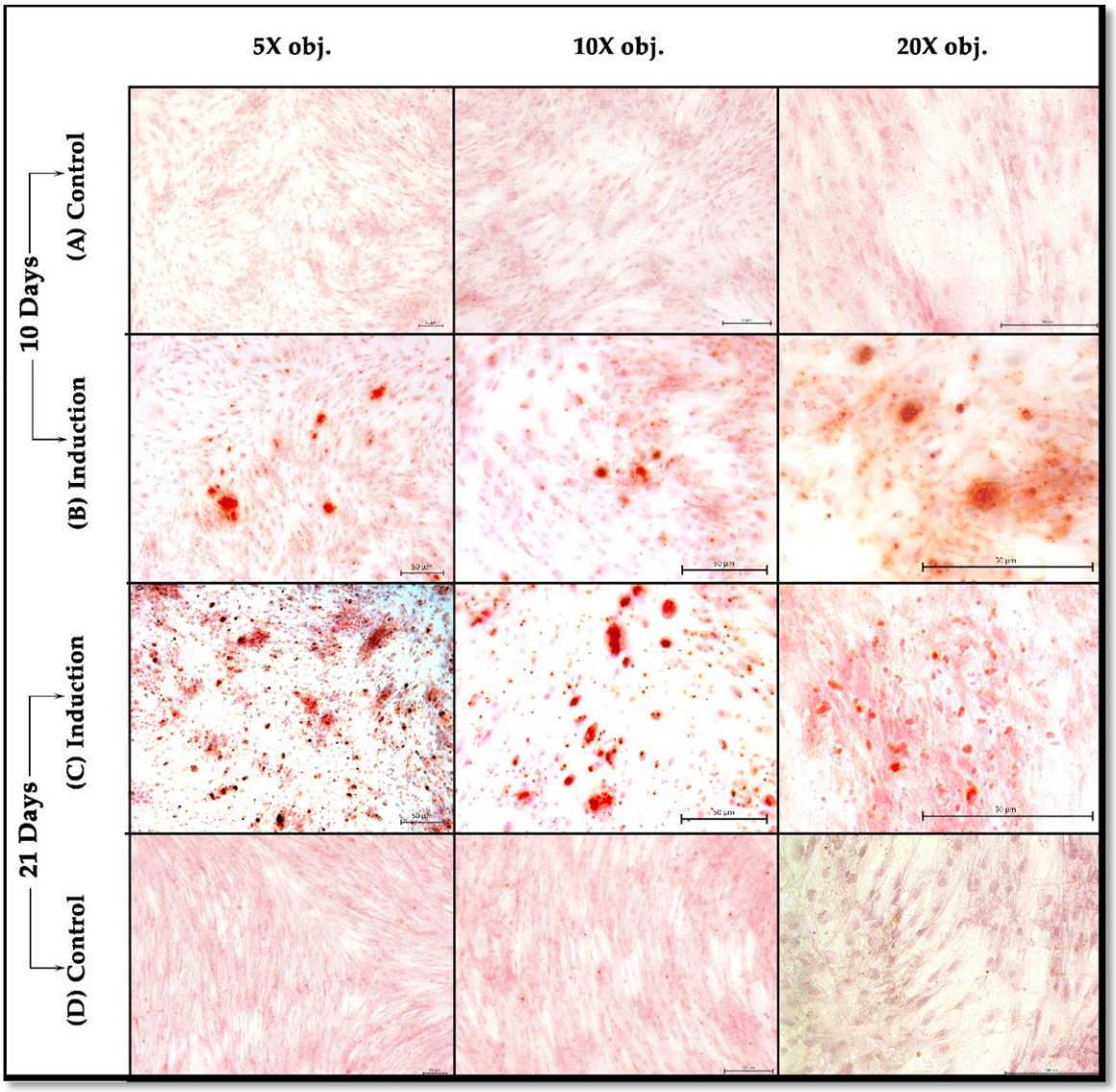

Figure 4.4. hAD-MSCs cultured in growth medium after (A, D) 10 and 21 days of culture (Negative controls). Mineralized nodules formed in osteogenic medium after (B) 10 days; and (C) 21 days of osteogenic induction and they were stained by Alizarin Red S.

4.2.2.2. Chondrogenic differentiation of hAD-MSCs

Sulfated proteoglycan deposits of hAD-MSCs were demonstrated by Alcian blue staining. The round morphology of chondrocytes after chondrogenic induction was demonstrated

after 10 and 21 days of incubation, as compared to control groups (Figure 4.5). Also, higher sulfated proteoglycan deposits were observed at the end of 21 days of incubation, when we compare it with the one at the end of 10 days of incubation.

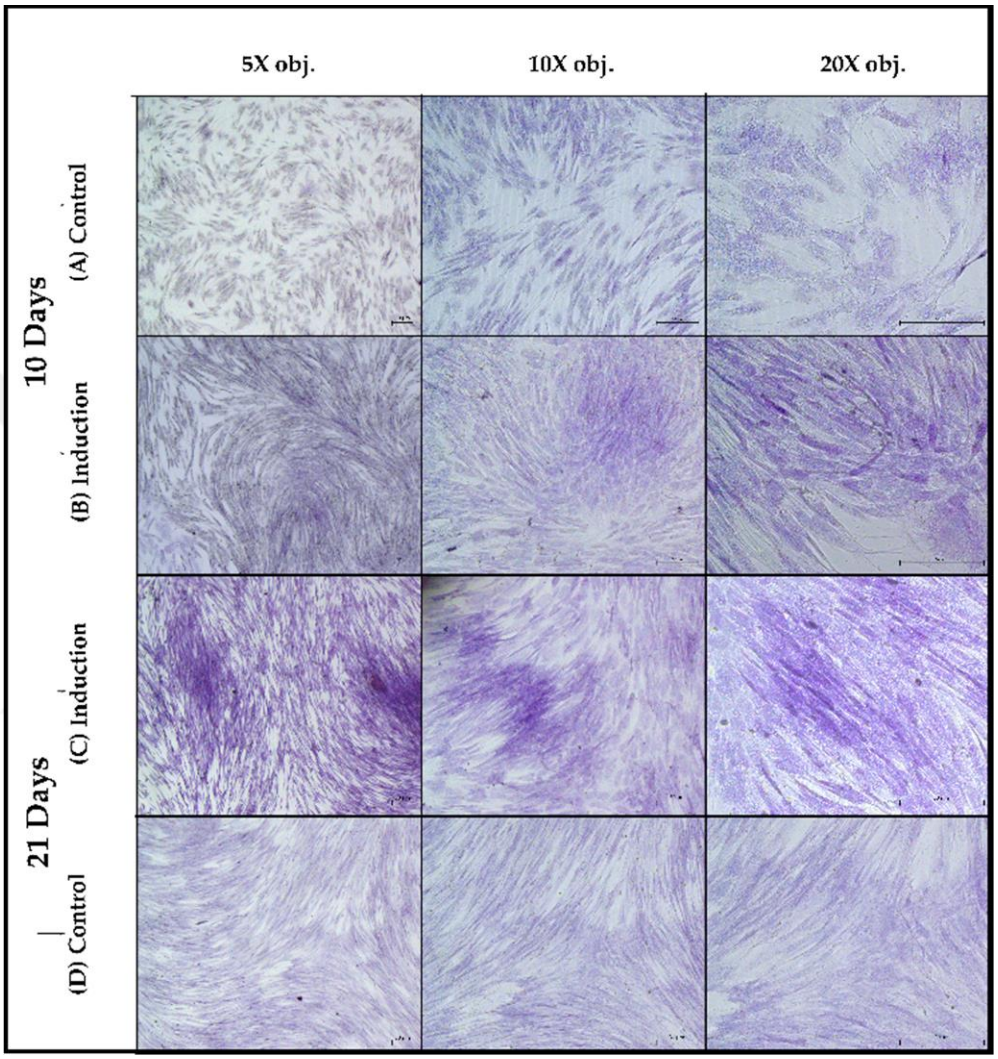

Figure 4.5. hAD-MSCs cultured in growth medium after 10 days (A); and (D) 21 days of incubation. Chondrogenic differentiation potential of hAD-MSCs under chondrogenic conditions after (B) 10 days; (C) 21 days of induction was investigated by Alcian Blue.

4.2.2.3. Adipogenic differentiation of hAD-MSCs

Fatty vacuole deposits of hAD-MSCs were investigated by Oil Red O after 10 and 21 days of adipogenic induction. Lipid accumulation at the end of 21 days of incubation was higher than that of at the end of day 10 (Figure 4.6).

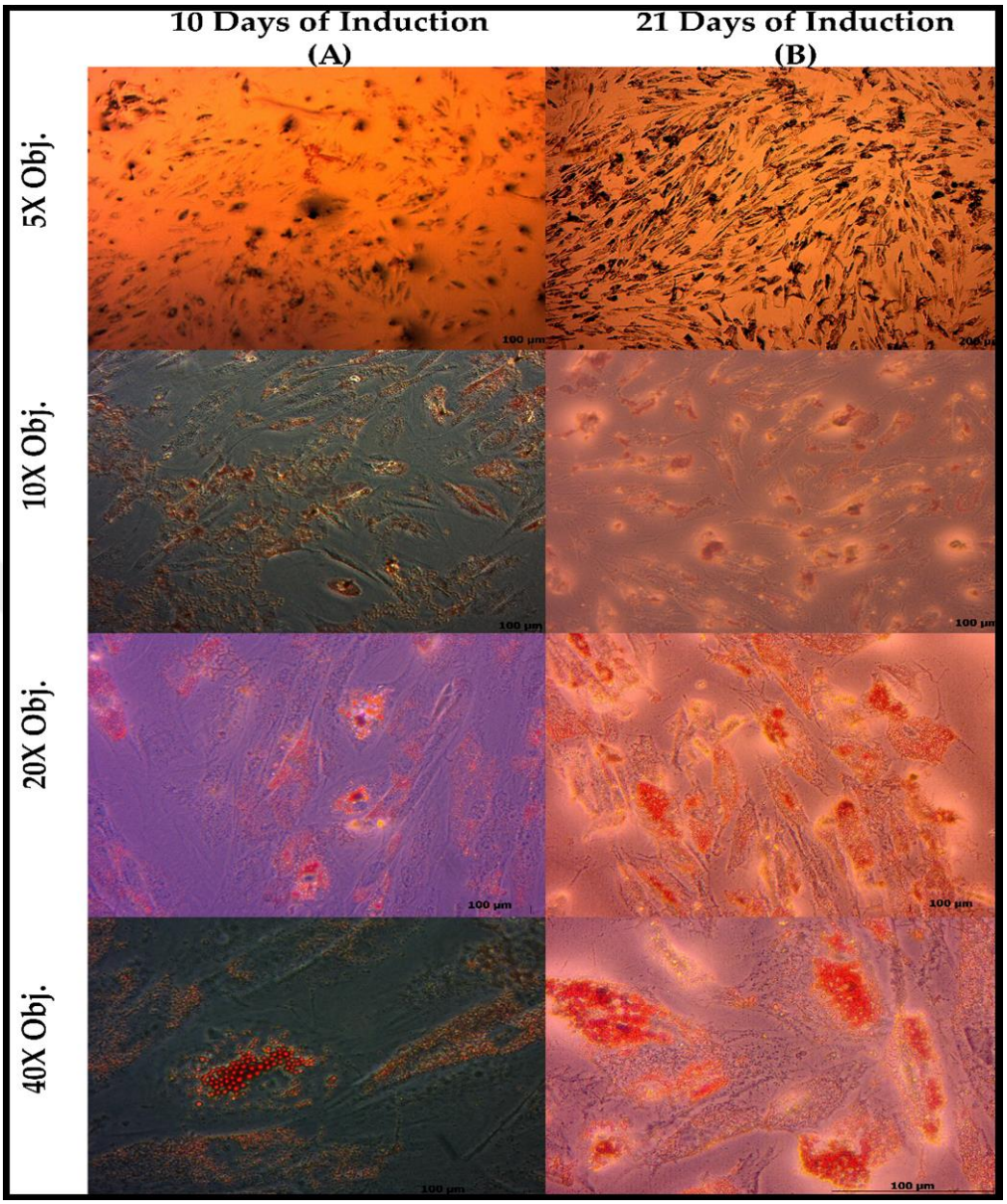

Figure 4.6. Adipogenic differentiation of hAD-MSCs in the adipogenic medium after (A) 10 days and (B) 21 days of induction. Oil red O was used to determine fat droplets.

4.3. MTS CELL VIABILITY ASSAY

MTS cell viability assay was carried out to detect the proliferative capacities of hAD-MSCs grown in BCPs cultured in either OM or BM after 1, 7, 14, and 21 days of incubation. Standard curve of hAD-MSCs was determined at 490 nm (Appendix 1). As shown in Figure 4.7 proliferation rate of hAD-MSCs in BM with BCPs was significantly higher than the ones in OM with BCPs throughout 14 days of incubation ($^{**}P<0.01$).

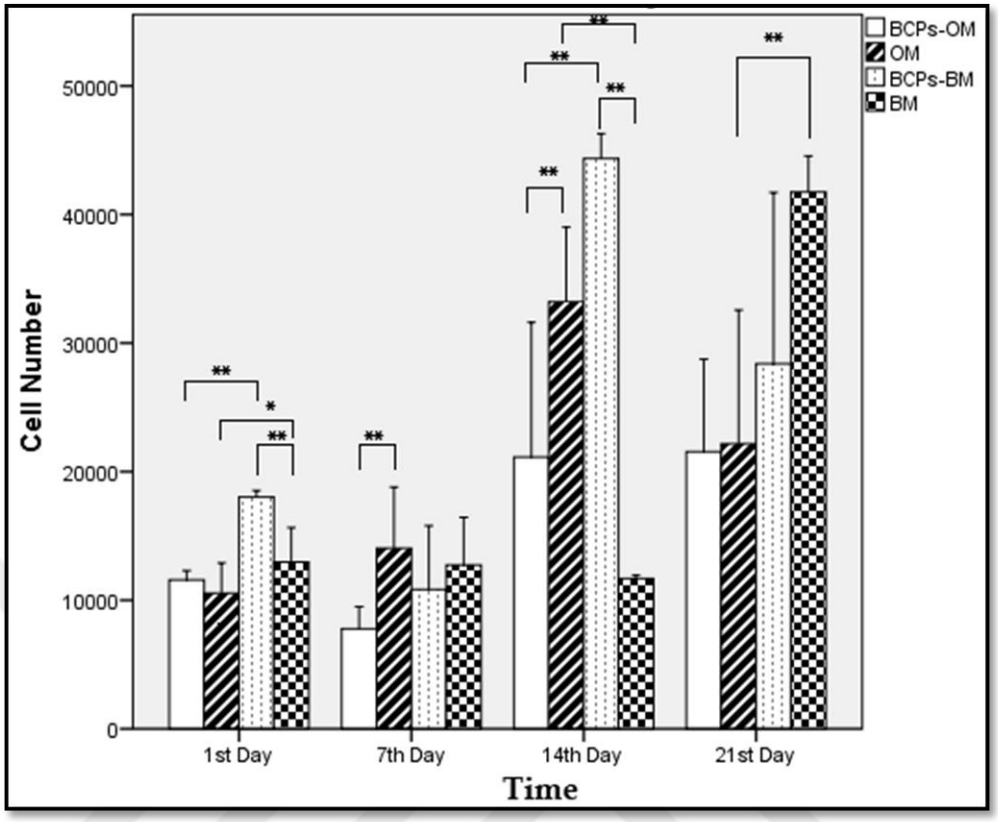

Figure 4.7. Cell proliferation of hAD-MSCs cultured in either OM or BM with/without BCPs. Proliferation profiles of the cells were investigated by MTS cell viability assay. Results exhibited ± SD calculated from at least 3 independent experiments in triplicate. $^{**}p < 0,01$ and $^{*}p < 0,05$ showed statistically significant differences (BCPs-OM: hAD-MSCs grown in bone chip secretions cultured in osteogenic medium; OM: Osteogenic medium; BCPs-BM: hAD-MSCs cultured in basal medium with the addition of bone chip secretions; BM: Basal medium).

MTS analysis displayed no significant difference in proliferation capacity of hAD-MSCs cultured in OM either with or without BCPs after 1 and 21 days of incubation. Nevertheless, hAD-MSCs in OM with BCPs decreased their rate of proliferation relative to the ones in OM at day 7 and 14 ($^{**}P<0.01$). Surprisingly, hAD-MSCs in OM represented significantly higher proliferation profiles in comparison with hAD-MSCs in BM at day 14 ($^{**}P<0.01$). Proliferative potential of hAD-MSCs in BM with BCPs was significantly higher than the others at day 14; while hAD-MSCs in BM exhibited an increase in the rate of growth compared to the ones in OM and BM with BCPs at day 21 ($^{**}P<0.01$).

4.4. ALKALINE PHOSPHATASE ASSAY

Intracellular alkaline phosphatase activity was investigated at days 1, 3, 7, 14, and 21. Standard curve of ALP activity was detected using p-nitrophenyl phosphate (nmol/µl) at 405 nm (Appendix 2). The total protein content was used to normalize the ALP values (Appendix 4). Figure 4.8 exhibited that there was no significant difference between the differentiated and undifferentiated hAD-MSCs grown in BCPs (BCPs-OM and BCPs-BM) and their controls (OM and BM) at days 1 and 3. However, ALP activity significantly reduced in undifferentiated hAD-MSCs with BCPs (BCPs-BM) at day 7 as compared to its control (BM). Moreover, ALP activity of hAD-MSCs cultured in OM was significantly higher than the ones with BCPs (BCPs-OM) at days 7 and 14; whereas hAD-MSCs cultured in BM with BCPs (BCPs-BM) showed slightly higher ALP activity than that of its control (BM) at the end of 14 days of incubation.

4.5. DETERMINATION OF MINERALIZATION

4.5.1. Quantitative Calcium Assay

Calcium deposition of the each group was detected by colorimetric technique after 7, 14 and 21 days of incubation. Calibration curve of calcium concentration (mg/dl) was detected according to the manufacturer instructions (Appendix 3). Normalization was done by using total protein content (Appendix 4). Calcium deposits of hAD-MSCs in OM showed an increase in mineralized matrix deposition in comparison with hAD-MSCs in BM throughout 21 days of culture (Figure 4.9). It has been demonstrated that the calcium deposits of hAD-MSCs in OM was significantly greater than that of with BCPs after 14 and 21 days of incubation (** $p < 0,01$). hAD-MSCs in BM with BCPs displayed significantly higher calcium-rich deposits than the ones in BM after 21 days of culture.

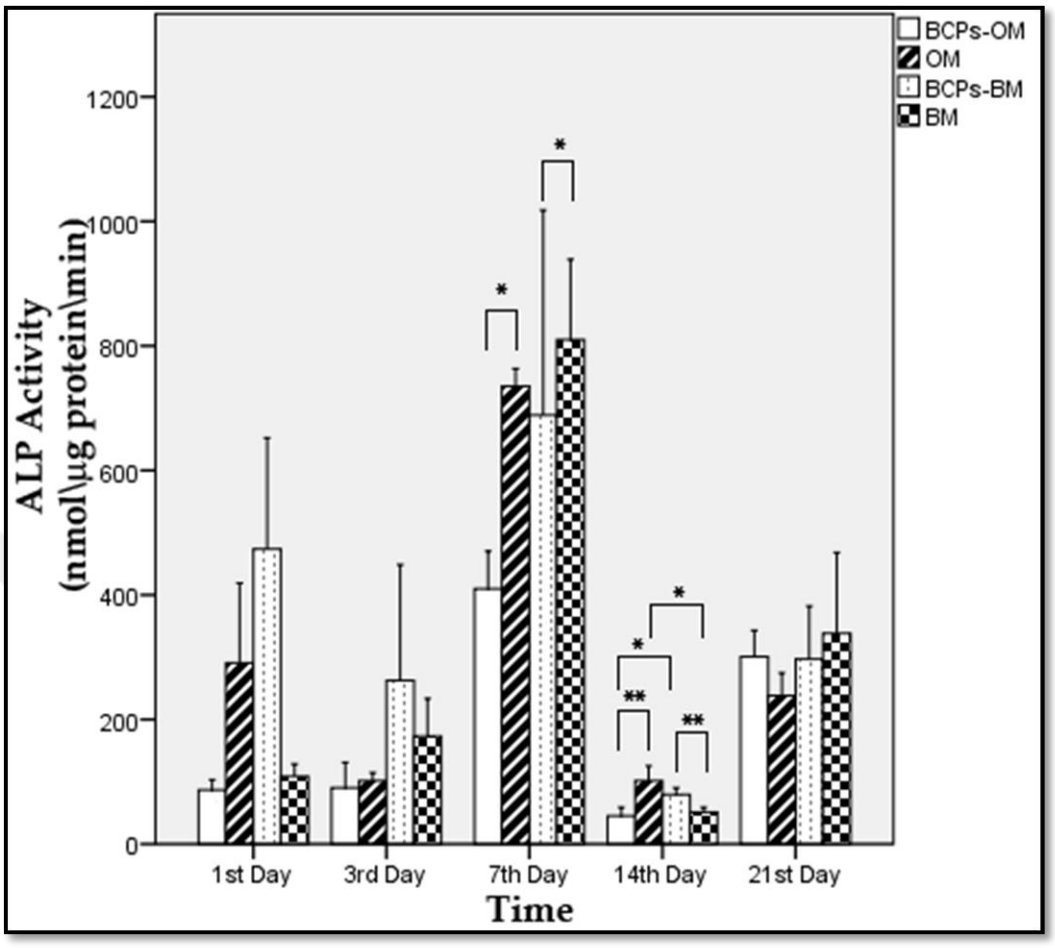

Figure 4.8. ALP activity of hAD-MSCs in the presence and absence of BCPs at 1, 3, 7, 14 and 21 days of incubation in the osteogenic and basal medium. ALP was normalized by the total protein content of the cells with units of (nmol pNpp/min) / (µg protein). ALP activity significantly reduced in hAD-MSCs with BCPs. Values are represented as the mean ± SD. Significance was showed at $^{*}p < 0,05$ and $^{**}p < 0,01$ when compared with the control groups (BCPs-OM: hAD-MSCs grown in bone chip secretions cultured in osteogenic medium; OM: Osteogenic medium; BCPs-BM: hAD-MSCs cultured in basal medium with the addition of bone chip secretions; BM: Basal medium).

4.5.2. von Kossa Staining

von Kossa staining was carried out for the investigation of mineralization on hAD-MSCs cultured in either OM or BM with or without BCPs following 7, 14, and 21 days of incubation (Figure 4.10). Mineralization was exhibited at the highest degree on hAD-MSCs

cultured in OM at the end of 21 days of the period in comparison with the ones with BCPs. Nevertheless, hAD-MSCs cultured in BM in the presence of BCPs didn't show mineralization nodules significantly as compared to the ones cultured in BM at days 14 and 21. Mineralized bone modules were displayed as dark-brown regions.

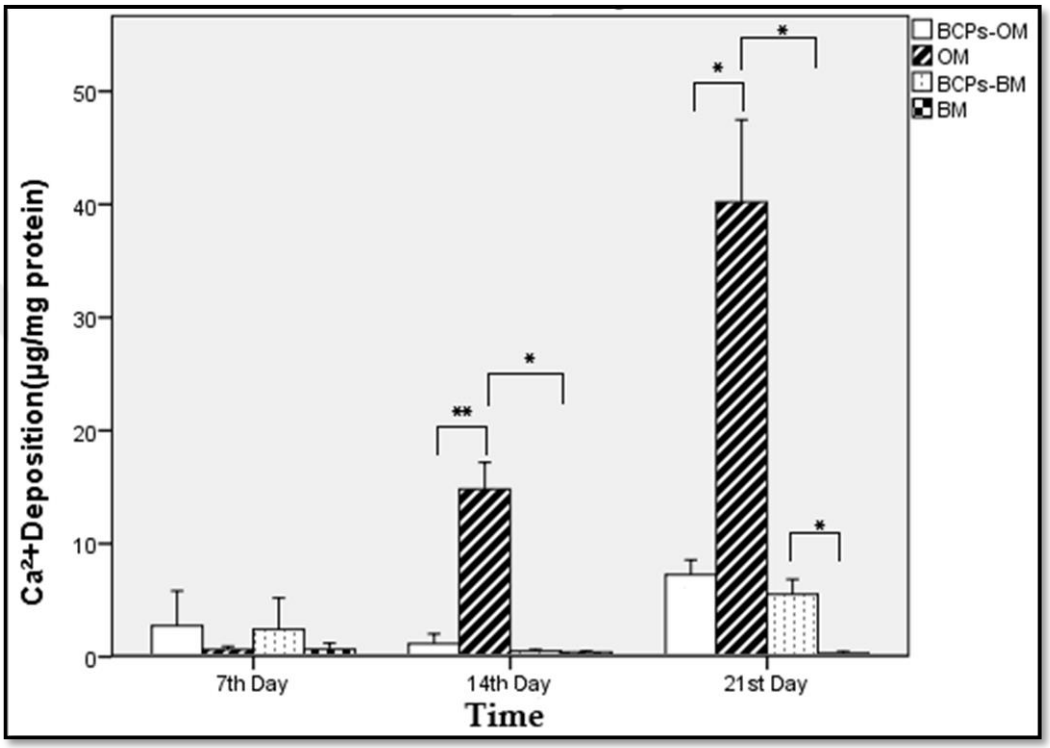

Figure 4.9. Calcium deposition of hAD-MSCs cultured in either OM or BM with/without BCPs following 1, 7, and 14 days of incubation period. Calcium content of hAD-MSCs cultured in OM with BCPs was lower than their control groups throughout 21 days of the period. Values are the means ± SD, ** $p < 0,01$ represents statistically significant difference (BCPs-OM: hAD-MSCs grown in bone chip secretions cultured in osteogenic medium; OM: Osteogenic medium; BCPs-BM: hAD-MSCs cultured in basal medium with the addition of bone chip secretions; BM: Basal medium).

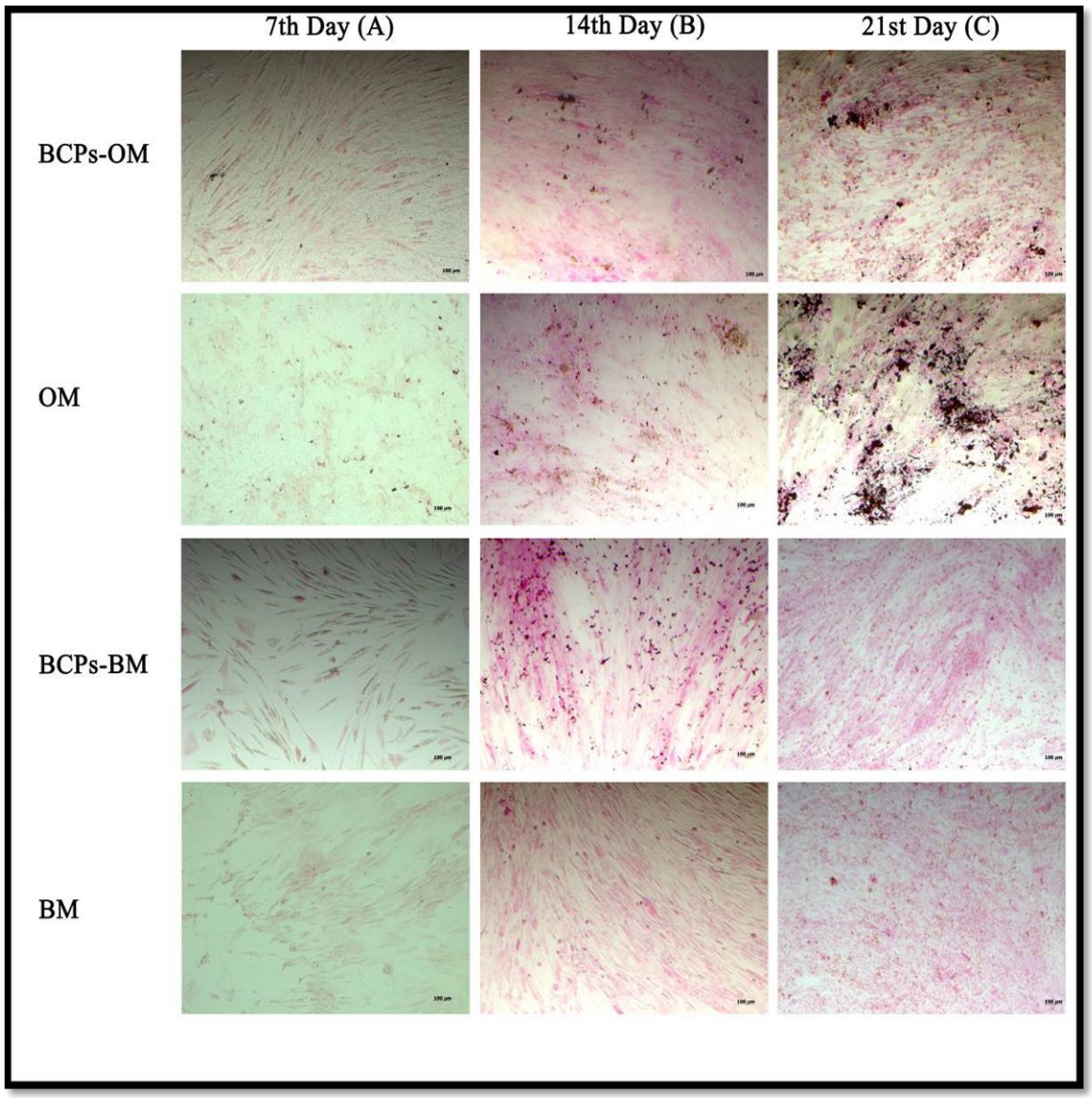

Figure 4.10. von Kossa staining of hAD-MSCs cultured in either OM or BM with/without BCPs at day (A) 7, (B) 14, and (C) 21 days of incubation. Mineralization exhibited a significant reduction in the presence of BCPs when compared to their control groups after 21 days of culture. Bars show 100 μm. Images are acquired 10X (BCPs-OM: hAD-MSCs grown in bone chip secretions cultured in osteogenic medium; OM: Osteogenic medium; BCPs-BM: hAD-MSCs cultured in basal medium with the addition of bone chip secretions; BM: Basal medium).

4.6. REAL-TIME PCR ANALYSIS

Real-time PCR was performed to investigate the expression profiles of bone-specific genes: bone morphogenetic protein-2 (BMP-2), collagen type I (Col1A1), sclerostin, dickkopf-1 (Dkk-1), osteocalcin (OCN), osteonectin (ON), osteopontin (OPN), and osteoprotegerin (OPG). Target gene levels were normalized against the housekeeping gene, beta-actin expression levels.

In this study, hAD-MSCs with BCPs cultured in OM exhibited a stimulatory effect on both sclerostin and BMP-2 expressions throughout 21 days of incubation (Figure 4.11). Specifically, a significant increase in the level of BMP-2 expression was demonstrated throughout 21 days of culture. Besides, a stimulatory effect of BCPs was observed on sclerostin expression after 7, 14 and 21 days of incubation. The expression of sclerostin of hAD-MSCs cultured in OM in the presence of BCPs was significantly higher than that of its control from day 7 to 14 but a significant decrease in the level of sclerostin was investigated from day 14 to day 21. However, it was still upregulated comparing with its control. Interestingly, the level of Dkk-1expression was observed without any significant difference between hAD-MSCs cultured in OM with/without BCPs. OPG production was also evaluated by real-time PCR. According to the results, the level of OPG expression was significantly increased from day 7 to day 14. However, a significant reduction in its level was detected after 21 days of incubation (78 % knockdown).

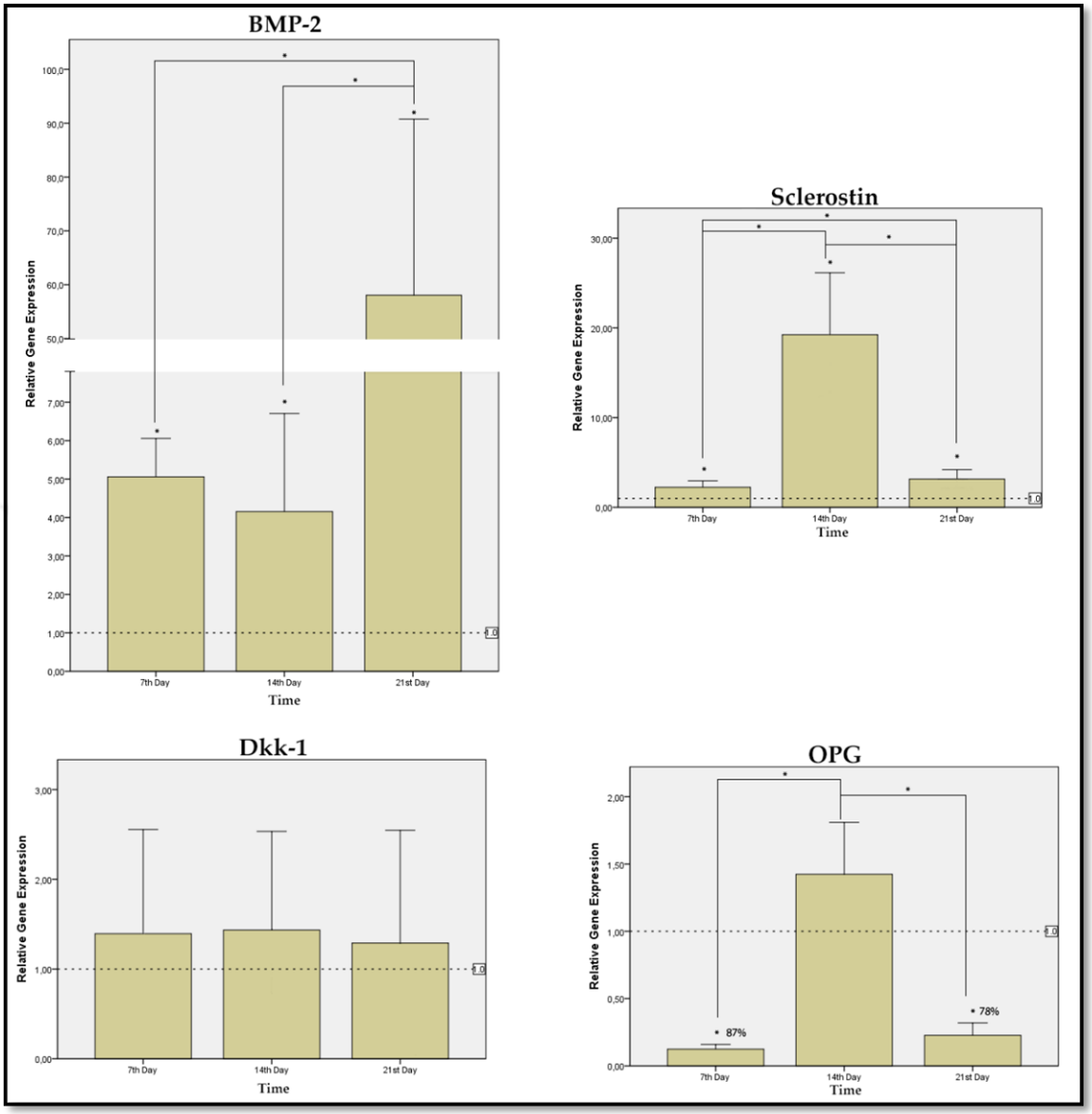

Figure 4.11. Effect of BCPs was detected using real-time PCR analysis. Gene expression profile of bone-related genes, including BMP-2, sclerostin, Dkk-1 and OPG in hAD-MSCs with/without BCPs cultured in osteogenic medium for 7, 14 and 21 days of incubation. Each target gene mRNA levels were normalized against beta-actin expression levels.* $p <$ 0,05 shows the significant difference between differentiated hAD-MSCs in the presence of the BCPs and their control groups. The Pfaffl's method was used to calculate relative gene expression levels. The means of at least 2 independent experiments in duplicate are plotted and the each value is ± SD.

Expressions of osteoblastic phenotype genes, including Col1A1, OCN, OP, OCN, and ON were also analyzed by real-time PCR on days 7, 14 and 21 after osteogenic induction of

hAD-MSCs with/without BCPs (Figure 4.12). Our data indicated that Col1A1 production was not significantly different in hAD-MSCs cultured in OM with BCPs than the ones without BCPs at days 7 and 14. Nevertheless, the level of Col1A1 expression was significantly reduced from day 7 to 14. Furthermore, the significantly lowest Col1A1 expression was demonstrated in hAD-MSCs cultured in OM with BCPs at day 21 (87 % knockdown).

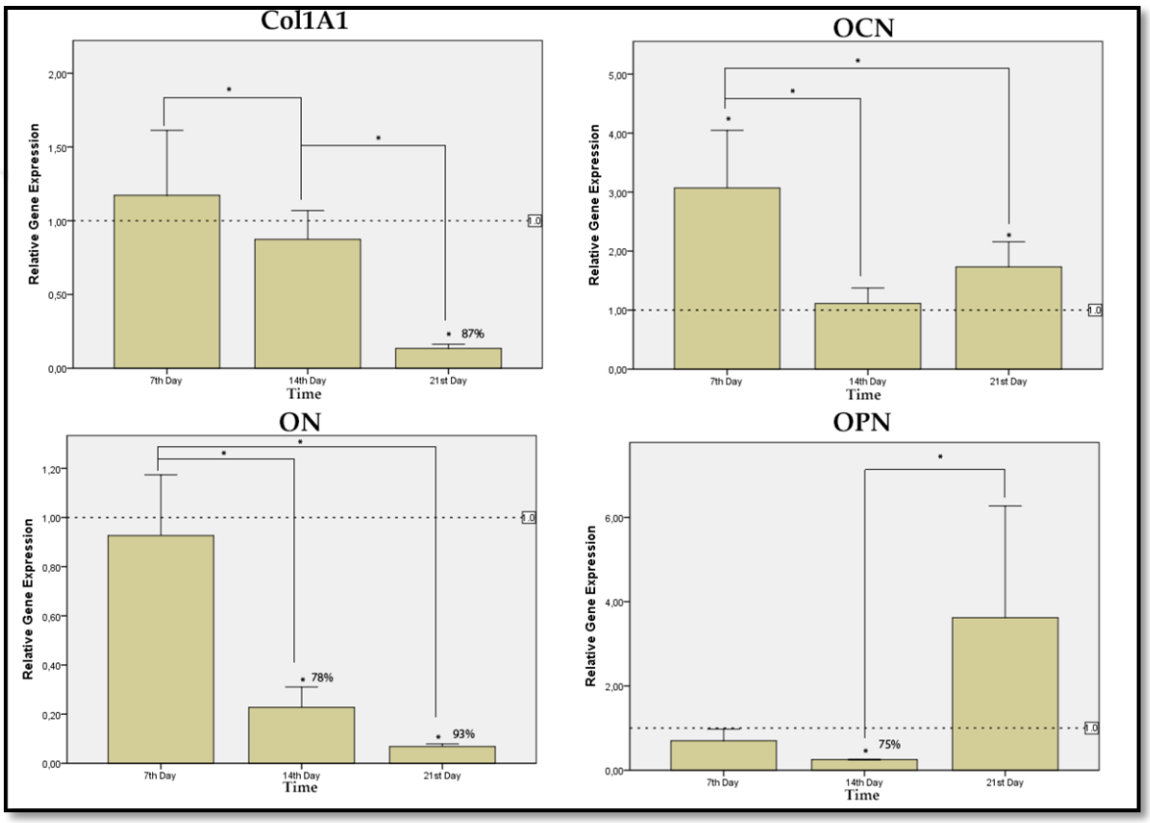

Figure 4.12. Gene expression profile of bone specific genes, including Col1A1, OCN ON, and OPN in hAD-MSCs with/without BCPs cultured in osteogenic medium for 7, 14 and 21 days of incubation. Each target gene mRNA levels were normalized against beta-actin expression levels.* $p < 0,05$ shows the significant difference between differentiated hAD-MSCs in the presence of the BCPs and their control groups. The Pfaffl's method was used to calculate relative gene expression levels. The means of at least 2 independent experiments in duplicate are plotted and the each value is ± SD.

Additionally, the level of OCN expression was significantly increased with the addition of BPCs at day 7; whereas the ones without BPCs (OM) exhibited lower expression level of OCN. Besides, the level of OCN expression in hAD-MSCs cultured in OM with BCPs

displayed a significant decrease in comparison with its control from day 7 to 14. OCN expression level of hAD-MSCs in OM with BCPs was significantly increased at day 21. Expression of ON was significantly reduced from day 7 to day 14 in hAD-MSCs cultured in OM with BCPs. The level of ON expression was significantly decreased at days 14 and 21. Similarly, downregulation of OPN was detected at day 14 (75 % knockdown) that was followed by a significant increase after 21 days of incubation in hAD-MSCs cultured in OM in the presence of BCPs.

Gene expression profiles of hAD-MSCs cultured in BM with BCPs was also determined to investigate whether BCPs affected the cell differentiation in BM. As shown Figure 4.13, BCPs exhibited an inhibitory effect on the BMP-2 and sclerostin expressions of hAD-MSCs cultured in BM at day 7. The level of BMP-2 expression was significantly decreased (83 % knockdown) (* $p < 0,05$). Nevertheless, BMP-2 expression was significantly upregulated in the cells with BCPs in comparison with the ones without BCPs (BM) from days 14 to 21. In the same way, hAD-MSCs cultured in BM in the presence of BCPs showed an inhibitory effect on sclerostin expression when compared to the ones without BCPs (BM) at day 7 (46% knockdown). However, the level of sclerostin expression was significantly upregulated from day 7 to day 14. Moreover, a significant increase in the level of sclerostin expression in the presence of BCPs group was detected at day 21 (* $p < 0,05$).

According to the results, Dkk-1 expression was significantly downregulated in hAD-MSCs cultured in BM with BCPs at days 7 and 21 (71 % and 61 % knockdown, respectively). Nevertheless, there was no significant difference between the time points. Furthermore, OPG expression was constantly decreased by BCPs during 21 days in culture (* $p < 0,05$).

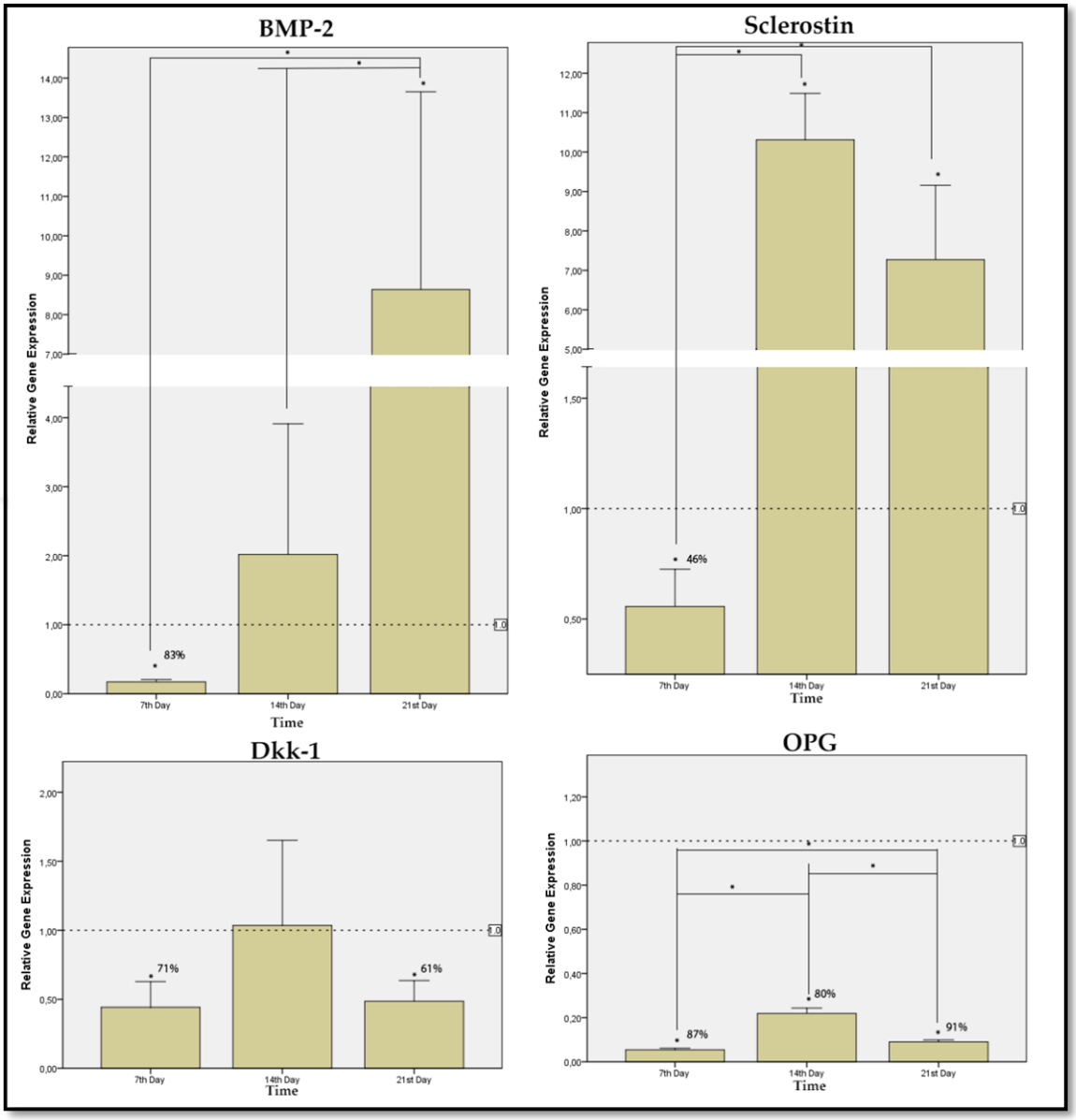

Figure 4.13. BMP-2, sclerostin, Dkk-1 and OPG mRNA expressions in hAD-MSCs with/without BCPs cultured in BM at days 7, 14 and 21. Beta-actin expression levels were used to normalize the target genes. All values of at least 2 independent experiments in duplicate were plotted. The relative mRNA expression was computed with the Pfaffl method. Error bars indicate as means ± SD. $^{*}p < 0,05$ displays hAD-MSCs treated with BCPs relative to their controls.

For bone-related genes, the level of Col1A1 expression was constantly decreased throughout 21 days of incubation (Figure 4. 14). Furthermore, expression of OCN was significantly decreased at day 7 (33 % knockdown) but a significant increase in the expression of OCN was investigated at day 14. Similarly, the level of OPN expression in hAD-MSCs cultured

in BM with BCPs was significantly reduced at day 7(52 % knockdown). However, after 14 days incubation, OPN expression was significantly increased in comparison with the ones without BCPs (BM). Besides, ON expression was significantly decreased in hAD-MSCs cultured in BM with BCPs throughout 21 days of incubation (* $p < 0,05$).

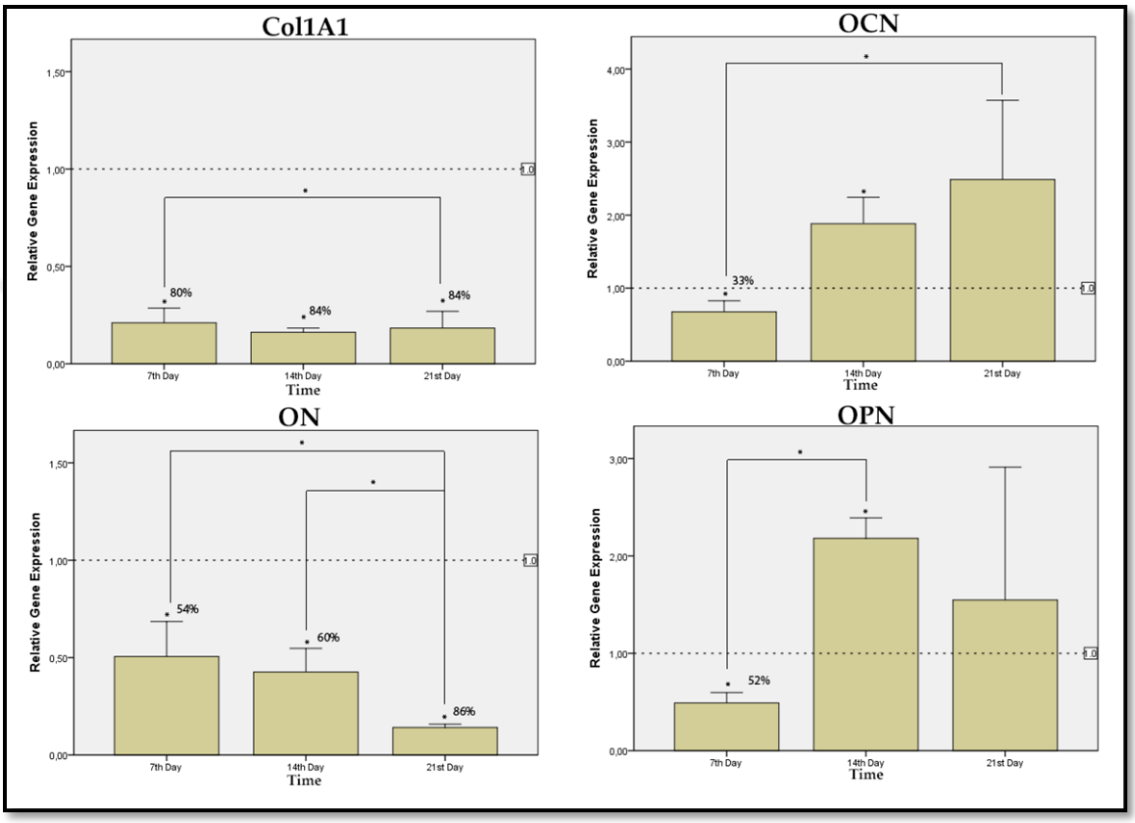

Figure 4.14. Col1A1, ON, OP, OCN and OPG mRNA expressions in hAD-MSCs with/without BCPs cultured in BM at days 7, 14 and 21. Beta-actin expression levels were used to normalize the target genes. All values of at least 2 independent experiments in duplicate were plotted. The relative mRNA expression was computed with the Pfaffl method. Error bars indicate as means ± SD. *p < 0,05 displays hAD-MSCs treated with BCPs relative to their controls.

4.7. MEASUREMENT OF CYTOKINE LEVELS BY ELISA ASSAY

TNF-α, IL-6 and IL-1β were investigated by using quantitative sandwich enzyme immunoassay technique. Calibration curve of each cytokine level was investigated by regression analysis using log-log four paramater logistic curve-fit λ_{max}:450 nm (Appendix 5,

6, 7). Production of IL-6 was significantly induced by BCPs in hAD-MSCs cultured in either BM or OM throughout 21 days ($^{**}p < 0,01$) (Figure 4.15).

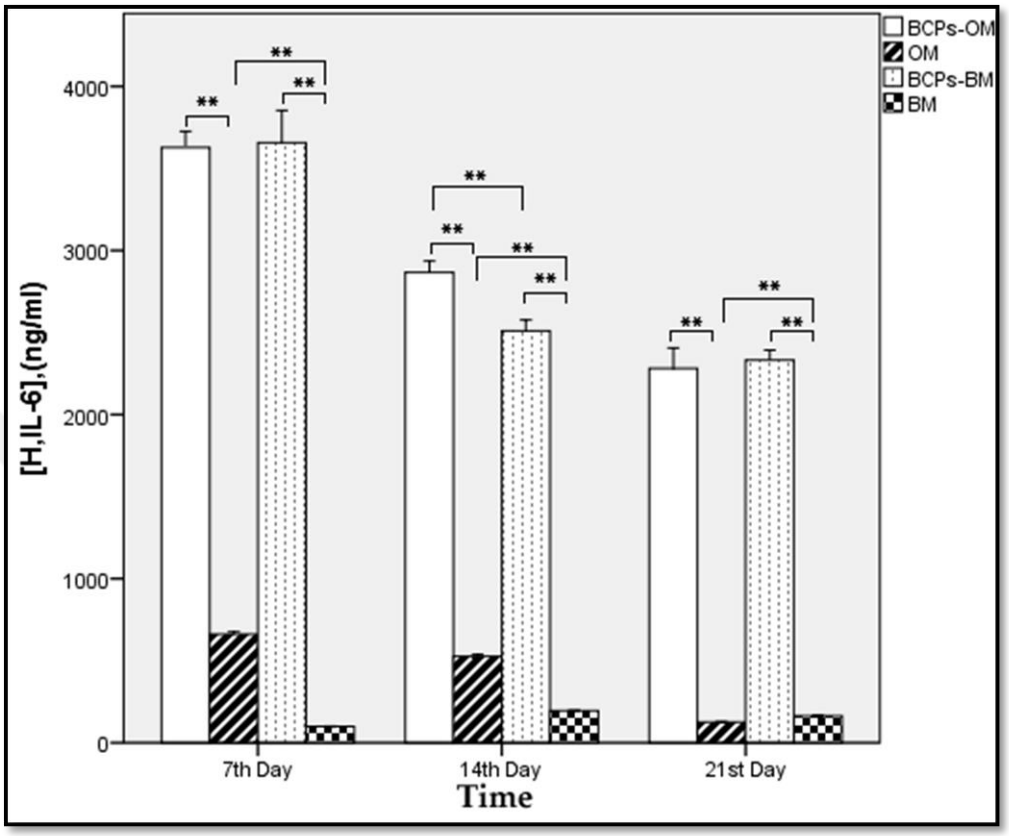

Figure 4.15. Stimulatory effects of BCPs were observed on IL-6. The level of IL-6 production of hAD-MSCs in the presence of BCPs was significantly different from its control. Error bars represent ± SD of the means of triplicates in the same experiment. $^{**}p< 0,01$ displays significance between BCPs treated groups and their controls. (BCPs-OM: hAD-MSCs grown in bone chip secretions cultured in osteogenic medium; OM: Osteogenic medium; BCPs-BM: hAD-MSCs cultured in basal medium with the addition of bone chip secretions; BM: Basal medium).

Similarly, the stimulatory effects of BCPs on the level of IL-1β and TNF-α displayed the same profiles after 7, 14 and 21 days of culture ($^{**}p < 0,01$) (Figure 4.16). Production of IL-1β and TNF-α were significantly increased in the presence of BCPs on 7 days. Nevertheless, inhibition effects of BCPs were observed the productions of IL-1β and TNF-α of hAD-MSCs cultured in either OM or BM at day 14 in comparison with in the absence of BCPs (Figure 4. 17).

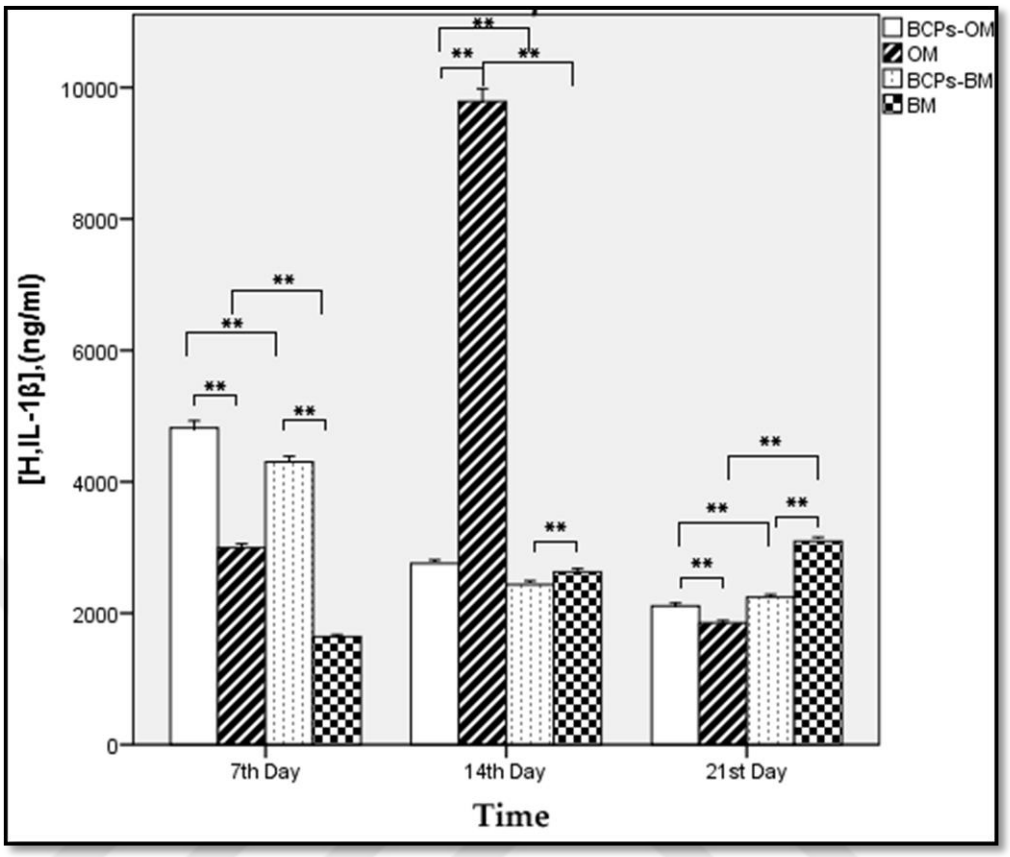

Figure 4.16. IL-1β was determined by Elisa Assay. BCPs displayed induction effects on the level of IL-1β production of hAD-MSCs at the initial and later stages of bone formation. Error bars represent ± SD of the means of triplicates in the same experiment. $^{**}p< 0,01$ displays significance between BCPs treated groups and their controls. (BCPs-OM: hAD-MSCs grown in bone chip secretions cultured in osteogenic medium; OM: Osteogenic medium; BCPs-BM: hAD-MSCs cultured in basal medium with the addition of bone chip secretions; BM: Basal medium).

Furthermore, hAD-MSCs cultured in OM with BCPs displayed significantly greater levels of either TNF-α or IL-1β than its control (OM) at day 21; while the reduce levels of IL-1β and TNF-α of hAD-MSCs cultured in BM in the presence of BCPs was observed at day 21 ($^{**}p < 0,01$).

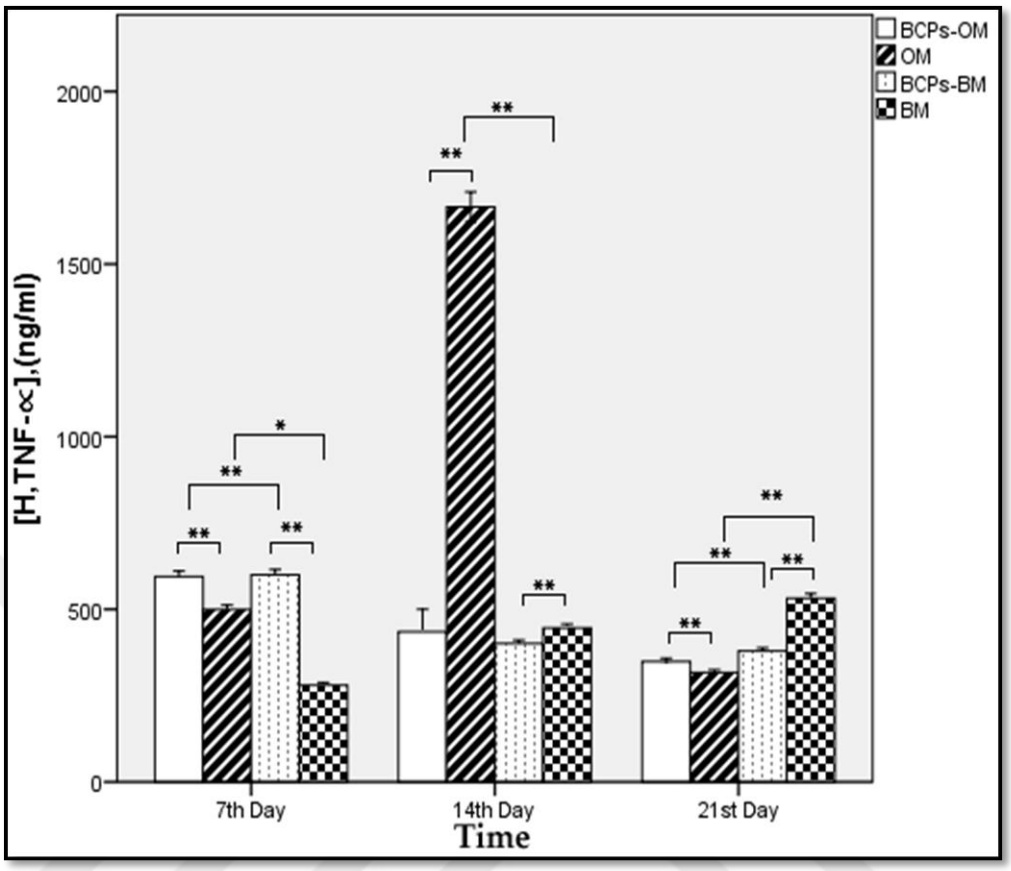

Figure 4.17. The level TNF-α production of hAD-MSCs in the presence of BCPs was significantly different from its control. Culture supernatants were used to measure each cytokine level by Elisa Assay. Error bars show ± SD of the means of triplicates in the same experiment. $^{**}p< 0,01$ represents significance between BCPs treated groups and their controls. (BCPs-OM: hAD-MSCs grown in bone chip secretions cultured in osteogenic medium; OM: Osteogenic medium; BCPs-BM: hAD-MSCs cultured in basal medium with the addition of bone chip secretions; BM: Basal medium).

5. DISCUSSION

Although the physiological process of bone tissue is very well known phenomena, fracture healing mechanism has not still been clearly enlightened. In spite of regenerative capacity of bone tissue, a physiological and biological processes are sometimes unsuccessful during fracture repair resulting in non-union, delayed-union and pseudo-arthrosis [253]. The purpose of this study was to demonstrate the influence of human cancellous bone chips soluble molecules onto hAD-MSCs cultured in osteogenic and basal media. Here, we showed that the influence of BCPs on the cells at the fracture site by carrying out an *in vitro* models of osteogenesis assay for the first time in the literature.

Firstly, cell proliferation of each group was evaluated. The influence of BCPs on the viabilities of hAD-MSCs in both basal and osteogenic media was determined by MTS cell proliferation assay after 1, 7, 14 and 21 days of culture. According to the results, hAD-MSCs survived in both basal and osteogenic media in the presence and absence of the BCPs throughout 21 days of incubation. Low proliferation rates are usually observed during the differentiation process. It may explain why the cells cultured in OM showed lower proliferative capacity than the one in BM. Nevertheless, a significant reduction in cell viabilities of hAD-MSCs cultured in BM after day 14 was observed due to cell death related to lack of available space. Furthermore, it has been showed that BCPs treatment caused an adverse effect on mesenchymal stem cell proliferation during bone formation.

hAD-MSCs differentiate into osteoprogenitor cells that develop into pre-osteoblasts, which ultimately become mature osteoblasts [322, 323]. The commitment of hAD-MSCs and differentiation of hAD-MSCs towards osteogenic lineage depend on various transcription factors and signaling molecules. The main regulatory transcription factor controlling osteoblast maturation is Runt-related transcription factor 2 (Runx2) that binds to promoter regions of bone-related genes, such as ALP, Col1A1, OCN, ON and OPN and regulates their expression. Nevertheless, expression levels of those genes vary according to the osteoblast maturation stages (Abay et al., 2016). ALP is expressed at the initial stage of bone formation. ALP activity is a well-known early osteogenic marker for osteogenesis and plays a key role in extacellular matrix mineralization process. As mineralization starts, ALP activity reduces [324, 325]. The present data showed that hAD-MSCs grown in BCPs cultured with OM displayed a decrease of ALP activity at days 7 and 14. The cells possessing significant ALP

activity formed an extracellular mineralized matrix. Our findings indicated that ALP activity which is a necessary enzyme for matrix mineralization was inhibited by BCPs. Concerning the results in ALP activity and the matrix mineralization based on calcium deposition and von Kossa staining, a drastic decrease of extracellular matrix mineralization was detected in the presence of BCPs at the early stage of osteoblasts maturation.

According to real time-PCR results, the expression of bone-related genes, including BMP-2, sclerostin, Dkk-1, OCN, Col1A1, OPG, ON and OP were analyzed. We reported that an increase level of BMP-2 expression was detected in hAD-MSCs cultured in OM with BCPs after 21 days of incubation period. BMPs are important growth factors that lead to stimulating various molecular cascades. They regulate both osteoblasts and osteoclasts activities. They are secreted by several cell types such as osteoprogenitor cells, osteoblasts and chondrocytes and capable of stimulation of subsequent signaling pathways involved in osteogenesis, adipogenesis and chondrogenesis. There are various clinical circumstances which need an enhancement of bone repair depending on fracture healing potential. BMPs have been the most widely studied key molecules that regulate bone regeneration [326]. They are responsible for induction of mesenchymal stem cell proliferation and osteoblast formation. Osteoinductive capacity of BMPs leads to the use of BMPs as therapeutic agents in clinical applications to enhance fracture repair. Recombinant human BMP-2 (rhBMP-2) and BMP-7 (rhBMP-7) have been approved by FDA for clinical practice [167] and used in a large number of clinical conditions such as non-union, bone necrosis and critical bone defects [169].

In addition to BMPs role on osteoblasts activity, they are necessary for osteoclast differentiation. BMP receptors and their ligands are also expressed by osteoclasts and they directly affect formation and maturation of osteoclasts. Even though BMPs exhibit multiple effects to enhance osteoblasts formation at the initial stage of bone formation, a major role of BMP signaling in the osteoclast maturation is not well defined. Previous *in vitro* studies showed that resorptive activity of osteoclasts was induced by treatment of exogenous BMP-2 [327-329]. Furthermore, other studies suggested that BMPs indirectly regulate osteoclast cells by increasing expression of osteoclast-promoting factors via osteoblast cells [330-332]. We showed that in the presence of BCPs, the highest level of BMP-2 expression was detected during the even late stage of bone regeneration.

Previous pre-clinical animal studies have exhibited a strong response to fracture healing in the presence of BMPs. Nevertheless, results of various human clinical studies have displayed that absence of a powerful response seen in animal models leads us to take into consideration about the regulation of clinically effective doses needed in humans [333]. Stimulation of cancer cells and ectopic bone formation have occurred concerning the use of supraphysiological doses. Unfortunately, optimal doses have still been under investigation. Additionally, osteoclast-mediated bone resorption and unsuitable adipogenesis have been considered as other adverse effects of the clinical use of BMP-2 [334, 335]. This study exhibits the influence of the level of BMP-2 expression of hAD-MSCs cultured in OM in the presence of BCPs. According to the results, there was an increase in BMP-2 expression of hAD-MSCs in OM with BCPs throughout 21 days of incubation. Expression of BMP-2 was upregulated approximately 5 fold after 7 and 14 days of culture period in the cells in OM with BCPs followed by a significant enhancement in its levels (app. 54 fold). We demonstrated that BCPs induced expression of BMP-2 throughout 21 days.

Canonical Wnt/β-catenin signaling possesses a significant role MSCs differentiation, cells growth and functions at the early stage of bone regeneration [50]. A balance between osteoblast and osteoclast formation affects bone mass at the early stage of fracture repair. Wnt/β-catenin signaling cascade induces osteoblastogenesis. However, osteoblastogenesis is blocked by Dkk-1 which is one of the major antagonists of canonical Wnt/β-catenin signaling cascade. An inactivation of Wnt/β-catenin signaling cascade by Dkk-1 significantly inhibited fracture healing [60]. Several groups have studied the effect of anti-Dkk-1 antibodies in numerous animal models and they investigated promising influences on bone mass density [336].

Sclerostin, like Dkk-1, is an inhibitor of canonical Wnt/β-catenin signaling cascade. Sclerostin which is a secreted protein is synthesized mainly by osteocytes and premature osteoclasts. Recent reports showed that high bone mass density in both human and animal models was caused by inactivating mutations in *SOST* gene. Consequently, the sclerostin-neutralizing antibody was evaluated to cure various bone loss models. It was exhibited that anti-sclerostin treatment enhanced bone mineral density and osteoblast formation in cynomolgus monkeys [337] and post-menopausal women who injected a single subcutaneous dose of anti-sclerostin had enhanced bone formation markers for three weeks following treatment [338, 81]. Although BCPs did not have any significant influence on the

level of Dkk-1 expression in the cells cultured in OM in our study, BCPs were able to stimulate sclerostin expression throughout 21 days of incubation period. Additionally, the highest level of sclerostin expression was detected at day 14, and then there was a reduction at day 21. It may explain the sclerostin mechanism that is different from classical BMP-2 antagonists. Integration of BMPs with Wnt/β-catenin signaling increases bone formation. BMPs increase differentiation of mesenchymal stem cells towards osteogenic lineages while preventing proliferative signals enhance by Wnt/β-catenin signaling [51]. We investigated that BCPs treatment strongly increased sclerostin expression. Inactivation of Wnt/β-catenin signaling antagonists stimulate bone mass balance and such an effect may possess therapeutic implications in the healing of fractures.

Additionally, other Wnt/β-catenin signaling molecules have been investigated because of their remarkable roles during bone regeneration in fracture healing. RANK, RANKL and OPG are the key molecules that regulate osteoclastogenesis. Binding of RANKL to RANK induces differentiation of pre-osteoclasts following activation of mature osteoclasts. Conversely, OPG which is a competitive inhibitor of RANKL causes suppression of bone resorption by binding to RANKL. Consequently, OPG/RANKL ratio affects the rate of bone resorption.

The relative ratio of the OPG/RANKL is the main factor of resorption stage in bone repair by suppressing osteoclasts activities [339]. Our findings indicated that OPG expression was significantly decreased after 7 days of incubation (87 % knockdown) and then it was upregulated from day 7 to day 14 in the hAD-MSCs cultured in OM in the presence of BCPs. Nevertheless, OPG expression was significantly downregulated from day 14 to 21. It was showed that OPG mRNA expression was inhibited by BCPs during bone remodeling, particularly at the initial and late stage of bone remodeling. BMP signaling was included not only in osteoblasts activity but also in osteoclastogenesis. Even though, the effect of BMPs signaling in MSCs enhances bone mass; BMP signaling in osteoclast activity is more intricate than osteoblasts activity. BMPs also induce osteoclastogenesis via OPG/RANKL pathway. Specifically, BMP-2 directly increased differentiation of osteoclasts. Several studies have reported that bone marrow macrophages treated with BMP-2 led to upregulation of RANKL. BMP signaling cross-talk with RANKL-mediated signaling and may contribute to the increased bone loss [340].

About 90 % of bone matrix protein is collagen that provides a construction in which mineral is deposited [341]. In this study, expression of Col1A1 was significantly downregulated from day 7 to 14. However, BCPs had no significant effect on the level of Col1A1expression after 14 days of the culture period. As expected, Col1A1 expression was downregulated at day 21. Expression of Col1A1 supports matrix mineralization. Nevertheless, our findings exhibited that BCPs treatment didn't affect the expression of Col1A1 at the early stage of bone repair.

Expressions of osteocalcin, osteonectin and osteopontin were also evaluated to determine the late stage of bone matrix formation. Osteocalcin is a hydroxyapatite-binding protein that is synthesized by osteoblasts. It is vitamin-K dependent and its gamma-carboxyglutamic acid (Gla) residue is capable of binding calcium. This function of osteocalcin is responsible for osteoid mineralization and it is expressed primarily during the stage of bone formation. Furthermore, osteocalcin plays a major role in avoiding excessive matrix mineralization [342, 9]. It is used as biochemical late markers of bone remodeling. However, several studies suggested that osteocalcin are released even during bone resorption [343, 344]. Our findings indicated that the level of OCN expression in hAD-MSCs cultured in OM in the presence of BCPs was increased at day 7. Nonetheless, it was significantly downregulated from day 7 to day 14. An increased level of osteocalcin expression was also observed after 21 days of culture.

Osteonectin and osteopontin are the substances of the mineralized extracellular matrix and expressed by different stages of osteoblastic maturation. Even though their functions are still unknown, they are mainly responsible for regulation of cell-matrix interactions, osteoblasts proliferation and intermediation of hydroxyapatite deposition [341]. According to the results, osteonectin expression was significantly downregulated at day 14. Likewise, osteopontin was significantly inhibited throughout 21 days by BCPs. All these results exhibited that BCPs displayed inhibition effects on bone matrix proteins during bone remodeling.

The influence of BCPs on hAD-MSCs cultured in BM is required to be investigated. BCPs suppressed expressions of both BMP-2 and sclerostin at the early stage of bone formation. They were significantly downregulated at day 7. Nevertheless, an increase level of sclerostin expression was observed at day 14. Sclerostin was upregulated 12 fold at day 14; whereas

the level of BMP-2 expression was not significantly changed during this period. Furthermore, the level of OPG expression in hAD-MSCs cultured in BM in the presence of BCPs was continuously downregulated throughout 21 days of culture.

The expressions of bone-related genes, such as Col1A1 (80 % knockdown), OCN (37 % knockdown), OP (52 % knockdown) and ON (54 % knockdown) were also significantly downregulated by the influence of BCPs. Moreover, Dkk-1 was significantly reduced at day 7. As taking into account the molecular mechanisms of mesenchymal stem cells differentiation into osteogenic lineage, the emphasis of canonical Wnt/β-catenin signaling pathway in the bone regulation has appeared. Dkk-1 is a critical regulator of mesenchymal stem cells proliferation. Even though Wnt/β-catenin signaling cascade is known as an improvement of osteoblasts activity throughout autocrine mechanisms, it has still been under investigation in mesenchymal stem cells. Various studies suggested that Dkk-1 leads to mesenchymal stem cell proliferation rather than their differentiation by suppressing Wnt/β-catenin signaling. Inhibition of the Wnt/β-catenin signaling decreases nuclear β-catenin that is capable of activation of transcription factors. For that reason, Dkk-1 reduces the cell to cell contacts that are essential for mesenchymal stem cell differentiation [345]. In this study, Dkk-1 mRNA expression was significantly decreased during initial and late stages of bone repair.

For matrix genes, Col1A1 expression was continuously downregulated throughout 21 days of incubation. Furthermore, the level of osteocalcin expression was also decreased at day 7 (37 % knockdown). Nonetheless, osteocalcin production was significantly increased at day 14. Similarly, osteopontin mRNA expression was significantly downregulated at day 7 (52 % knockdown) that was followed by a significant increase at day 14. Additionally, the level of osteonectin expression was significantly decreased with the usage of BCPs throughout 21 days of incubation.

OPG mRNA expression was also continuously downregulated within a period of 21 days. Moreover, hAD-MSCs cultured in BM with BCPs showed a suppressive effect on sclerostin expression compared with the ones in which no BCPs was added at day 7. Nonetheless, the level of sclerostin expression was significantly upregulated from day 7 to day 14. In the same way, BCPs treatment exhibited a suppressive effect on BMP-2 expression at day 7 (83 %

knockdown). However, an increase level of BMP-2 expression was observed from day 14 to day 21.

The influence of BCPs on the production of cytokines such as TNF-α, IL-1β and IL-6 was also determined by Elisa assay. Our findings from this study indicated that treatment of hAD-MSCs with BCPs induced the production of TNF-α, IL-1β and IL-6 during bone remodeling. Adaptive and innate immune systems are essential during fracture healing. Inflammatory cells near the injury site secrete cytokines which play remarkable roles initiating repair cascade. Cytokines, TNF-α, IL-1β and IL-6 act as chemotactic effects on other inflammatory cells for stimulation of downstream responses resulting in enhancement of matrix synthesis, induction of angiogenesis and gathering of mesenchymal stem cells at the fracture site. IL-1β was produced less than IL-6 and TNF-α throughout fracture repair. However, it stimulates the secretion of IL-6. IL-1β and IL-6 are the most important cytokines for bone repair [253].

The expression of IL-1β and TNF-α in fracture healing demonstrates a biphasic pattern. Their activities peak within 24h during the early stage of bone healing followed by a decline during cartilaginous callus tissue formation at the 3rd day of post-fracture before increasing for a second at the changeover from chondrogenesis to osteogenesis during endochondral ossification [293]. TNF-α production regulates osteoclastogenesis. It is responsible for resorption of calcified cartilage tissue. Consequently, new bone formation is delayed without TNF-α. In addition to the stimulation of osteoclastogenesis, TNF-α also increases the recruitment of MSCs and stimulates apoptosis of hypertrophic chondrocytes. In one of the study, early cartilage formation was increased by overexpression of TNF-α in diabetic fracture repair [258].

The rate of complications, including delayed-union, non-union and fracture healing time might be enhanced by overexpression of pro-inflammatory cytokines. According to the results, as expected, TNF-α displayed similar expression pattern of the production of IL-1β. Inductive effect of BCPs was detected on mesenchymal stem cells at day 7 and day 14. Moreover, IL-6 concentration in hAD-MSCs in either BM or OM with BCPs were significantly higher than their control groups for a period of 21 days. Our findings suggested that BCPs caused over-stimulation of productions of pro-inflammatory cytokines. This might be osteoclastogenesis rather than osteoblastogenesis.

6. CONCLUSION

Regulation of BMPs and their antagonists are critical for bone regeneration in fracture healing. The rate of BMPs signaling is a primary parameter in identifying the balance between bone formation and resorption during bone remodeling. Approximately 10 % of fractures have incapacitated healing because of non-unions. Bone grafts are the gold standard to cure non-union fracture. Additionally, BMP-2 and BMP-7 are also used for the treatment of non-union fractures. The efficiency of BMPs has been evaluated in various clinical testing. Those studies suggested that patients treated with BMP-2 had enhanced fracture. Besides, usage of mesenchymal stem cells transfected with BMPs has also been explored for the treatment of the non-union fracture. Regardless in the use of BMPs in fracture healing, side effects of rhBMP in the treatment of non-union were also reported. Because of high dose requisite, unstable carrier molecules, safety concerns and restrictions of the clinical trials finished to date, the use of rhBMPs in fracture repair have been frustrating. Besides, molecular mechanisms of BMPs in bone repair are also required to proceed with confidence as using rhBMPs in orthopedic surgeries.

As a result, the findings of this study indicated that BCPs displayed an inhibitory effect on the cell viability, proliferation and differentiation of hAD-MSCs in fracture healing. Moreover, BCPs treatment resulted in suppressed bone repair with decreased ALP activity and extracellular matrix mineralization when it was compared with the controls. hAD-MSCs differentiation into mineralized bone was also inhibited by preventing the upregulation of bone-specific genes, such as Col1A1, OCN, OP, and ON due to BCPs secretion. However, BCPs significantly stimulated BMP-2 expression and its antagonist sclerostin during bone remodeling. Regarding Dkk-1 expression, we did not significantly observe differences between the cells with/without BCPs.

BMP-2 mediated differentiation of osteoprogenitor cells is necessary to enhance fracture repair. Nevertheless, sclerostin suppresses this process and might be the main element controlling bone cell differentiation. Our findings suggested that considerable potential and durable therapeutic effects of BMP-2 in clinical use should be noticed to enhance fracture repair. There is a need to optimize BMP-2 concentration for a suitable induction of bone healing. Moreover, the balance between BMP-2 and sclerostin expression could be a remarkable molecular agent for innovative therapeutic approaches for healing fractures. As

found in this study, BCPs induced the production of cytokines including Il-6, TNF-α and IL-1β that regulate immune response during fracture repair. Therefore, BCPs may cause overexpression of immune response at the injury site.

Overall, the main reason to reveal the influence of BCPs onto hAD-MSCs cultured in either OM or BM during bone repair is to provide a knowledge for the development of novel therapeutic targets that could be concerned in the regulation of the molecular mechanisms of bone remodeling during fracture repair. Results provide a better understanding of the biology of the fracture repair to improve new strategies and concepts in terms of augmentation of host bone graft incorporation which contributes the elimination of histopathological circumstances.

7. FUTURE PROSPECTS

Fracture repair operations are the most commonly performed orthopedic surgeries in the world and this study will contribute a novel knowledge of fracture healing in terms of molecular biology aspects and the development of numerous potential treatment options. Future assessments of the effect of BCPs on the cells during bone regulation in fracture repair are required to investigate the molecular and cellular interactions of bone chip soluble molecules with the cells differentiated towards osteoblastic lineage by the way of signal transduction pathway.

For further studies, it is important to reveal internal mechanism of proteasomes in bone tissue and their functions in the healing of fractures. Mass spectrometry is an excellent instrument for the identification of proteins. Mass spectrometry proteomic analysis of differentiated hAD-MSCs under the influence of BCPs will provide a broad understanding of the biology of fracture healing.

Results obtained from these experiments may guide us to investigate bone formation around the fracture site by electrospray ionization quadrupole time-of-flight mass spectrometry (ESI/QUAD MS). Thus, it will be possible to observe whole proteome profile of differentiated hAD-MSCs under the influence of BCPs.

Identification and quantification of intracellular and secreted proteins, soluble molecules and cytokines in bone repair will provide potential biomarkers. Moreover, when a better understanding of the molecular background of fracture healing evolves, we expect to find novel applications to overcome clinical problems. Investigation of new proteins, soluble molecules and cytokines presented in fracture site will provide novel treatment strategies for the future.

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APPENDIX A: STANDARD CURVES

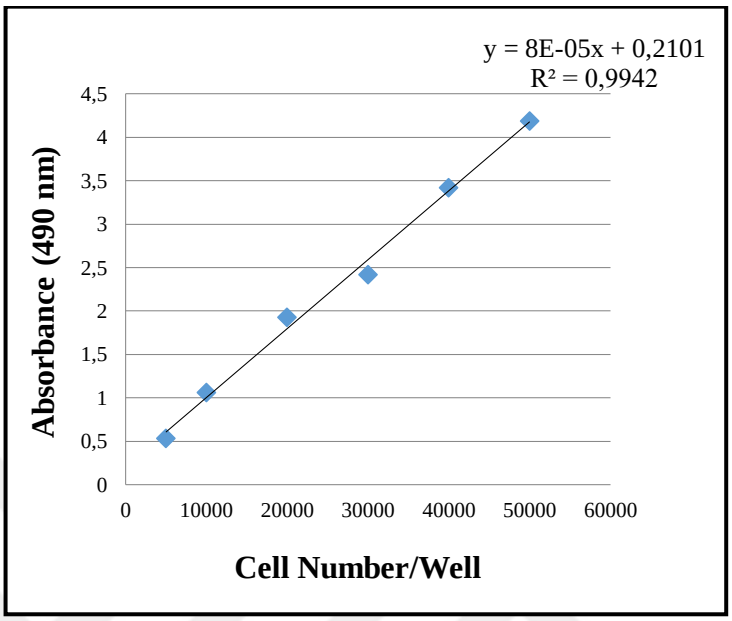

Figure A.1. Standard curve of hAD-MSCs absorbance at 490 nm versus cell number per well.

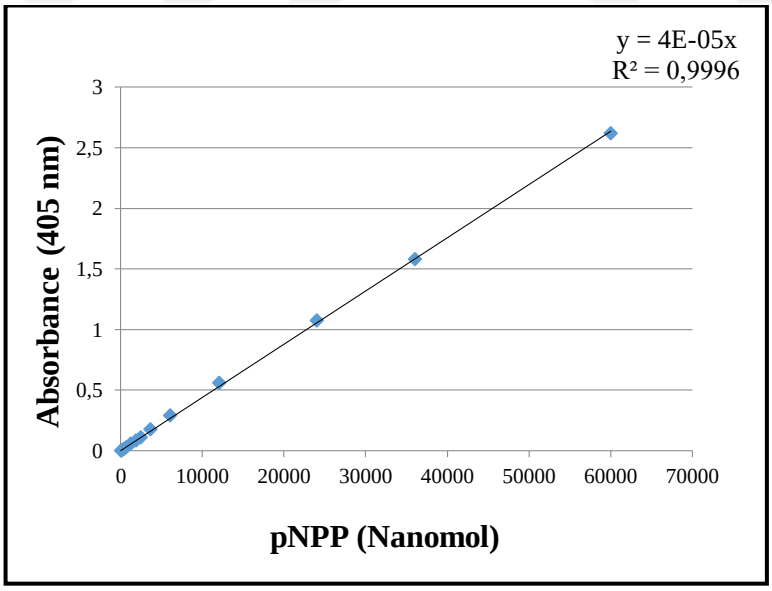

Figure A.2. Standard curve of alkaline phosphatase activity using p-nitrophenyl phosphate (pNPP) (nmol/µl) as a phosphates substrate that converts yellow (OD: 405 nm) when dephosphorylated by alkaline phosphatase.

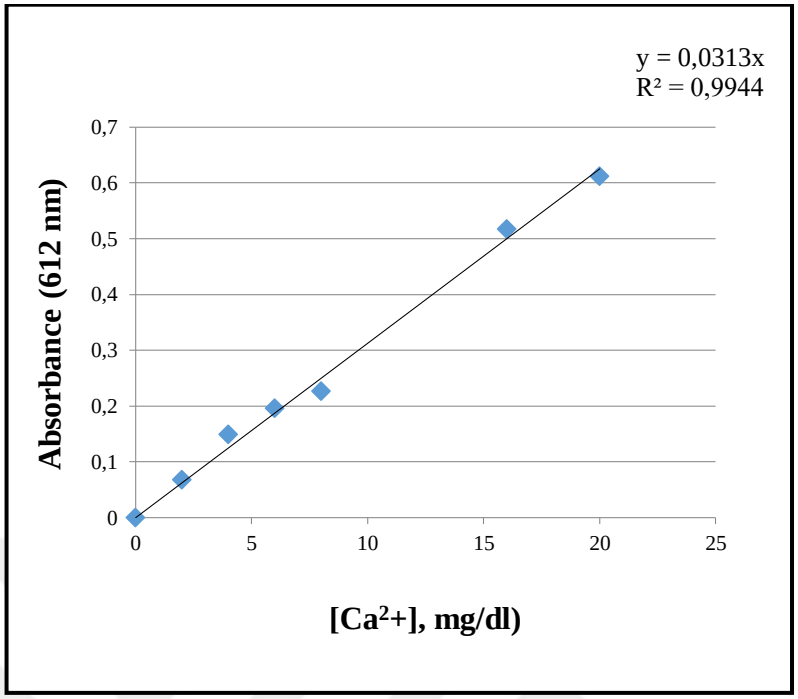

Figure A.3. Calibration curve of calcium concentration (mg/dl) according to the manufacturer instructions.

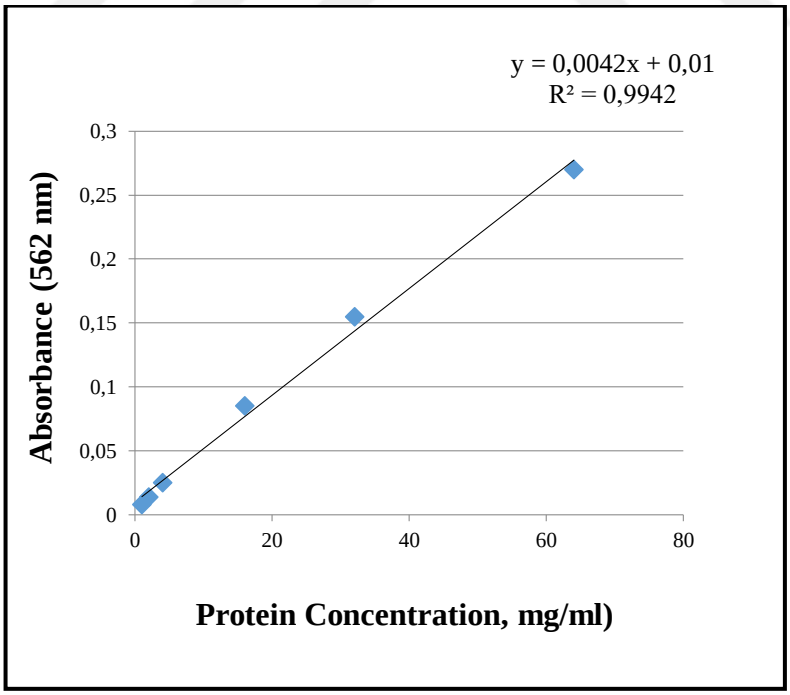

Figure A. 4. Standard curve for the Smart™ BCA protein assay (20-2,000 µg/ml), bovine serum albumine (standard) λ_{max}: 562 nm corrected for blank.

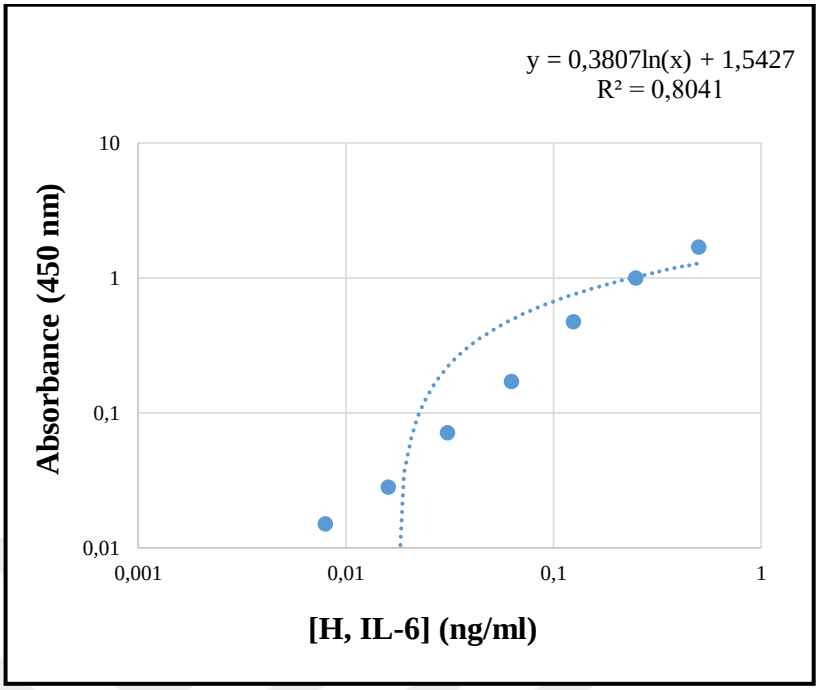

Figure A.5. Concentration of human IL-6 standard (ng/ml). Standard curve was generated by regression analysis using log-log four-parameter logistic curve-fit. λ_{max}: 450 nm.

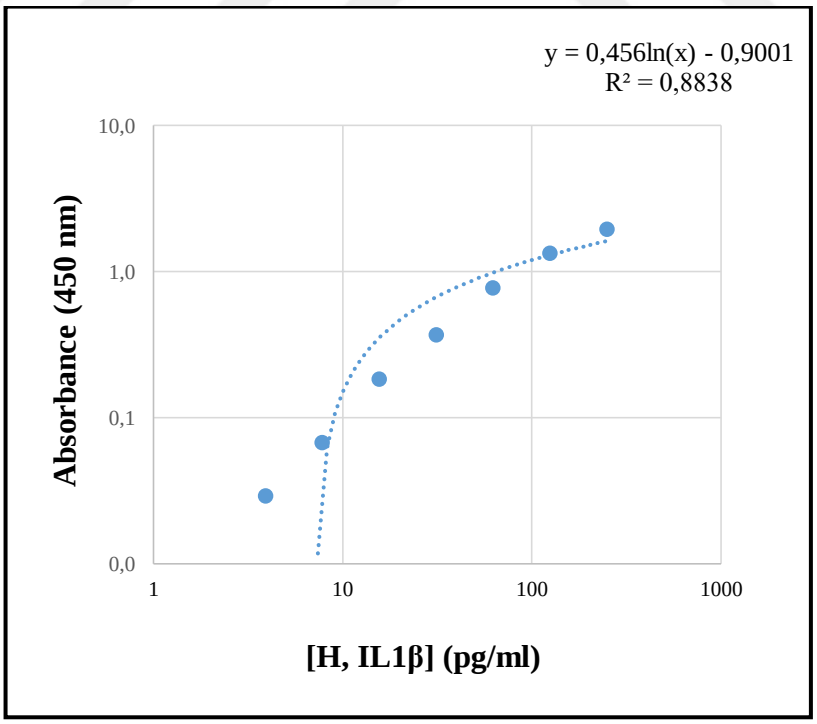

Figure A.6. Human IL-1β standard curve (pg/ml). Calibration curve was determined by regression analysis using log-log four-parameter logistic curve-fit. λ_{max}: 450 nm.

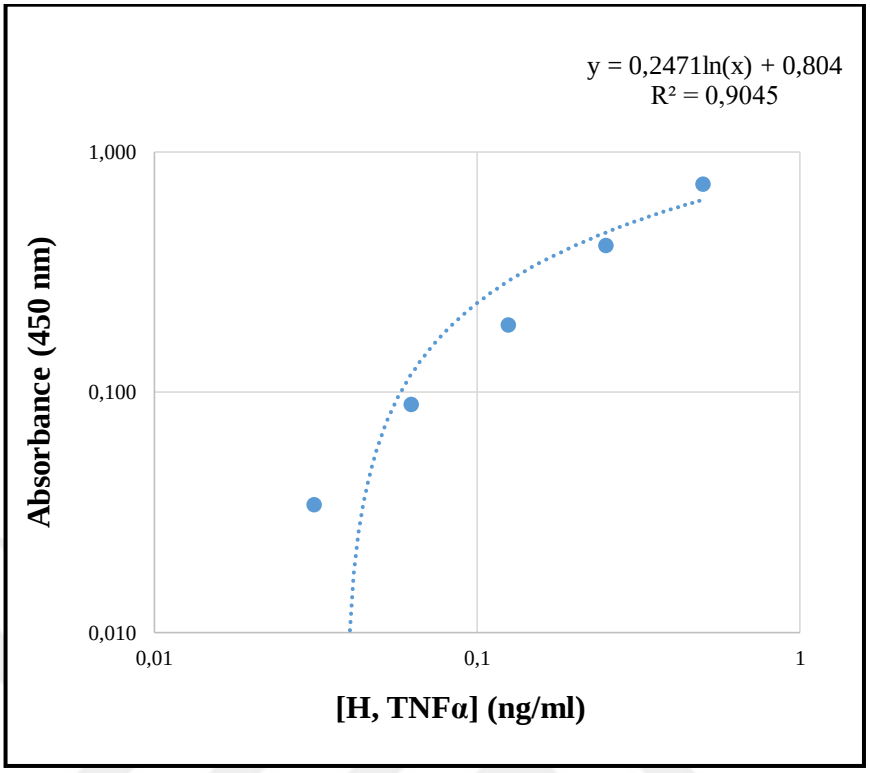

Figure A.7. Concentration of human TNFα standard (ng/ml). Standard curve was determined by regression analysis using log-log four-parameter logistic curve-fit. λ_{max}: 450 nm.