UNIVERSITY OF CALIFORNIA

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# Mandible versus Long Bone Marrow Cells

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Oral Biology

by

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ABSTRACT OF THE DISSERTATION

Mandible versus Long Bone Marrow Stromal Cells

by

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Doctor of Philosophy in Oral Biology

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The jaw and long bone (LB) are part of bones that make up the skeleton. Although the jaw is, to some extent, analogous to long bone and other bones of the body, they have a distinct developmental origin and mode of ossification. Systemic diseases affect the jaw differently compared to other bones, and the existence of jaw-specific bone pathologies suggests that it employs a different bone homeostatic mechanism. Marrow cells are the functioning orchestrators that maintain the balance between bone formation and bone resorption. The apparent distinction of mandible (MB) and its specific diseases justify that the understanding of MB cell functions and differentiation cannot be concluded from studies of other bone sites. Thus our overall *objective* is to study MB *vs.* LB marrow cell characteristics and functions. We, first, established a protocol for rat MB marrow cell isolation, including bone marrow stromal cells (BMSCs) and

osteoclast (OC) precursors. Characterization of BMSCs uncovered an enhanced ability of the MB *vs.* LB BMSCs to induce bone formation both *in vitro* and *in vivo*. Taking molecular differences into consideration, we assessed the potential clinical relevance of the MB *vs.* LB BMSC bone regeneration potential in the critical-sized intramembranous calvarial and endochondral femoral defects. MB BMSCs could regenerate both types of bone and produced better quality bone in intramembranous bone defects. Next, differences in osteoclastogenesis were investigated. Our data demonstrate that although the MB marrow contains an increased number of OC precursors, under parathyroid hormone and 1,25 dihydroxyvitamin$D_3$ stimulation, the LB marrow has a higher osteoclastogenic potential. This appears to be, at least in part, due to the higher RANKL stimulation and OPG inhibition of LB *vs*. MB BMSCs by these hormones. The differences in the MB *vs.* LB at the cellular level elucidate the existence of jaw specific diseases, particularly bisphosphonate-related osteonecrosis of the jaw (BRONJ). The jaw intrinsically has reduced osteoclast formation ability and cannot counteract the inhibitory effect of bisphosphonates to reestablish the normal bone resorption process. The increased sensitivity in nature of the jaw to antiresorptive treatments could explain, at least in part, the pathophysiology of BRONJ and its exclusive clinical manifestations in the jaw bone.

The dissertation of Thawinee Chaichanasakul is approved.

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# DEDICATION

To the loving memory of my grandparents.

To my family, who has shared in and has shaped my life's journey

To my parents, Larry and Dee Dee, who have been a constant source of inspiration and encouragement.

To my sister, Chalisa, who I look up to for strength of character and a positive perspective of life.

To my brother, Andy, who always puts family first and always makes me proud.

And to Yang, my husband and my best friend, who loves me unconditionally and believes in me more than I ever believe in myself.

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| | |
|---|---|
| $1,25D_3$ | $1\alpha,25$-dihydroxyvitamin$D_3$ |
| ALP | Alkaline phosphatase |
| BMD | Bone mineral density |
| BMPs | Bone morphogenetic proteins |
| BMSCs | Bone marrow stromal cells |
| BRONJ | Bisphosphonate related osteonecrosis of the jaw |
| BV | Bone volume |
| CD36 | Cluster differentiation 36 |
| CFU | Colony-forming unit |
| CFU-F | Colony-forming unit fibroblast |
| Col IIa1 | Collagen II alpha 1 |
| Col Xa1 | Collagen X alpha 1 |
| CPs | Cysteine proteinases |
| CSD | Critical-sized defect |
| CV | Calvaria |
| DBM | Demineralized bone matrix |
| DPP | Dentin phosphoprotein |
| DSP | Dentin sialoprotein |
| DSPP | Dentin sialophosphoprotein |
| FBS | Fetal bovine serum |
| FISH | Fluorescence in situ hybridization |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| H&E | Hematoxylin and eosin |
| HSCs | Hematopoietic stem cells |
| IGF | Insulin-like growth factor |
| LB | Long bone |
| LPL | Lipoprotein lipase |

| | |
|---|---|
| M-CSF | Macrophage stimulating factor |
| MB | Mandible |
| MMPs | Matrix metalloproteinases |
| MNCs | Multinucleated cells |
| MSCs | Mesenchymal stem cells |
| N.Oc/B.Pm | Number of osteoclasts per bone perimeter |
| OCN | Osteocalcin |
| OCs | Osteoclasts |
| ONJs | Osteonecrosis of the jaws |
| OPG | Osteoprotegrin |
| ORN | Osteoradionecrosis |
| PBS | Phosphate buffered saline |
| $PGE_2$ | Prostaglandin $E_2$ |
| PPARγ | Peroxisome proliferator-activated receptor-γ |
| PTH | Parathyroid hormone |
| qPCR | Real-time (quantitative) polymerase chain reaction |
| RANKL | Receptor of activator of NFκB ligand |
| RBCs | Red blood cells |
| RT | Reverse transcriptase |
| SEM | Standard error of mean |
| Sox 9 | SRY-box containing gene |
| TGFβ | Transforming growth factor-β |
| TRAP | Tartrate resistant acid phosphatase |
| TRAP | Tartrate resistant acid phosphatase |
| TV | Tissue volume |
| $V_D$ | Vehicle of 1α,25-dihydroxyvitamin$D_3$ |
| veh | Vehicle |
| $V_P$ | Vehicle of parathyroid hormone |
| α-MEM | Alpha minimum essential medium |

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## PUBLICATIONS AND PRESENTATIONS

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# CHAPTER 1

# INTRODUCTION

## 1.1 Mandible versus Long bone

### *1.1.1 Development and Mode of Ossification*

The complete vertebrate skeleton is composed by the axial and appendicular skeletons. The axial skeleton is the core of the body that consisted of the spine, sternum, ribs, and skull (also known as craniofacial bones). Attached to the core bones is the appendicular skeleton, which consists of bones of upper and lower limps (Karaplis 2008). Skeletogenesis of these bones is achieved by unique embryonic skeletal cell populations. The craniofacial skeleton originates from neural crestal cells of neuroectodermal germ layer, while other axial and appendicular bones arise from cells of mesoderm layers (Chai and Maxson 2006; Karaplis 2008).

The bone formation process involves two primary mechanisms: endochondral and intramembranous ossification. Endochondral ossification requires a cartilaginous template for calcification of bone that involves a series of formation and degradation processes of the cartilaginous framework. Intramembranous ossification, on the other hand, relies on the condensation of mesenchymal cells at the ossification center without the requirement of the cartilaginous element (Karaplis 2008). The mandible (MB) is an integral bone structure involved in mastication, an essential process affecting the quality of life. Together the maxilla and mandible make up the jaw bone. The jaw bone is considered to be a part of the craniofacial bones, and it follows similar embryological development. A study utilized a two-component mouse transgenic model to follow the migration and differentiation of neural crestal cells and

confirmed, besides craniofacial skeletal structures, these cells also contributed to the formation of the mandible as well as Meckel's cartilage (Chai et al. 2000).

Mandible bone formation employs both chondrogenesis and osteogenesis. During early development, the mandible is formed primarily by intramembranous ossification. At the later stage, secondary cartilages, which includes coronoid, condylar, and angular cartilage, at the mandible proximal end undergoes endochondral ossification. Meckel's cartilage presents briefly during mandible development. It has an important role in mandibular morphogenesis and possibly acts as a template for mandible formation (Chai et al. 2000; Chai and Maxson 2006; Ramaesh and Bard 2003). Long bones (LBs) such as femur and tibia bones, are part of appendicular skeleton. Functionally, they are the weight bearing bones that involve in movement and locomotion. These bones are derived from the mesoderm and exclusively undergo endochondral ossification. This involves the process where bone calcifies over a preformed cartilage (Mackie et al. 2008).

### *1.1.2 Homeostatic Mechanism*

Besides differences in developmental origins and the mode of ossification, the MB possesses a distinctive homeostatic mechanism, a maintenance process under the influence of mechanical and non-mechanical stimuli to achieve a balance between bone formation and bone resorption. It has been well understood that the lack of mechanical loading or immobilization of bone disturbs the homeostatic equilibrium that results in bone loss and deterioration of bone structures (Jaworski et al. 1980; Sievänen 2010; Uhthoff and Jaworski 1978). Mechanical loading varies in different bone types with respect to amplitudes, rates and frequencies. A

previous study recorded a long term *in vivo* daily habitual bone strain history of the tibiofibula and MB in rabbit. In comparison to the LB strains, the MB strain differed in frequency featuring a strong rhythmic or cyclic pattern simulating repetitive masticatory functions (de Jong et al. 2010). In fact, mechanical loading from masticatory force directly affects bone mineral density (BMD) of the mandible. Several studies compared the effect of the alteration in masticatory force on the mandible and found that its BMD was greatly reduced in masticatory hypofunction (i.e. soft food diet and removal of molar teeth) while the opposite pattern was observed when animals received increased masticatory demand (i.e. bite-block insertion) (Mavropoulos et al. 2004, 2005; Patullo et al. 2009; Rawlinson et al. 2009b).

Non-mechanical stimuli such as local and systemic factors play a major role in bone homeostatic mechanism. These factors include: 1) calcium-regulating hormones such as parathyroid hormone (PTH), 1,25-dihydroxyvitamin $D_3$ and calcitonin, 2) systemic hormones such as growth hormone (IGF), estrogen and glucocorticoids, and 3) local factors such as prostaglandin $E_2$ ($PGE_2$), insulin-like growth factor (IGF) and transforming growth factor-β (TGF-β) among others. Abnormality in the secretion or production of these factors contributes to the development of metabolic bone diseases, notably osteoporosis, hyperparathyrodism and Paget's disease of bone. Osteoporosis is a disease in which a decrease in bone mass and loss of bone structure increase risk of fractured. It is an age-related disease commonly affecting women after menopause when production of estrogen is reduced. Hyperparathyroidism is caused by an over production of PTH that causes more calcium to be released from the bones by increasing bone resorption activity. The compensatory mechanism for a hypocaemic condition, however, causes osteitis fibrosa cystica, a diffuse bone loss with fibrous replacement that weakens bone

structures (Adams 2006). Unlike, the first two metabolic bone conditions, the causation of Paget's disease of bone or osteitis deformans is unknown. It is characterized by abnormal bone remodeling with excessive increase in bone resorption and bone formation, resulting in weak bone structure (Roodman 2005). All three examples of those metabolic bone diseases systemically affect all bones of the body including the mandible. More evidence has emerged emphasizing the correlation between mandibular bone density and osteoporosis (Jeffcoat et al. 2000; Kribbs et al. 1989; Lerner 2006; Mavropoulos et al. 2007). The susceptibility of the mandible to estrogen deficiency, modeling osteoporosis, was further investigated in an ovariectomized rat model and found that the mandible was far less affected by this bone loss induced model in comparison to the tibia and femur (Kuroda et al. 2003; Yang et al. 2003). Considering the multifaceted nature of osteoporosis etiology, a study that combined the effect of estrogen depletion and protein malnutrition found the MB loses significantly less bone than the proximal tibia (Mavropoulos et al. 2007). Similarly, hormones important in bone metabolism exert a distinctive effect on MB and LB mineralization. Mineralization of the MB was affected by $1,25D_3$ deficiency but unaltered by the abolishment of PTH in contrast to LB that was affected by both hormones (Liu et al. 2009). Clearly, the MB is far less affected by these systemic changes in comparison to the LB and also suggests different homeostatic mechanisms of the two bones.

### *1.1.3 Cellular Heterogeneity*

Bone marrow contains two main populations of adult stem cells: bone marrow stromal cells (BMSCs) and hematopoietic stem cells (HSCs). Endothelial progenitor cells which give

rise to blood vessels have been found in circulating blood and identified as originating from the bone marrow (Asahara et al. 1997; Shi et al. 1998). These cells have been considered as a third population of adult bone marrow stem cells, but for simplicity the two main populations will be discussed in this section.

***Bone Marrow Stromal Cells***

Bone marrow stromal cells are mixture of multipotential cell populations that are capable of self-renewal and form colonies from a single cell called colony forming unit-F (CFU-F). These colonies can differentiate into mesodermal cell lineages such as osteoblasts, chondrocytes, and adipocytes (Bianco et al. 2001; Friedenstein et al. 1966, 1970; Owen 1988). There have been reports that BMSCs also have the capacity to transdifferentiate into ectodermal and endoderm cell lineages including epithelial cells, neurons, muscle cells and hepatic cells (Kopen et al. 1999; Petersen et al. 1999). Mesenchymal stem cells (MSCs) present in bone marrow also have the ability to differentiate into these cell lineages, and they are the common predecessors of the BMSCs (Pittenger et al. 1999; Uccelli et al. 2008). In fact, these nomenclatures have been used interchangeably because the pluripotency or the stemness of these cells is still a subject of controversy. For that reason, we refer to these cells as bone marrow stromal cells, which is also widely accepted by the majority (Prockop 2009).

At physiological conditions, BMSCs have an important function in maintaining bone homeostasis, and the efficacy of this function depends on their response to stimulation by various systemic and local mediators (Osyczka and Leboy 2005; Yang et al. 2009; Zhou et al. 2010). Human orofacial (mandible and maxilla) BMSCs have an enhanced response to osteogenic

differentiation media in comparison to iliac crest-derived BMSCs from the same individuals (Akintoye et al. 2006; Matsubara et al. 2005). Consistent with these reports, rodent and murine mandible BMSCs were also found to have a higher osteogenic differentiation potential in comparison to those derived from long bones (**Chapter 3)** (Aghaloo et al. 2010; Yamaza et al. 2011). Among many other osteoinductive growth factors, bone morphogenetic proteins (BMPs), especially BMP-2, promotes osteoblast differentiation in vitro and facilitates in vivo bone formation (Fromigué et al. 1998; Thies et al. 1992; Wang et al. 1990). In a similar trend, mandible and maxilla BMSCs were found to have an enhanced osteogenic responsiveness to BMP-2 compared to iliac crest BMSCs (Osyczka et al. 2009).

Besides normal maintenance functions in physiological conditions, BMSC response to external insults such as drugs or radiation is considerably more significant. Bisphosphonate related osteonecrosis of the jaw (BRONJ) is a side effect of nitrogen-containing bisphosphonate such as pamidronate and zoledronic acid. Those drugs are used for the treatment of osteoporosis, bone metastasis, multiple myeloma and other bone conditions (Russell and Rogers 1999). Clinical manifestation of BRONJ presents exclusively in maxilla and mandible bones, indicating that bisphosphonates may favor these bone sites. Although bisphosphonates are potent inhibitors of osteoclasts, they might exert some action on BMSCs. In the presence of pamidronate, decrease in proliferation rate and lower alkaline phosphatase activity were observed in human MB BMSCs. Though the underlying mechanism is unknown, pamidronate affected cell survival and osteogenic properties of human mandible BMSCs more than iliac crest BMSCs (Stefanik et al. 2008). Another instance of differential BMSC response has been observed through osteoradionecrosis (ORN). ORN is a nonhealing exposed irradiated bone of at least 3 months in

duration without a residual or recurrent tumor (Madrid et al. 2010). It is a severe complication of radiation therapy with higher incidence in the head and neck compared to axial and appendicular bones, 3-14% and 0.44%, respectively (Feltl et al. 2006; Morrish et al. 1981). The variable incidence of ORN in different skeletal sites led to a speculation that there might be a disparity in BMSC response and recovery from irradiation in different bone sites. Surprisingly, orofacial BMSCs were found to survive higher radiation doses and recover quicker compared to iliac crest BMSCs (Damek-Poprawa et al. 2010). Evidently, there is a disparity in BMSC response between mandible and axial/appendicular bones at physiological conditions and in the presence of external insults. The strikingly higher osteogenic property of MB BMSCs put an emphasis on skeletal-site specific disease and regeneration.

***Bone Marrow Derived Osteoclasts***

Discovered in the early 1960s, hematopoietic stem cells (HSCs) are capable of self-renewal, give rise to all the different types of blood cells and can be isolated from circulating blood or bone marrow (Till and Mcculloch 1961). They are indeed another main population residing in the bone marrow either next to the osteoblast lining of the endosteal bone surface or next to endothelial cells of sinusoidal vessels in the marrow cavity (Yin and Li 2006). Specifically, HSCs give rise to two cell types: myeloid (monocytes, macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/ platelets, dendritic cells) and lymphoid lineages (T cells, B cells, and NK cells) (Yin and Li 2006). Monocytes, granulocyte/macrophage colony-forming unit precursors of the common myeloid lineage, eventually give rise to macrophages and osteoclasts (Gordon and Taylor 2005). Osteoclasts (OCs) are multinucleated

cells whose differentiation and maturation requires macrophage stimulating factor (M-CSF) and receptor of activator of NFκB ligand (RANKL), cytokines supplied among others by BMSCs (Boyle et al. 2003). The importance of RANKL in osteoclast differentiation is demonstrated in RANKL null mice, which portrayed osteopetrotic phenotype (Odgren et al. 2003). Interestingly, when RANKL knockout mice were rescued with lymphocyte-promoter driven RANKL expression, osteoclast formation was recovered only in the long bones but not in the MB. This finding suggests that the MB preosteoclasts respond differently to RANKL presented by the immune cells. Therefore, MB osteoclast precursors might also respond differently to RANKL supplied by BMSCs.

The hallmark of osteoclastic function is bone resorptive ability, which involves sequential steps of mineral dissolution and degradation of collagen matrix (Teitelbaum 2000). The latter process is accomplished by collagenolytic proteinases, notably cysteine proteinases (CPs) and matrix metalloproteinases (MMPs) (Delaisse et al. 2000). Of these proteinases, Cathepsin K, belonging to the CP family, is well established as a rate-limiting enzyme important in osteoclast-mediated organic matrix resorption (Teitelbaum 2000). The MMPs synthesized by OCs are MMP-3, -9, -10, -12, and -14, but the MMP key enzyme important in collagenolysis is still undefined (Andersen et al. 2004; Delaiss et al. 2003). Substantial evidence supports skeletal-site specific divergency in osteoclastic function due to differential usage of proteinases in organic matrix resorption (Everts et al. 1999a, b, 2006, 2009; Shorey et al. 2004; Zenger et al. 2010). For example, calvarial OCs were shown to depend on MMPs in collagen matrix degradation more than CPs, in contrast, LB OCs were shown to depend primarily on CPs (Everts et al. 1999b). Additionally, the cathepsin K knockout mouse model demonstrated that, in absence of

cathepsin K, remodeling in the calvarial bone was altered to a lesser extent in comparison to long bone (Everts et al. 2009). Though MMP expression in MB osteoclasts has not been explored, MMP was found to participate in the regulation of the MB development and formation (Chin and Werb 1997). Interestingly, the pattern of proteinase usage was also mirrored in the comparison of osteoclasts derived from long bone and scapula, another intramembranous bone (Shorey et al. 2004). Aside from the proteinases above, tartrate resistant acid phosphatase (TRAP), an enzyme that is highly expressed in all osteoclasts, also showed differential expression in calvarial and LB OCs, but results are inconclusive (Perez-Amodio et al. 2006; Zenger et al. 2010). Undoubtedly, there exist bone site-specific differences in OCs. Recently, murine jaw and LB marrows were investigated for their osteoclastogenic potential. In the presence of M-CSF and RANKL, more osteoclasts were formed in LB cultures in an early differentiation period than the jaw cultures, but this difference was not observed in the later time point. LB marrow contained more osteoclast precursors, particularly myeloid blasts. Additionally, TRAP expression was found to be higher in LB cultures, however, the resorptive activities were comparable between the two cultures (Souza Faloni et al. 2010). The substrate on which OCs are generated has a pivotal influence on the formation of OCs. More OCs formed from LB marrow cells on bone substrate compared to dentin, while this pattern is reverse with jaw marrow cells (Azari et al. 2011). The culturing substrates also influence the shape of osteoclasts. The jaw cultures contained larger OCs on plastic and on dentin, while LB cultures formed a higher percentage of multi-compartmented osteoclasts (Azari et al. 2011; Souza Faloni et al. 2010). Bone-site specific differences in OCs became apparent between the jaw and long bone; however, more evidence

and further studies are needed to elucidate the disparity in osteoclastogenesis of the two bone sites.

## 1.2 Significance of Mandible Study

The divergency in the development, mode of ossification, and homeostatic mechanism of the MB and LB is well supported. Growing evidence suggests the differences in cellular response as another crucial factor distinguishing the MB and LB as separate entities. Yet little is known about the cellular and molecular basis for mandibular *vs.* long bone divergent homeostasis. The sparseness of supporting data might be due to the fact that the orofacial bones are small and complex in anatomy, rendering a challenge to bone cell isolation (Sodek and McKee 2000). In any case, there are clear distinctions between the MB and LB, therefore conclusions about the MB cellular function and homeostatic mechanism should not be drawn from the cells of other skeletal sites. Crucially, there exist jaw specific diseases such as periodontitis, cherubism (Ueki et al. 2001), hyperparathyroid jaw tumor syndrome (Simonds et al. 2002), and recently revealed bisphosphonate related osteonecrosis of the jaw (BRONJ) (Khosla et al. 2007; Ruggiero et al. 2009) that make the mandible unique and in need of further studies.

## 1.3 Bone Remodeling

Bone remodeling is a lifelong process that occurs via the coupling of bone formation to bone resorption. An intricate balance of these processes maintains skeletal homeostasis. Osteoblasts, originated from multipotent BMSCs, are the predominant players of the bone

formation process, while the bone resorption process relies on osteoclasts, cells from hematopoietic precursors of the monocyte-macrophage origin.

Examining the stem cell niches within the bone marrow cavity, a portion of HSCs resides next to the endosteal bone surface which is lined primarily by osteoblasts (Gong 1978; Yin and Li 2006). Osteoblasts and osteoclasts function in synergy to conserve this equilibrium, and their anatomic location is suggestive of their reciprocal communication (Yin and Li 2006). Evidence for this interaction comes from studies demonstrating that osteoblasts/stromal cells produce hematopoietic factors such as RANKL and macrophage colony stimulating factor (M-CSF) that control the differentiation of osteoclasts (Boyle et al. 2003; Calvi et al. 2003; Martin and Ng 1994; Taichman et al. 1996). Mechanistically, RANKL and M-CSF, expressed by osteoblasts/stromal cells, bind to RANK (RANKL receptor) and c-FMS (receptor of M-CSF) on osteoclast precursors that leads to osteoclast activation and maturation (Teitelbaum 2000). M-CSF is important as a determinant factor of osteoclast precursor cells, but RANKL is required for differentiation, fusion into multinucleated cells, activation and survival of osteoclastic cells (Hsu et al. 1999; Khosla 2001; Udagawa et al. 1990). Moreover, RANKL-deficient mice exhibit a complete lack of osteoclasts, signifying the importance of RANKL in osteoclastogenesis (Kong et al. 1999; Odgren et al. 2003). The RANKL-RANK activation maintains normal bone resorption but in pathological conditions its activation is associated with excessive bone loss (Wada et al. 2006). Neutralizing the activity of RANKL, osteoblasts/stromal cells also produce osteoprotegerin (OPG), a decoy receptor that competes with RANKL for RANK binding (Lacey et al. 1998; Simonet et al. 1997; Yasuda et al. 1998). Thus, the OPG/RANKL/RANK system has emerged as a dominant mediator of osteoclastogenesis, and many metabolic bone diseases are

caused by or are related to the alterations of this system (Hofbauer et al. 2004; Khosla 2001; Theoleyre et al. 2004).

### *1.3.1 Regulation of Bone Remodeling*

#### *Systemic and Local Regulators*

Bone remodeling is regulated by systemic hormones and local factors, which affect both osteoblastic and osteoclastic cell lineages and exert their effects on the differentiation and functions of these cells. Systemic regulators of bone remodeling include calciotropic hormones such as PTH, $1,25D_3$, calcitonin, and other hormones such as growth hormone, glucocorticoid, thyroid hormones, and sex hormones (Hadjidakis and Androulakis 2006). The two systemic regulators of interest are the PTH and $1,25D_3$. They act directly on their receptors expressed on osteoblast/stromal cells to control the bone formation process, while their effects on osteoclast and bone resorption process are mediated by osteoblast/stromal cells (Mee et al. 1996; Rodan and Martin 1981). PTH is considered the most important humoral regulator of bone homeostasis because it exerts both anabolic and catabolic effects on bone depending upon the exposure duration. Continuous PTH exposure has been shown to decrease osteoblast differentiation and bone resorption, whereas intermittent PTH increases osteoblast differentiation and increase bone formation (Jilka et al. 2010; Lotinun and Sibonga 2002; Lotinun et al. 2004; Miller et al. 1997; Yang et al. 2009). $1,25D_3$ has dual roles in bone remodeling. It enhances osteoclast formation and bone resorption via action mediated by osteoblasts. It can also stimulate bone formation and augments osteoblast precursors recruitment (Erben et al. 1997; Zhou et al. 2010). Finally, local

factors also play a key role in regulating the bone remodeling process. They are molecules synthesized by bone cells such as insulin-like growth factors (IGFs), prostaglandins, tumor growth factor-beta (TGF-β), bone morphogenetic proteins (BMP), and cytokines (Hadjidakis and Androulakis 2006).

***Regulation of the OPG/RANKL/RANK system***

Since the discovery of the OPG/RANKL/RANK system, the emphasis of bone remodeling has been on the control of osteoclastogenesis through this triad. The systemic and local factors plus additional compounds also modulate OPG, RANKL and RANK expression. A complete detailed list of all modulators of this system has been thoroughly reviewed (Theoleyre et al. 2004). Some examples of these modulators and their actions are: 1) PTH, increased RANKL/decreased OPG production, 2) $1,25D_3$, increased RANKL production, 3) glucocorticoids, increased RANKL/decreased OPG production, and TGFβ and estrogen, increased OPG production (Hofbauer et al. 1999; Huang et al. 2003; Kitazawa et al. 1999; Khosla 2001; Saika et al. 2001; Thirunavukkarasu et al. 2001).

The interdependence of osteoblasts and osteoclasts is governed by BMSC support. Besides providing progenitor cells for osteoblast differentiation, BMSCs also mediate osteoclastogenesis via the RANKL/OPG system. A recent study found that the ability of preosteoblastic/stromal cells to support osteoclastogenesis is rapidly lost due to a decrease in RANKL and an increase in OPG production during osteoblast differentiation (Gori 2000). Abnormalities of bone remodeling often result from aberrant production of the systemic and local factors. Thus, BMSC response to perturbations via the expression of RANKL/OPG is important in osteoclastogenesis and the overall maintenance of normal bone remodeling process.

## 1.4 Skeletal Defect and Regeneration

### *1.4.1 Skeletal Regeneration Mirroring Skeletal Development: Skeletal Site-Specific Stem Cells from Craniofacial vs. Appendicular Bones*

Bone formation is a continual process that begins during embryonic development of bone and persists through life in a form of bone remodeling and bone repair. The skeletal repairment is a fascinating process because the healed tissue is indistinguishable from the adjacent normal tissue while other adult tissues heal with scar tissue formation (Colnot et al. 2003). The indistinction between healed and normal bones is probably due to the fact that the same molecular signaling of chondrogenesis and osteogenesis are shared between skeletal development and bone repair (Colnot et al. 2003; Ferguson et al. 1998). Since the MB and LB form with different modes of ossification, differential reparative mechanisms are also expected. Comparing molecular mechanisms responsible for the healing of different bone sites, however, is a challenge because endochondral bones actually share similar regulatory molecules as intramembranous bones during cartilage-to-bone replacement. Likewise, some of the intramembranous bones may develop partly with the chondrogenic phase (Eames et al. 2003). Indeed, when comparing cartilage and bone tissue of the limb and head skeleton, the molecular markers of cartilage and bone are practically identical (Eames and Helms 2004).

Another speculation has been raised whether there is a different developmental cell lineage which could contribute to unique stem cell populations for site-specific bone regeneration. Previously introduced in the earlier section, the MB and LB arise from neural crestal and mesodermal cells, respectively. To investigate these two cell populations, a study utilized an exquisite cell labeling approach, *WntCre::Z/EG* transgenic mouse model, which

resulted in GFP labeling of *Wnt1*-expressing neural crest cells and mesodermal cells that are positive for β-galactosidase activity. The mandibles and tibiae from *WntCre::Z/EG* mice were then examined after the introduced skeletal injuries healed. At the injury site of each bone, GFP positive neural crest cells were only observed in the mandible, while cells positive for β-galactosidase activity were seen only in the tibia. Next they investigated if these cell lineages were able to interchangeably heal injury sites of the other cell lineage by grafting mesodermal cells into mandibular defects and vice versa. The results indicated that skeletal stem cells maintained their 'positional memory' that dictates the fate of cell differentiation (i.e. mesodermal cells in mandibular defects differentiated into chondrocytes). Furthermore, the Hox gene expression, determinants of the pattern and morphogenesis fetal skeletal development, might influence the fate of adult skeletal stem cells at the injury sites (Leucht et al. 2008). A comprehensive gene expression analysis of bone organs and adult bone-derived cells from rodent skull and limb found that the adult bone and bone cells maintain a site-specific gene expression associated with their development (Rawlinson et al. 2009a). Together, this notion indicates that skeletal defects heal through recruitment of progenitor cells of their own origin and reinforces that there exists bone site-specific skeletal stem cells from craniofacial *vs.* appendicular bones that are responsible for adult bone regeneration (Eames and Helms 2004; Helms and Schneider 2003; Leucht et al. 2008).

### *1.4.2 Bone Grafting Models*

Bone is an important organ that protects and provides structural support for many organs of the body. Substantial loss or deformity of the bone tissue is devastating, both physically and psychologically, and adversely affects the quality of life. Such large osseous defects may be the results of severe trauma, infection, cancer, tumor resection or congenital malformations and post challenges to reconstructive surgeons. Although bones posses their own healing ability, large bony defects often require bone grafts or bone graft substitutes to aid in completion of the bone replacement. Bone grafting has been a conventional treatment modality for many years which include autogenous bone, allografts, and xenografts (Damien and Parsons 1991; De Long et al. 2007). Each type of bone grafting and bone grafting substitutes involves three essential elements: osteogenesis, osteoinduction and osteoconduction (De Long et al. 2007; Giannoudis et al. 2005). Osteogenesis is the generation of bone from bone-forming cells. Osteoinduction is defined as a process that supports the mitogenesis of undifferentiated mesenchymal cells, leading to the formation of osteoprogenitor cells that form new bone. While osteoconduction denotes a property of a matrix that supports the attachment of bone-forming cells (De Long et al. 2007; Giannoudis et al. 2005).

Autogenous bone or autograft has long been regarded as the gold standard graft for augmentation of bone healing. This is because autogenous bone possesses all three elements by providing osteoinductive growth factors, osteogenic cells, and osteoconductive scaffold (Giannoudis et al. 2005). Comparing to allograft and xenograft, autograft has a greater osteogenic capability with the following common donor sites: iliac crest, tibia, femur, and ribs (Damien and Parsons 1991). Additionally, the inherent compatibility with host tissues yields

little to no immunologic reaction. Although the benefits of autograft are paramount, there is a limited availability of host bone supply, and harvesting autogenous bone is an invasive procedure that increases the morbidity risk to the donor site (Arrington et al. 1996; Damien and Parsons 1991; Ross et al. 2000).

Allograft is a common alternative to autograft and is prepared in fresh, frozen or freeze-dried forms (Giannoudis et al. 2005). Benefits of allograft include sparing the donor site morbidity, being abundantly available, and in some forms, having a long shelf-life (De Long et al. 2007; Giannoudis et al. 2005). However, disease transmission and immunologic response are major risks and disadvantages of allograft, especially when using the fresh preparation. The immunologic reaction is dampened by freezing or freeze-drying the bone graft. These processes and further sterilization destroy osteogenic cells and lessen osteoinductive property (De Long et al. 2007; Giannoudis et al. 2005). This type of graft also exhibits a higher resorption rate and a lower revascularization ability (Damien and Parsons 1991).

Xenograft, a bone graft from another species, carries histocompatibility antigens different from the recipient and can cause immunologic response with higher risk of rejection (Damien and Parsons 1991). Processes such as deproteination and defatting decrease the antigenic response from the xenograft but, similar to allografts, osteoinductive proteins are also removed during the course of treatment (Damien and Parsons 1991). With these disadvantages, xenograft is not a common choice of bone graft used in bone augmentation.

Donor site morbidity and limited amounts of autogenous bone are the major drawbacks of autogenous bone grafting. Similarly, the use of allografts and xenografts may be associated with an increased risk of rejection, disease and infection. To circumvent the limitations of bone

grafting, several bone graft substitutes have been developed and categorized based on the three essential characteristics of bone graft: osteoinductive, osteoconductive and osteogenic bone graft substitutes (De Long et al. 2007; Giannoudis et al. 2005). Common osteoinductive bone graft substitutes include demineralized bone matrix (DBM) and BMPs. DBM is produced by acid extraction of human cadaver bone or allograft and contains type-1 collagen, non collagenous proteins and osteoinductive growth factors (De Long et al. 2007; Friedlaender 1983; Giannoudis et al. 2005). The demineralization process does not remove bone growth factors making DBM an excellent osteoinductive material. Commercially, it is available in many forms such as freeze-dried powder, gel, putty and strips that are convenient to use. Though DBM is often used as a bone graft substitute, it is commonly used in conjunction with bone grafts (Giannoudis et al. 2005; Sassard et al. 2000; ). The osteoinductive property of DBM is due to an osteogenic protein within the bone matrix, which was discovered and identified as BMPs (Urist 1965). BMPs function by inducing uncommitted progenitor cells into chondroblastic, osteoblastic and/or adipocytic cells depending on the timing of administration and the differentiation stage of the target cells (Asahina et al. 1996). Clinically, large dosages of BMPs are required to induce adequate bone formation in human (Govender et al. 2002). Additionally, functional heterogeneity and nonspecificity of BMPs lead to many side effects such as bone resorption, ectopic bone formation, adipogenic differentiation, and tumor growth (Kaneko et al. 2000; Luo et al. 2008; Moerman et al. 2004). Osteoconductive bone graft substitutes are generally calcium phosphate synthetic substrates that are an excellent osteoconductive matrix for host osteogenic cells (De Long et al. 2007). However, they do not have high structural integrity, and because of the lack of osteoinductive factors, they often cannot be used alone as a pure source of bone graft

substitutes (De Long et al. 2007). To date, osteogenic bone graft substitutes with sufficient evidence to support potential efficacy is bone marrow aspirate (De Long et al. 2007). Within the bone marrow aspirate, an important population of stem cells capable of bone formation and other therapeutic potential is BMSCs.

### *1.4.3 Bone Tissue Engineering: BMSCs as Ideal Alternative to Autogenous Bone*

With limited supply and several disadvantages of bone grafting, recently, bone tissue engineering has emerged as a promising approach for bone defect regeneration, and BMSCs are the most reliable cell source for tissue engineering. As alluded to in the previous section, BMSCs are multipotential cells residing within the bone marrow and are capable of differentiating into various mesenchymal lineages, including osteoblasts, chondrocytes and adipocytes (Bianco et al. 2001; Pittenger et al. 1999). They are an excellent source of osteoprogenitors and their osteogenic potential has been well described *in vitro* (Friedenstein et al. 1970, 1987; Owen and Friedenstein 1988). BMSC osteogenic ability has been further demonstrated *in vivo*, as evidenced by bone formation following ectopic implantation of human marrow stromal cells in immunocompromised mice (Kuznetsov et al. 1997; Mendes et al. 2004; Niemeyer et al. 2006). BMSCs can be isolated with ease and can be expanded by subculturing. In fact, human BMSCs survive as many as 15 passages without losing their differentiation potential (Tsuchida et al. 2003). BMSCs are resilient as their growth or differentiation is relatively unaffected by the cryopreservation and thawing process (Jafarian et al. 2008; Tsuchida et al. 2003). In addition to these excellent characteristics, BMSCs possess immunosuppressive

and anti-inflammatory properties which make them a good candidate for bone defect regeneration and an ideal alternative to the gold standard autogenous bone grafting (Kagami et al. 2011; Uccelli et al. 2007, 2008).

Interest in the use of BMSCs in bone defect regeneration has grown since the realization of their beneficial properties. Since then, the use of BMSCs from animal and human sources have shown success in healing craniofacial and long bone defects (Krebsbach et al. 1999). Axial and appendicular bones are common donor sites for obtaining autogenous graft used in all types of bone defects including oral and craniofacial bone defects. Studies have demonstrated that healing of the oral and craniofacial bone defects are more successful with bone grafts from the common anatomical sites (Jackson et al. 1986; Oklund et al. 1986). Evidence indicates that matching a donor to the defect skeletal site might affect the outcome of the bone defect repair. A question has been raised whether skeletal sites, where BMSCs are obtained, would play a role in the healing of bone defects. Indeed, the enhanced osteogenic potential of orofacial BMSCs in human, rodent and murine demonstrated a possible superior cell source for bone defect regeneration compared to LB **(Chapter 3)** (Akintoye et al. 2006; Aghaloo et al. 2010; Yamaza et al. 2011).

### *1.4.4 Scaffolds/Carriers*

BMSCs have demonstrated excellent bone regeneration ability *in vivo* and success in healing bone defects. However, in larger size bone defects, appropriate scaffolds or carriers mimicking BMSCs embryonic environment are necessary to improve the clinical success of bone regeneration (Caplan 1991). Studies have demonstrated that autologous BMSCs cultured on

scaffolds can induce new bone formation *in vivo* and improve the healing of critical-size defects (Khojasteh et al. 2008; Meinel et al. 2005). Fabrication of suitable scaffolds has been a challenge in the bone tissue engineering field as several characteristics are required to achieve ideal scaffolds. Some of these characteristics include biocompatibility, biodegradability, osteoinduction, osteoconduction, and mechanical stability (Chapekar 2000; Rose and Oreffo 2002). Scaffolds for BMSCs necessitate further requirements for optimal interaction between the transplanted cells, scaffolds, and the host environment. The scaffolds should allow BMSC adhesion, proliferation and differentiation to enable the bone formation process. The porosity, pore size distribution, and the continuity of the scaffolds are also important for vascularization because ample vascular supply is critical for osteogenesis (Petite et al. 2000). Several materials have been employed as cell carriers. Hydroxyapatite, calcium phosphate and ceramic are osteoconductive and can stimulate cell differentiation. However, they have several problems including the lack osteoinductive characteristic, porous interconnectivity, and biodegradability (Rose and Oreffo 2002). Most importantly, their brittleness is prone to fracture and may not be suitable to use in large skeletal defects (Grundel et al. 1991; Moore et al. 1987). To avoid these problems, biodegradable composite scaffolds poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and their co-polymer poly(lactic-co-glycolic acid) (PLGA) have been developed. These scaffolds can be modified to the desired porous structures and can be used alone or in combination with other osteoconductive materials. Despite the benefits, they lack mechanical competence (Rose and Oreffo 2002).

Natural biodegradable degradable polymers such as gelatin and collagen have surfaced as another form of the scaffolds. Gelatin, specifically, is a denatured collagen of bovine or porcine

skin, bone or tendon that has been through a hydrolysis process (Djagny et al. 2001). Gelatin-based sponge prepared from pork skin is commercially available as Gelfoam®. They are biodegradable, biocompatible, porous and flexible in shape. In addition, they provide excellent cellular support, and have a high affinity to other matrix proteins and hemostatic properties. With these excellent benefits, gelatin scaffold is a promising carrier for BMSC-based bone tissue regeneration (Rohanizadeh et al. 2007). A study has shown that Gelfoam® has the ability to support osteoblastic cell penetration, growth, proliferation and differentiation (Rohanizadeh et al. 2007). BMSCs transplanted with gelatin sponge carrier has demonstrated success in repairing alveolar cleft and critical- and noncritical- sized cranial defects (Ben-David et al. 2011; Gimbel et al. 2007; Krebsbach et al. 1998; Zhao et al. 2007; Zou et al. 2011).

### *1.4.5 Critical-sized Defect Models*

Large osseous defects have been a devastating problem for affected patients, as well as, pose clinical challenges for reconstructive surgeons. Enhancing bone regeneration within the defects has been the goal of bone tissue engineering. The appropriate bony defect models with clinical relevant wound size are required in testing bone regenerative treatment and materials. Critical-sized defect (CSD) has been developed as a model to study bone defect healing. It is defined as the smallest size intraosseous wound that will not heal during the life time of the animal (Schmitz and Hollinger 1986). When CSDs are left untreated, they repair by fibrous connective tissue formation (Hollinger and Kleinschmidt 1990). For proper evaluation of bone repair efficacy, CSD model use should be considered against the following criteria: animal, age, size and site (Frame 1980; Bosch et al. 1998). In general, CSDs have been used in various bone

models to assess oral and craniofacial bones such as the calvaria and mandible, and long bones such as the femur (Drosse et al. 2008; Hollinger and Kleinschmidt 1990; Schmitz and Hollinger 1986). The diameter of critical-sized rodent calvaria defects ranges from 2, 4, 5, to 8 mm depending on the rat species, age, and the healing time of the model (Bosch et al. 1998; Freeman and Turnbull 1973; Mulliken and Glowacki 1980). The rat mandible CSD model has been well-established as 4 mm in diameter or more (Higuchi et al. 1999; Kaban et al. 1979; Kaban and Glowacki 1981; Park et al. 2003; Schmitz and Hollinger 1986). In the long bone model, the diameter of rat critical-sized femoral segmental defect ranges from 5 to 8 mm (Chen et al. 2002; Einhorn et al. 1984; Feighan et al. 1995; Lee et al. 1994; Lieberman et al. 1999; Oakes et al. 2003; Yasko et al. 1992).

## SPECIFIC AIMS AND HYPOTHESES

The jaws, to some extent, are analogous to long bone and other bones of the body; however, they have a distinct developmental origin, mode of ossification and homeostatic mechanisms. Furthermore, there are specific diseases that only affect the jaw, thus conclusions about its character and function cannot be deduced from the knowledge obtained from other bone sites. Our *overall objective* is to study the characteristic and function of mandible marrow cells and compare them to those derived from long bone.

**SPECIFIC AIM 1: Characterization of bone marrow stromal cells**

**Rationale**: Bone homeostasis involves the processes of bone formation and bone resorption. The unique homeostatic mechanism of the mandible (MB) is reflected in the discrete characteristics of the main cells of the bone formation and bone resorption. BMSCs are the main orchestrator of the bone formation, as they are the source for osteoprogenitors. Data from the MB BMSC study is sparse due to difficulty in cell isolation.

### 1.1 Isolation and characterization of rat MB *vs.* LB BMSCs

In this aim, we will establish a protocol to isolate MB BMSCs, evaluate cell characteristics and investigate the lineage differentiation potential: osteoblastic, chondrogenic and adipogenic.

### 1.2 Comparison of *in vivo* bone formation of BMSCs from rat MB *vs.* LB BMSCs

In this aim, the ability of MB *vs.* LB BMSCs to induce ectopic bone formation will be analyzed.

**Hypothesis:** We hypothesize that BMSCs isolated from the two distinct skeletal sites in the rat would display diverse differentiation potential. Specifically, MB BMSCs would have an enhanced osteogenic potential in accordance with previous findings (Akintoye et al. 2006; Matsubara et al. 2005; Yamaza et al. 2011)

**SPECIFIC AIM 2**: **Comparison of rat MB *vs.* LB osteoclastogenic potential at basal and stimulated conditions**

**Rationale:** In maintaining bone homeostasis, bone resorption is the complementary process to bone formation. Osteoclasts are cells that carry out the actual action of the bone resorption process. The MB distinctive homeostasis mechanism could be due to its unique osteoclast formation process. The difference in osteoclastogenesis could be due to osteoclast precursor characteristics or osteoclastogenic regulation of BMSCs via RANKL/OPG system. It has been shown that systemic diseases and systemic calciotropic hormones affect the MB differently (Adams et al. 1999, Mavropoulos et al. 2007). Therefore, it is important to investigate MB *vs.* LB osteoclastogenesis in hormone-stimulated conditions.

**2.1 Investigation of osteoclastogenic potential of MB *vs.* LB derived osteoclast precursors**

In this aim, the ability of MB *vs.* LB derived osteoclast precursors to differentiate into mature osteoclasts will be evaluated under RANKL and M-CSF induction.

**2.2 Analysis of MB *vs.* LB BMSC ability to mediate osteoclastogenesis through the expression of RANKL and OPG**

In this aim, the MB *vs.* LB BMSC mRNA expression of RANKL, OPG and RANKL/OPG will be compared with and without the induction of calciotropic hormones.

**2.3 Evaluation of *in vitro* osteoclastogenesis of MB *vs.* LB marrows under basal and hormone-stimulated conditions**

In this aim, the ability of MB *vs.* LB whole marrows to generate mature osteoclasts will be evaluated with and without the induction of calciotropic hormones.

**2.4 Validation of *in vivo* osteoclastogenesis of rodent mandible *vs.* tibia under continuous infusion of parathyroid hormone and 1,25 dihydroxyvitamin $D_3$.**

In this aim, a rodent model will be continuously infused with a vehicle control or a combination of parathyroid hormone and 1,25 dihydroxyvitamin$D_3$ via osmotic pumps. The osteoclast formation will be compared between mandible and tibia bones of the same animal.

**Hypothesis:** We hypothesize that MB *vs.* LB marrows have disparate osteoclastogenic potential at basal and stimulated conditions.

**SPECIFIC AIM 3: Evaluation of MB *vs.* LB derived BMSC bone regeneration potential in intramembranous *vs.* endochondral critical-sized bone defects**

**Rationale:** When a bone injury occurs, the skeletal stem cells, especially BMSCs, are recruited to the injury site to initiate the bone repair process. The same molecular mechanism of skeletogenesis continues through adulthood in the form of skeletal remodeling and repair (Ferguson et al. 1998). The mandible and long bone are different in their developmental origins and employ different molecular mechanisms of skeletogenesis. The fact that human orofacial BMSCs have enhanced response to osteogenic factor and form more bone than iliac crest cells in an ectopic bone formation model led us to investigate MB *vs.* LB BMSC bone regeneration potential. Accouning for the differences in the developmental origin, a study demonstrated that skeletal defects heal through the recruitment of progenitor cells of their own origin (Leucht et al. 2008). Thus, in the bone repairment, skeletal sites where BMSCs are obtained from and anatomical sites of the injury must be taken into consideration for successful bone defect regeneration.

### 3.1 Investigation of MB *vs.* LB BMSC bone regeneration potential in rat critical-sized calvarial *vs.* critical-sized femoral defects

In this aim, critical-sized calvarial and critical-sized femoral defects represent intramembranous and endochondral bone defects, respectively.

**Hypothesis:** We hypothesize that MB BMSCs will induce greater and more mature bone regeneration than LB BMSCs with a better outcome in the intramembranous calvarial defect.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Isolation and Culture of Bone Marrow Cells

All animals and surgical procedures were handled in accordance with the guidelines of the Chancellor's Animal Research Committee at UCLA. Soft tissue was dissected from one-month-old Sprague-Dawley rat mandibles, and the third molar was extracted (Charles River Laboratories, Wilmington, MA). Using a 26-gauge needle on the buccal cortex, we flushed bone marrow from the superior alveolar ridge with alpha minimum essential media (α-MEM, Mediatech, Herndon, VA, USA) and collected it through the extraction socket. Long-bone marrow cells were isolated from the tibiae of the same animals (Javazon et al. 2001; Yoshimura et al. 2007). Harvested whole bone marrow cells, containing a mixture of BMSCs and osteoclast precursors, were pooled in a single suspension, filtered through a 40-μm strainer, and red blood cells (RBCs) were lysed using an RBC Lysis Solution (BioLegend ®, San Diego, CA, USA). RBC-free bone marrow cells were counted (Vi-CELL$^{TM}$ cell viability analyzer, Beckman Coulter, Fullerton, CA, USA), and plated at 1 x $10^6$ cells/ml (2.6 x $10^5$ cells/cm$^2$ for 12-well plate and 5.3x$10^5$cells/cm$^2$ for 24-well plate) in α-MEM supplemented with 10% FBS, and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin).

Three populations of cells were cultured in our study, BMSCs, osteoclast precursors, and whole marrow, containing both BMSCs and osteoclast precursors. For BMSC culture, suspended cells were discarded after 6 days. Confluent adherent BMSCs were cultured in various differentiating and stimulated conditions, and fresh media with appropriate

supplementation were replaced every 3-4 days. For whole marrow experiments, cells were cultured without the removal of suspension cells, and half of the media were replaced every 3-4 days. For osteoclast precursors, bone marrow cells were cultured according to osteoclastogenesis protocol in **Chapter 2.5.1**.

## 2.2 Lineage Differentiation Potential of Bone Marrow Stromal Cells

### *2.2.1 Osteogenic Potential*

Confluent BMSCs were supplemented with osteogenic differentiation media (α-MEM+10% FBS with 50 μg/μL ascorbic acid and 4 mM β-glycerophosphate), which was replaced every 3-4 days. The osteoblast phenotype of osteogenic differentiated BMSCs was analyzed at 7- and 14- day of the differentiation period (see assays below).

#### *Colony-forming unit (CFU)*

BMSCs were washed twice with phosphate-buffered saline (PBS), and then stained with Giemsa dye in methanol solution for 10 minutes (Ladd Research, Williston, VT). Colony-forming unit fibroblast (CFU-F) derived colonies, at least 50 cells per group) were enumerated.

#### *Alkaline Phosphatase Staining and Activity*

BMSCs were fixed with fixative solution, containing citrate solution, acetone and 37% formaldehyde, and stained with colorimetric alkaline phosphatase (ALP) kit according to the manufacturer's protocol (Sigma, St. Louis, MO). The numbers of ALP positive colonies, stained reddish violet color, were visualized at 3, 7, 14 and 21 days of differentiation. Additionally,

total, ALP-positive and ALP-negative colonies were quantified by comparing Giemsa stained total colonies to ALP-positive colonies. Next, ALP activity was determined by conversion of *p*-nitrophyl phosphate to *p*-nitrophenol in whole-cell lysates of 3 and 7 days of differentiation. BMSCs were washed with cold phosphate buffered saline (PBS), and lysis buffer containing 0.2% NP-40 was added to each well. Lysate was sonicated, collected, and activity was measured using the phosphatase substrate in alkaline buffer solution. Absorbance was read on a microplate reader at 405 nm.

### *$^{[45]}$Calcium Assay*

BMSCs were cultured in osteogenic media for 21 days. Matrix mineralization was determined by $^{[45]}$Calcium Assay as previously described (Parhami et al. 1997). Briefly, 1 μCi/ml of $^{45}CaCl_2$ (ICN Biomedicals, Inc.) was added and incubated for 48 hours. After incubation, the medium was removed, cells were washed with PBS and scraped in PBS. Cells were placed in glass scintillation vials containing perchloric acid. 3% $H_2O_2$ was added to each vial, mixed and incubated for 60 min at $80^0$C. After incubation, the mixture was dissolved in ethyleneglycol monoethyl ether. $^{45}$Calcium was measured by scintillation counting.

### *von Kossa Staining*

BMSCs were cultured in osteogenic media for 14 and 21 days. Cells were fixed with 1-2% formaldehyde in 0.1M cacodylate buffer, washed twice with cacodylate buffer pH 7.4. Cells were incubated in saturated $LiCO_3$ to remove urates and subsequently washed with deionized water. $AgNO_3$ was added to the wells, and cells were incubated in ultraviolet light for 1 hour.

After rinsing with deionized water, cells incubated with 1 ml sodium thiosulfate for 5 min, rinsed again with water and air-dried.

***Osteoblastic Gene Expression***

To further investigate osteoblastic potential of BMSCs, mRNA expression of osteoblastic genes, including ALP and osteocalcin (OCN) were analyzed. The isolated BMSCs were also assessed for dentin sialophosphoprotein (DSPP) expression. All gene expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). **Chapter 2.4** described RNA isolation and real-time polymerase chain reaction (qPCR) methods and all primer sequences used for characterizing osteoblastic potential of BMSCs were listed in **Table 2-1.**

***In vivo Osteogenesis: Surgical Bone Marrow Stromal Cell Implantation***

To evaluate osteogenesis *in vivo*, we isolated BMSCs as previously described in **Chapter 2.1**. For scaffold preparation, LB or MB BMSCs (20 x $10^6$ cells each) were incubated with 6x6 mm gelatin sponge (Gelfoam, Pfizer, New York, NY, USA) in a 5% CO2/95% air atmosphere at 37°C for 3 days. On the day of surgery, BMSC suspension along with gelatin sponge carrier were centrifuged, and the media were discarded. The scaffolds were kept at 37°C until ready for implantation. Following anesthesia, scaffold with BMSCs were subcutaneously implanted into immunocompromised 4 week-old male nude mice (NIH III Nude; Charles River Laboratories, Wilmington, MA) at the intrascapular area (Pettway et al. 2005). Sponges alone were utilized as controls. After 6 weeks, transplants were placed in 10% formalin for 48 hrs and stored in 70% ethanol. The samples underwent with μCT imaging and histological analysis. μCT imaging was

performed at 12 μM isotropic voxel resolution, and tissue and bone volume analysis was performed. We used Dolphin Software (Dolphin Imaging, Chatsworth, CA, USA) to generate 3D and multiplanar reconstructed images. Then, transplants were decalcified (Fisher, Pittsburgh, PA, USA), paraffin-embedded, sectioned, and H&E- stained. Photomicrographs were taken with a Leica DMLB microscope (Leica Microsystems, Wetzlar, Germany) with BioQuant software (R&M Biometrics, Nashville, TN, USA). Two independent experiments, utilizing cells from different animals and performed at a different time, were performed, for a total of 8 transplants for each group.

### *2.2.2 Adipogenic Potential*

Confluent BMSCs were supplemented with adipogenic differentiation media, consisted of high-glucose Dulbecco's modified Eagle's medium (Cellgro, Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), 10 μM Dexamethasone, 0.5 mM IBMX, and 1.7 μM insulin. The adipogenic potential of differentiated BMSCs was analyzed at 7- and 14- day of the differentiation period (see assays below).

#### *Oil Red O Staining*

Adipocyte-derived BMSCs were analyzed with oil red O staining which revealed red colored lipid droplets accumulated in cytoplasmic vacuoles of differentiated cells. Briefly, cells were fixed with 10% formalin solution, washed with water followed by 60% isopropanol. Cells then stained with oil red O working solution which consisted of 3 parts of 3.5mg/ml oil red O in

isopropanol solution and 2 parts deionized water for 20 minutes. Oil red O dye was removed and rinsed with water and let dry.

### *Adipogenic Gene Expression*

The adipogenic potential of BMSCs was further investigated by analyzing the expression of adipogenic markers, including lipoprotein lipase (LPL), peroxisome proliferator-activated receptor-γ (PPARγ) and cluster differentiation 36 (CD36). All gene expression was normalized to GAPDH. **Chapter 2.4** described RNA isolation and real-time polymerase chain reaction (qPCR) methods and all primer sequences used for characterizing adipogenic potential of BMSCs were listed in **Table 2-1.**

## *2.2.3 Chondrogenic Potential*

For chondrogenesis, isolated BMSCs were cultured in chondrogenic media consisted of alpha minimum essential media (Cellgro, Mediatech, Inc., Manassas, VA) supplemented with 1% FBS, 1% P/S, 50 μg/ml ascorbic acid, 6.25 μg/ml insulin, and 10 ng/ml transforming growth factor beta (TGFβ). BMSCs were analyzed for chondrogenicity at 7- and 14- day of the differentiation period (see assays below).

### *Alcian Blue Staining*

At the appropriate differentiation period, differentiated chondrocytes were fixed with 10% formalin and washed several times with PBS. Cells were then stained with 1% Alcian Blue

in 0.1 N HCl, pH 1.0. Cells were washed with water and let dry. The ability of BMSC to form a cartilage matrix, stained blue, was analyzed in this assay.

***Chondrogenic Gene Expression***

The chondrogenic potential of BMSCs was further investigated by analyzing the expression of chondrogenic markers, including collagen II alpha 1 (Col IIa1), collagen X alpha 1 (Col Xa1), and SRY-box containing gene 9 (SOX9). All gene expression was normalized to GAPDH. **Chapter 2.4** described RNA isolation and real-time polymerase chain reaction (qPCR) methods and all primer sequences used for characterizing chondrogenic potential of BMSCs were listed in **Table 2-1**.

## 2.3 RNA Isolation and Real-Time Polymerase Chain Reaction

Total RNA from BMSCs was collected at appropriate day of differentiation period using Trizol following the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Briefly, 0.2ml of chloroform per 1 ml of Trizol reagent was added to cell lysates. Phase separation of the cell lysate was performed by centrifugation. The collected total RNA in aqueous phase, with the addition of glycogen as RNA carrier, was precipitated with 100% isopropanol, washed with cold 80% ethanol, dried, and resuspended in RNase-free water. The isolated RNA samples were purified using DNase from DNA-free system following manufacturer's protocol (Ambion, Austin, TX). Purified RNA samples were measured at the absorbance of 260 nm and 280 nm with spectrophotometry. For reverse transcriptase (RT) reaction, 3 μg of purified RNA was incubated with 0.3 μg of oligo dT, 4μl 5X RT buffer and RNase free water up to 11μl at 70oC

for 3 minutes and then on ice for 2 minutes. Then 4 µl of a solution consisted of dATP, dCTP, dGTP, dTTP, RNasin® Ribonuclease Inhibitor, 540 U M-MLV RT was added, and the final mixture was incubated for 1 h at 37oC. The reaction was terminated by incubating the samples at 80$^{o}$C for 10 minutes. All reagents used in RT reaction were purchased from Promega, Madison, WI, USA. Converted cDNA were used in real-time polymerase chain reaction (qPCR), performing in triplicate for at least 3 independent experiments, with iQ SYBR Green supermix (Bio-Rad, Hercules, CA, USA) and rat gene-specific, see **Table 2-1** for all primers used in qPCR). The amplification protocol was set as follows: 1 cycle of 95°C for 3 min followed by 40 cycles of 30 sec at 95°C, 20 sec at 60°C, and 30 sec at 72°C. Amplification of cDNA, product detection, and data analysis were performed using iCycler iQ real-time detection system (Bio-Rad, Hercules, CA, USA). Relative gene induction was determined by the 2-ΔΔCt method using GAPDH as a control and was expressed as either fold induction or percent maximum induction.

**Table 2-1.** Primer sequences used in real-time PCR

| Gene | Accession number | Forward and Reverse primers 5'→3' |
|---|---|---|
| Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) | NM_017008 | F: CGGCAAGTTCAACGGCACAGTCAAGG<br>R: ACGACATACTCAGCACCAGCATCACC |
| Alkaline phosphatase (ALP) | NM_013059 | F: GGACGGTGAACGGGAGAAC<br>R: TGAAGCAGGTGAGCCATAGG |
| Osteocalcin (OCN) | NM_013414 | F: GGACCCTCTCTCTGCTCACTCTG<br>R: ACCTTACTGCCCTCCTGCTTGG |
| Dentin sialophosphoprotein (DSPP) | NM_ 012790 | F: CGGTCCCTCAGTTAGT<br>R: TACGTCCTCGCGTTCT |
| Lipoprotein lipase (LPL) | NM_012598 | F: GATGGACGGTGACAGGAATG<br>R: CGATACAACCAGTCTACTACAATG |
| Peroxisome proliferator-activated receptor gamma (PPARγ) | AB011365 | F: TGCTCCACACTATGAAGACATC<br>R: GGACGCAGGCTCTACTTTG |
| Cluster Differentiation 36 (CD36) | NM_031561 | F: TATGGTGTGCTGGACATTGG<br>R: CTATGCTCATCTTCGTTAGGATTC |
| Collagen alpha 1 type II (Col IIa1) | AJ224879 | F: CCCTTTCTAAGAGACCTGAACTG<br>R: GGGCGTCTGACTCACACC |
| Collagen alpha 1 type X (Col Xa1) | AJ131848 | F: TATGTCAGCAACGCAGTATTAC<br>R: TGTCTATTCTGATGTCGTATAAGC |
| SRY-box containing gene 9 (Sox9) | XM_001081628 | F: TGGGAGCGACAACTTTACC<br>R: GGCGAGCACTTAGCAGAG |
| Receptor activator of nuclear factor kappa-B ligand (RANKL) | NM_057149 | F: GGAGAGCGAAGACACAGAAGCACTAC<br>R: CGAGCCACGAACCTTCCATCATAGC |
| Osteoprotegerin (OPG) | NM_012870 | F: TGTCCCTTGCCCTGACTACTCTTATAC<br>R: CCTTCCTCACATTCGCACACTCG |

## 2.4 Osteoclastogenesis of Bone Marrow Cells

### *2.4.1 Osteoclastogenesis of Bone Marrow-derived Osteoclast Precursors*

Bone marrow cells were isolated and harvested as previously described in **Chapter 2.1** . RBC-free cell suspension in complete media supplemented with 25ng/ml rat M-CSF (Prepotech, Rocky Hill, NJ) were plated in 100mm culture dish overnight to remove the adherent stromal cells. The nonadherent osteoclast precursors were collected and plated at the density of $1.5x10^5$cells/100ul ($4.7x10^5$cells/$cm^2$) in osteoclastogenic media, consisted of α-MEM supplemented with 10%FBS, 50ng/ml of rat M-CSF and 80ng/ml rat sRANKL (Prepotech, Rocky Hill, NJ), which was replaced every 2-3 days. After 6 days, osteoclast cultures were analyzed for osteoclastogenic ability.

### *2.4.2 Tartrate Resistant Acid Phosphatase Staining*

Osteoclast culture at the end of the culturing period was gently washed with PBS, fixed with fixative solution, containing citrate solution, acetone and 37% formaldehyde, and washed again with PBS. The cells were stained with tartrate resistant acid phosphotase (TRAP) staining, consisted of 37°C deionized water, diazotized Fast Garnet GBC solution, Naphthol AS-BI Phosphate solution, acetate solution and tartrate solution per manufacturer's protocol (387A TRAP kit, Sigma –Aldrich, St. Louis, MO, USA). $TRAP^+$ multinucleated cells (≥3 nuclei) were counted under light microscope.

### *2.4.3 Bone Resorption Assay*

Osteoclast precursors were cultured on calcium phosphate coated 16-well plate (BioCoat™ Osteologic ™ MultiTest Slides, BD Biosciences, Bedford, MA, USA) in α-MEM alone or osteoclastogenic media. After a 10 day culture, each well was washed with Milli-Q water and cells were removed with bleach. The osteoclast culture cells were removed with bleach solution and the total area of resorption pits was visualized with von Kossa stain. Using a light microscope camera, image of each well was captured at 2x magnification. The total resorbed area of each well was measured using the count and measure tool of cellSens® imaging software (Olympus, Center Valley, PA, USA).

### *2.4.4 Osteoclastogenic Potential of Bone Marrow Stromal Cells/ Osteoclastogenic Gene Expression*

Bone marrow cells were cultured at a density of $1x10^6$ cells/ml ($5.3x10^5$cells/$cm^2$) in complete α-MEM. After 6 days and upon confluency, suspension cells were discarded. To investigate the osteoclastogenic ability of BMSCs, the adherent cells were cultured in fresh osteogenic differentiation media (α-MEM+10%FBS with 50μg/μl ascorbic acid and 4 mM β-glycerophosphate) with the addition of vehicle (veh), PTH (10nM), or $1,25D_3$ (10nM), alone or in combination, which was replaced every 3-4 days. The osteoclastogenic potential of BMSCs was analyzed by the expression receptor of activator of NFκB ligand (RANKL), osteoprotegerin (OPG), and their ratio (RANKL/OPG). All gene expression was normalized to GAPDH. **Chapter 2.4** described RNA isolation and real-time polymerase chain reaction (qPCR) methods

and all primer sequences used for characterizing osteoclastogenic potential of BMSCs were listed in **Table 2-1.**

### *2.4.5 Osteoclastogenesis of Whole Bone Marrow Cells*

For osteoclastogenesis of whole bone marrow cells, cells were similarly cultured as in Chapter 2.4.4. but without the removal of suspension cells, and half of the media were replaced every 3-4 days. Osteoclastogenesis-induced whole marrow cultures were analyzed by TRAP staining (**Chapter 2.4.2**), and the total numbers of $TRAP^+$ multinucleated cells were compared between LB and MB derived cultures in each condition.

### *2.4.6 In vivo Continuous Infusion of Parathyroid Hormone and 1,25 dihydroxyvitamin $D_3$*

To evaluate in vivo osteoclastogenesis, 8 three-month-old, male Sprague-Dawley rats, weighing 400 - 470g were utilized in this experiment. Animals were divided into 2 groups of 4 animals (Charles River Laboratories, Wilmington, MA, USA). Alzet mini-osmotic pumps (model 1003D, Alza Corp., Palo Alto, CA, USA) were utilized as carriers for *in vivo* continuous infusion of PTH and $1,25D_3$. Under sterile condition, mini pumps were loaded with vehicle of PTH ($V_P$, 150mM NaCl, 1mM HCl, and 2% rat serum), vehicle of $D_3$ ($V_D$, ethanol), 40 μg/kg/day hPTH (1-34) (Bachem, Inc., Torrance, CA, USA), and $1,25D_3$ (Sigma-Aldrich, St. Louis, MO, USA). All filled mini pumps were incubated in sterile saline solution at 37°C overnight in order to equilibrate to their actual pumping rate prior to implantation. Following anesthesia, animals were subcutaneously implanted with Alzet mini-osmotic pumps in the subscapular region. Animals were given two pumps each, $V_P$ and $V_D$ pumps for the control

group and PTH and $1,25D_3$ pumps for the treatment group. After 3-day continuous infusion, animals were sacrificed at day 4, and their mandible and tibia bones were collected and placed in 4% formaldehyde solution for 48 hours and stored in 70% ethanol solution. The samples were further evaluated by histological and histomorphometric analyses (see **Chapter 2.8**).

**2.5 Animal Survival Surgery**

In order to evaluate bone healing potential of MB *vs.* LB BMSCs , 4-month-old, female athymic rats, weighing approximately 200 g, were utilized to create critical-sized calvaria and femoral defects representing intramembranous and endochondral bone defects, respectively. For each type of surgery, animals were randomly assigned to 3 groups: (1) control defect engrafted with gelatin sponge alone, (2) defect engrafted with MB BMSCs on gelatin sponge, and (3) defect engrafted with LB BMSCs on gelatin sponge.

Under 2-4% of isoflurane inhalation anesthetic, hair over the surgical site was removed, and the animals were given 0.01-0.05mg/kg of Buprenorphine subcutaneously. The surgical site was cleaned with betadine and 70% ethanol and opthalmic ointment was placed in the animal's eyes prior to surgery. For calvarial defect surgery (n=9 for each group), a 2cm long incision was made on the mid-calvaria, and the skin and underlying periosteum were reflected to expose the calvaria. A trephine drill was used to create 8 mm round calvarial defect under constant saline irrigation **(Fig. 2-1).** Eight millimeter round defect was selected because it has been deemed to be the acceptable critical-sized defect in rats (Schmitz and Hollinger 1986; Schmitz et al. 1990). Extreme care was taken to avoid injury to the underlying dura. Each defect was carefully inspected and flushed with saline to remove any remaining bone fragments or debris. After the

implantation, the periosteum and the skin over the defect were sutured in 2 layers with 4-0 polyglycolic acid suture (Polysyn, Angiotech Pharmaceuticals Inc, Vancouver, British Columbia, Canada). Calvaria were collected at the end of the 6-week healing period. Samples were fixed in 4% formaldehyde solution for 48 hours, rinsed with water and stored in 70% ethanol solution.

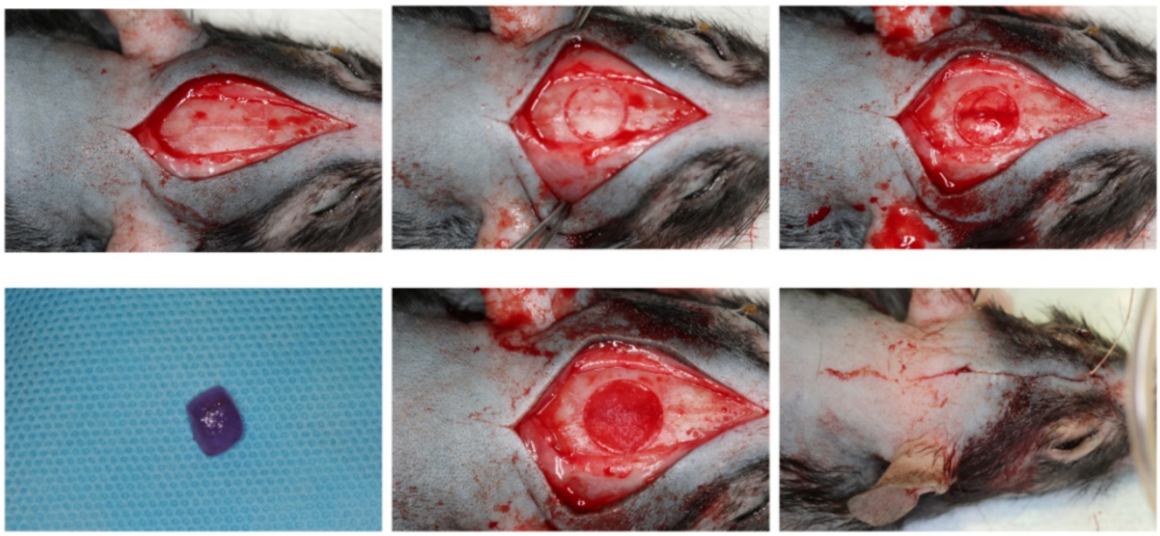

**Figure 2-1. Critical-sized rat calvarial defect surgery**

For the femoral defect (n=6 per group), animals were prepared for surgery under sterile conditions as above. A 6-mm critical-sized femoral defect was created following established protocols with some modifications (Chen et al. 2002; Oakes et al. 2003; Tsuchida et al. 2003). A 2-cm incision was made on the left lateral thigh overlying the femur, through skin and subcutaneous tissue. The vastus lateralis and biceps femoris were elevated through an anterolateral approach. Care was taken to keep the periosteum intact along the surface of the bone. Bone stabilizer consisted of a high-density polyethylene plate with six predrilled holes was secured to the anterolateral aspect of the femur using six 0.99 mm threaded Kirshner wires

**(Fig. 2-2).** Then, a 6-mm bone segment of central diaphysis was created using a reciprocating saw (Stryker, Kalamazoo, Michigan, USA) under continuous saline irrigation. After implantation, according to the groups assigned above, muscles and skin over the defect were sutured in 2 layers with 4-0 polyglycolic acid and 4-0 chromic gut sutures (Angiotech Pharmaceuticals Inc, Vancouver, British Columbia, Canada), respectively. New bone formation within the segmental defect was monitored with high resolution radiographs using Faxitron (Faxitron Bioptics, LLC, Tucson, Arizona) at the 2-, 4-, and 8-week healing period. Femurs with stabilizer still attaching along with the surrounding muscles were collected at the end of the 8-week healing period.

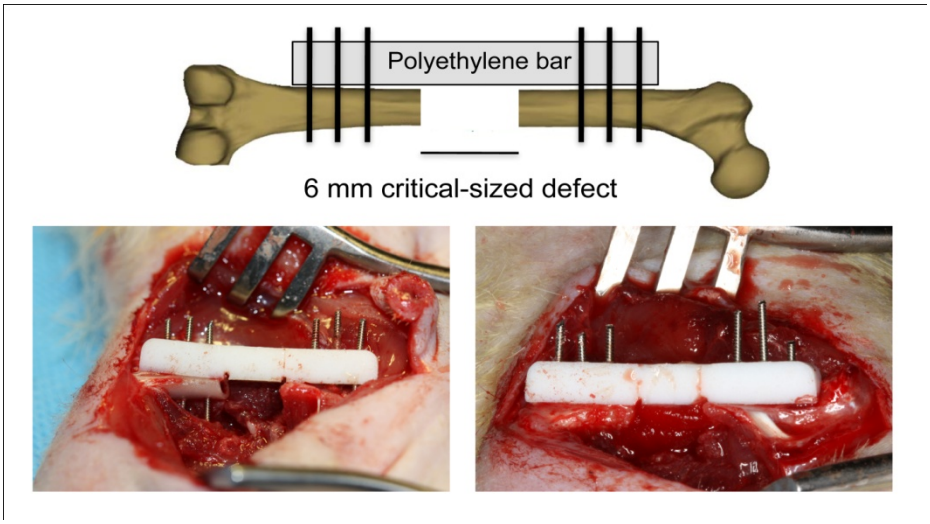


**Figure 2-2. Critical-sized femoral defect surgery**

## 2.6 High Resolution Micro Computed Tomography

Calvarial samples were imaged with high resolution microCT (SkyScan 1172 scanner; Skyscan, Kontich, Belgium) at source voltage of 70kV$_p$ and source current of 141 mA with the scanning resolution of 20 microns per pixel. Each scan was performed with 0.4 degrees per

rotation and a frame averaging of 10. 2D-images were reconstructed with NRecon program version 1.6.4, and were oriented in the transverse plane with DataViewer program version 1.4.4 (both from Skycan, Kontich, Belgium). Visualization, reconstruction and data analyses of area and volume of new bone formation covered within the defects were performed using CTAn version 1.11. Bone volume (BV) within an 8 mm, circular region of interest covering the calvarial defect was measured with a constant threshold for mineralized tissue and a fixed tissue volume (TV). New bone formation volume was quantified and expressed as percent BV/TV. Percent surface area of new bone formation over a fixed tissue surface area was calculated and expressed as percent bone healing (% bone healing) in order to determine the coverage of bone regenerated within the defect area. For femoral defects, femur bones were imaged in the same manner as above. Percent BV/TV and % bone healing were also calculated.

### 2.7 Histological and Histomorphometric Analyses

For *in vivo* osteoclastogenesis, bone samples were decalcified in 14.5% EDTA (pH 7.2) for 4 weeks. Paraffin embedded 4-μm-thick coronal sections of the interproximal area between the first and second mandibular molars and cross sections of proximal tibiae were obtained and stained with hematoxylin and eosin (H&E). To quantify the number of osteoclasts, H&E-stained slides were digitally scanned using the Aperio XT automated slide scanner and the Aperio ImageScope version 11 software (Aperio Technologies, Inc., Vista, CA, USA). Osteoclasts (≥2 nuclei) in contact with the bone surface were counted manually on the digital whole-slide image (bone perimeter or bone surface area measured with the ImageScope annotation tool) within the

alveolar bone region of the mandible and the trabecular bone region of the proximal tibia. The results are expressed as number of osteoclasts per bone perimeter (N.Oc/B.Pm).

For *in vivo* bone regeneration in critical-sized calvarial and femoral defects, bone samples were decalcified in 14.5% EDTA (pH 7.2) for 4 weeks. Paraffin embedded 4-μm-thick coronal sections of calvaria at the center of the defect and cross sections of tibiae at the center of the defect were obtained and stained with hematoxylin and eosin (H&E).

### 2.8 Statistical Analyses

Data were expressed as mean ± standard error of the mean (SEM) from at least 3 independent experiments. Data between groups were analyzed using Student's t-test. A $p<0.05$ was considered as significant.

# CHAPTER 3

# OSTEOGENIC POTENTIAL OF MANDIBULAR VS. LONG-BONE MARROW STROMAL CELL


## ABSTRACT

Although fundamentally similar to other bones, the jaws demonstrate discrete responses to developmental, mechanical, and homeostatic regulatory signals. Here, we hypothesized that rat mandible *vs*. long-bone marrow-derived cells possess different osteogenic potential. We established a protocol for rat mandible and long-bone marrow stromal cell (BMSC) isolation and culture. Mandible BMSC cultures formed more colonies, suggesting an increased CFU-F population. Both mandible and long-bone BMSCs differentiated into osteoblasts. However, mandible BMSCs demonstrated augmented alkaline phosphatase activity, mineralization, and osteoblast gene expression. Importantly, upon implantation into nude mice, mandible BMSCs formed 70% larger bone nodules containing three-fold more mineralized bone compared with long-bone BMSCs. Analysis of these data demonstrates an increased osteogenic potential and augmented capacity of mandible BMSCs to induce bone formation *in vitro* and *in vivo*. Our findings support differences in the mechanisms underlying mandible homeostasis and the patho-physiology of diseases unique to the jaws.


## INTRODUCTION

Despite being seemingly similar to other bones in the body, the maxilla and mandible serve distinct functions and demonstrate discrete responses to developmental, mechanical, and

homeostatic stimuli (Sodek and McKee 2000). Developmentally, the jaws, similar to other craniofacial bones, but distinct from the axial and appendicular skeleton, arise from neural crest cells of the neuroectoderm germ layer, and not from the mesoderm (Chai and Maxson 2006), and undergo intramembranous instead of endochondral ossification (Karaplis 2002). The mandible in particular is formed primarily by intramembranous ossification, while secondary cartilage at its proximal end contributes endochondral components at later stages. Meckel's cartilage, which precedes mandible formation but mainly disappears as the mandible develops, plays an important role in mandibular morphogenesis (Ramaesh and Bard 2003; Tsutsui et al. 2008). Although the same key regulators of osteoblastic differentiation, such as Runx2 and osterix, determine precursor commitment in intramembranous and endochondral bones (Ducy et al. 1997; Komori et al. 1997; Nakashima et al. 2002; Otto et al. 1997), several growth factors, receptors, and associated signaling cascades play distinct roles in the craniofacial *vs*. axial and appendicular skeleton (Abzhanov et al. 2007; De Coster et al. 2007; Kimmel et al. 2007). Systemic diseases, such as osteoporosis, hyperparathyroidism, or Paget's disease, affect all bones, including the jaws (White and Pharoah 2004). Indeed, there appears to be a correlation between mandibular bone density and osteoporosis (Jeffcoat et al. 2000; Kribbs et al. 1989; Lerner 2006; Mavropoulos et al. 2007). However, rat mandibles lose significantly less trabecular bone and bone mineral density at a lower rate than tibiae primary spongiosa after ovariectomy and malnutrition (Mavropoulos et al. 2007), suggesting different homeostatic mechanisms of the two bones.

The small size and anatomic complexity of the maxilla and mandible render bone cell isolation a challenge (Sodek and McKee 2000). Thus, little is known about the cellular and

particularly molecular basis for mandibular bone *vs*. long-bone divergent homeostasis. Most of our knowledge on mandible cell function and differentiation is derived from experimental models with cells from other skeletal sites (Lerner 2006). However, caution should be exercised in the extrapolation of such data to mandibular cell function. Indeed, human mandibular or maxillary marrow stromal cells demonstrate increased cell proliferation, delayed senescence, and stronger expression of osteoblastic markers compared with iliac-crest-derived marrow cells from the same patients (Akintoye et al. 2006), suggesting distinct functions and differentiation potential.

Here, we sought to compare the *in vitro* osteoblastic differentiation and capacity for *in vivo* bone formation of bone marrow stromal cells (BMSCs) derived from rat mandible *vs.* BMSCs derived from rat tibiae. We hypothesized that these marrow populations, derived from two distinct skeletal sites in the rat, would display diverse osteogenic potential. Indeed, mandible BMSCs demonstrated a more robust osteoblastic differentiation and induced significantly greater bone formation than their long-bone counterparts. Our findings demonstrate differences in osteogenic potential of mandible *vs.* long-bone BMSCs, and suggest an increased capacity of mandible BMSCs to induce bone formation *in vitro* and *in vivo.*

## RESULTS

### Isolation, Culture, and Characterization of Mandible BMSCs

To avoid contamination of bone marrow cells with incisor pulp, we inserted a 26-gauge needle into the buccal cortex, at the retromolar area and above the external oblique ridge, away from the central incisor root, and directed toward the alveolar ridge. A plain radiograph

demonstrates the needle position, superior to the incisor root and inferior to the molars **(Fig. 3-1 A).** Marrow was flushed from the mandible and collected through the extraction socket.

To confirm the absence of contamination from dental pulp/ odontoblasts, we extracted total RNA from confluent BMSC cultures, and determined dentin sialophosphoprotein (DSPP) expression by qPCR using incisor pulp RNA as a positive control. DSPP, a precursor of dentin phosphoprotein (DPP) and dentin sialoprotein (DSP), is highly expressed by odontoblasts and has been used widely to demonstrate odontogenic differentiation (Iohara et al. 2006; Yang et al. 2007). Bone cells express DSPP, but at a much lower level than odontoblasts (Qin et al. 2002). Pulp tissue expressed high DSPP levels that were > 42,000-fold than those in mandible BMSC cultures. In contrast, long-bone BMSCs expressed DSPP levels (1.12-fold) very similar to those in mandible BMSCs **(Fig. 3-1 B).** Analysis of these data suggests that mandible BMSC contamination with pulp cells, if any, was minimal. For all remaining experiments, DSPP levels were determined.

To begin characterizing CFU-F numbers in BMSCs from mandible *vs*. long bone, we grew cells for 1 wk in α-MEM and counted colonies. Mandible BMSC cultures displayed significantly more total colonies and more ALP-positive colonies than their long-bone counterparts **(Fig. 3-1 C),** suggesting increased CFU-F numbers in mandible marrow.

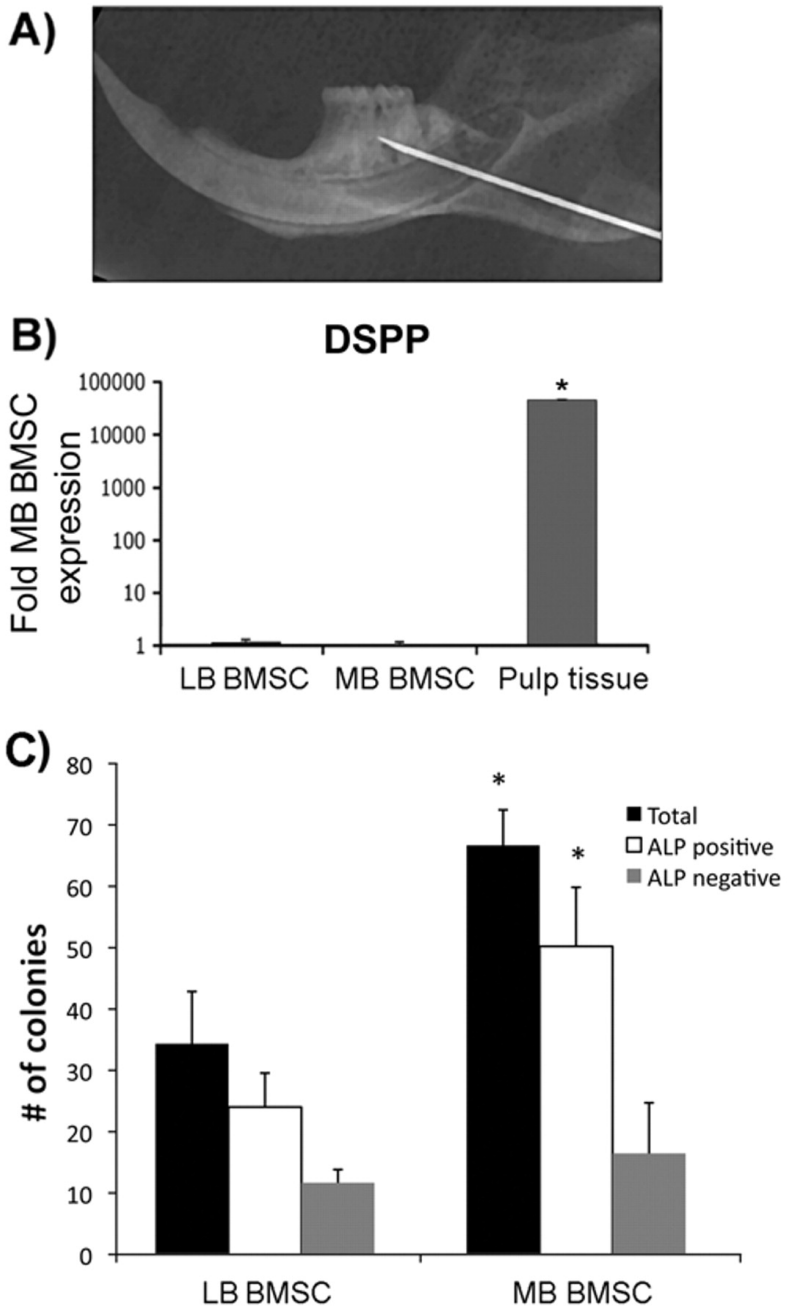


**Figure 3-1.** Isolation and culture of mandible BMSCs. **(A)** Radiograph of hemimandible showing the final position of the needle in the alveolar ridge superior to the incisor. The third molar has been extracted. **(B)** DSPP expression of long-bone (LB) BMSCs, mandible (MB) BMSCs, and pulp tissue by qPCR representative of more than 6 independent experiments. **(C)** Quantification of total, ALP-positive, and ALP-negative colonies formed by long-bone (LB) *vs*. mandible (MB) BMSC cultures (average of 3 independent experiments). *$p < 0.05$; error bars represent standard error of the mean.

**Mandible BMSCs Have a Higher Osteoblastic Potential Compared with Long-bone BMSCs**

To evaluate the osteogenic differentiation potential of mandible *vs.* long-bone BMSCs, we cultured confluent cells in osteogenic media for 0-21 days. ALP staining was stronger in mandible BMSCs from the beginning of the culture (3 days), continuing through the entire experiment (21 days; **Fig. 3-2 A**). Von Kossa assay showed significant mineral deposition in mandible BMSCs compared with long-bone BMSCs **(Fig. 3-2 B).** ALP activity paralleled ALP staining, demonstrating significantly higher levels for mandible *vs.* long-bone BMSCs **(Fig. 3-2 C)**. Mineralization quantification was also tested by [$^{45}$]Ca assays that, similar to von Kossa, showed higher calcium deposition in mandible BMSC cultures **(Fig. 3-2 D).** For further investigation of BMSC osteoblastic potential, ALP and osteocalcin (OCN) mRNA levels were examined. Mandible BMSCs demonstrated significantly higher ALP and OCN expression at 3 and 7 days **(Figs. 3-2 E, F).** Analysis of these data, collectively, demonstrates that mandible BMSCs possess higher osteogenic potential compared with their long-bone counterparts.

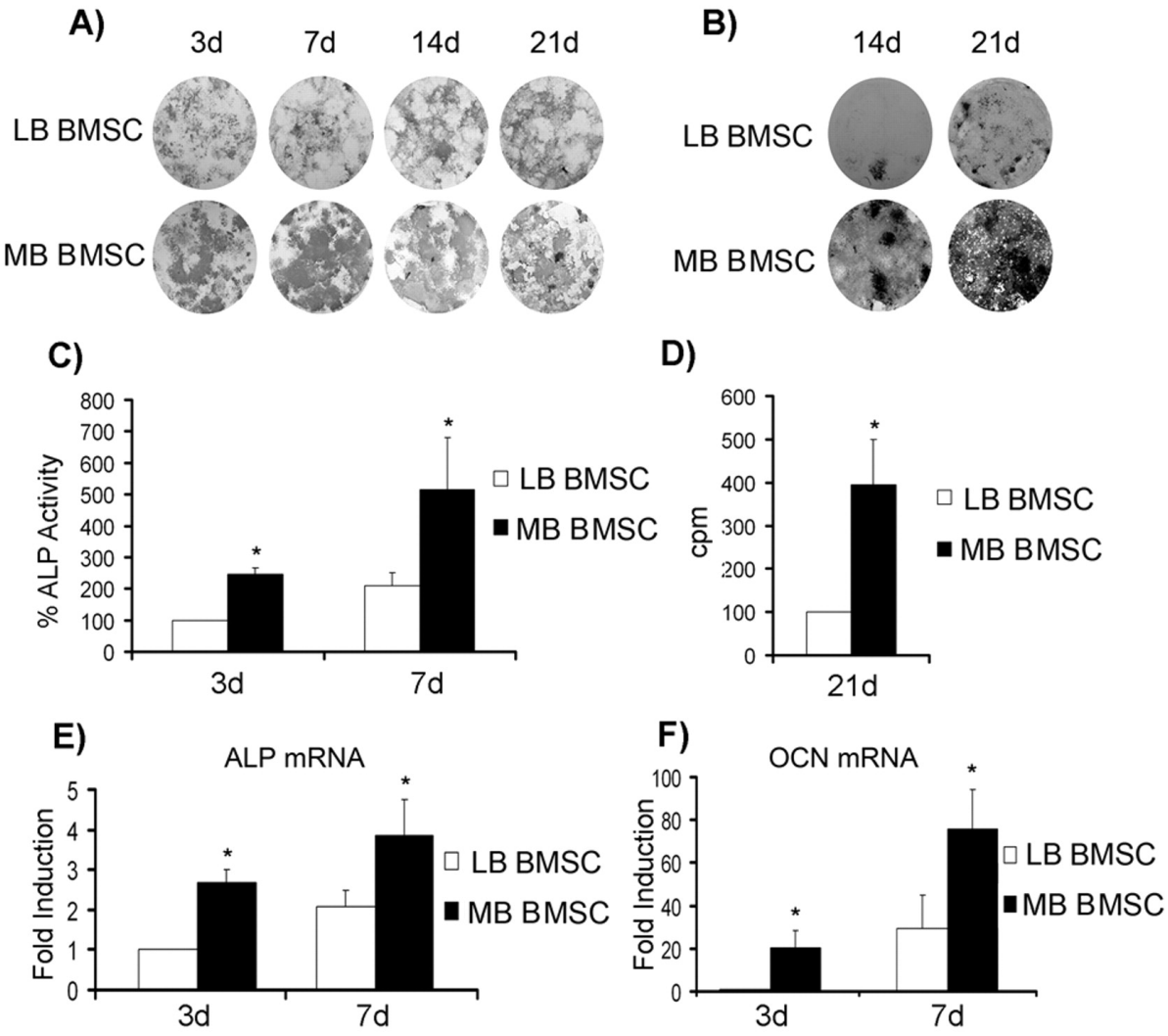


**Figure 3-2.** *In vitro* characterization of mandible *vs*. long-bone BMSCs. **(A)** Representative of 5 independent experiments of ALP staining at 3-, 7-, 14-, and 21-day cultures of long-bone (LB) *vs*. mandible (MB) BMSCs in osteogenic media. **(B)** Representative of 4 independent experiments of von Kossa staining at 14- and 21-day cultures of long-bone (LB) *vs*. mandible (MB) BMSCs in osteogenic media. **(C)** ALP activity assay at 3- and 7-day cultures of long-bone (LB) *vs*. mandible (MB) BMSCs in osteogenic media (average of 3 independent experiments). **(D)** $^{[45]}$Ca assay at 21 days' culture of long-bone (LB) *vs*. mandible (MB) BMSCs in osteogenic media (average of 3 independent experiments). **(E)** ALP and **(F)** OCN mRNA expression determined by qPCR of long-bone (LB) *vs*. mandible (MB) BMSCs cultured in osteogenic media for 3 and 7 days (average of 4 independent experiments).
*$p < 0.05$; error bars represent standard error of the mean.

**In vivo-increased Osteogenic Potential of Mandible *vs.* Long-bone BMSCs**

For determination of whether *in vitro* differences are recapitulated *in vivo,* BMSCs seeded on gelatin sponges for 3 days or gelatin sponges alone were implanted subcutaneously into nude mice for 6 wks (Pettway et al. 2005). At the end of the experiment, sponges alone did not produce a radiographic image (data not shown), suggesting absence of mineralization. Representative 3D-reconstructed microCT images of long-bone **(Fig. 3-3 A)** and mandible **(Fig. 3-3 B)** BMSC-seeded sponges are shown. Mandible BMSC sponges were consistently larger and more calcified than long-bone BMSC sponges. Tissue volume (TV) and bone volume (BV) quantification further demonstrate the significantly greater ability of mandible BMSCs to form larger and more calcified nodules than long-bone BMSCs *in vivo* **(Figs. 3-3 C, D).**

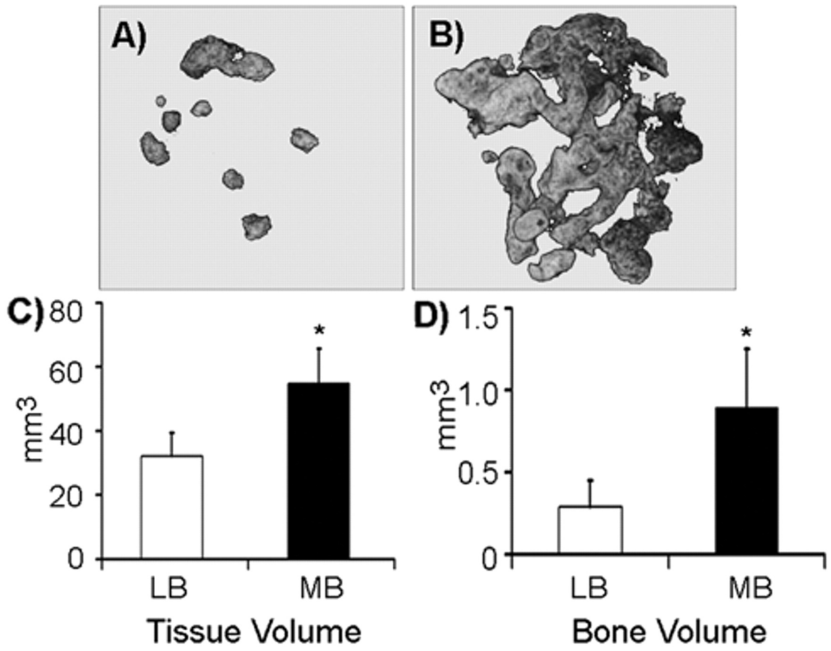


**Figure 3-3.** microCT analysis of gelatin sponge seeded with long bone or mandible BMSCs. 3D-reconstructed **(A,B)** microCT images of representative gelatin sponges seeded with (A) long-bone- or (B) mandible-derived marrow cells. **(C)** Tissue volume (TV) and **(D)** bone volume (BV) of long-bone (LB) *vs*. mandible (MB) marrow cell-seeded sponges, quantified by μCT (average of 8 individual transplants from 2 independent experiments). *$p < 0.05$; error bars represent standard error of the mean.

Then, transplants were decalcified, and H&E sections were performed **(Fig. 3-4).** Sponges without BMSCs demonstrated a thin fibrous capsule only, without any indication of bone formation (data not shown). All sponges seeded with long-bone or mandible marrow cells supported bone formation. Mandible BMSC sponges showed increased and more mature lamellar bone **(Fig. 3-4 D)** compared with long-bone BMSC sponges **(Fig. 3-4 A).** Marked osteoblastic rimming of bony trabeculae and bone marrow was seen only in mandible BMSC sponges **(Fig. 3-4 E).** On high magnification, orderly lines of lamellar bone formation with osteocytes were also observed **(Fig. 3-4 F).** In contrast, long-bone BMSC sponges showed a primarily cartilaginous matrix (purple in **Figs. 3-4 B, C**), with only peripheral bone formation. Analysis of these *in vivo* data further complements the *in vitro* data demonstrating that mandible BMSCs have higher osteoblastic potential compared with long-bone BMSCs.

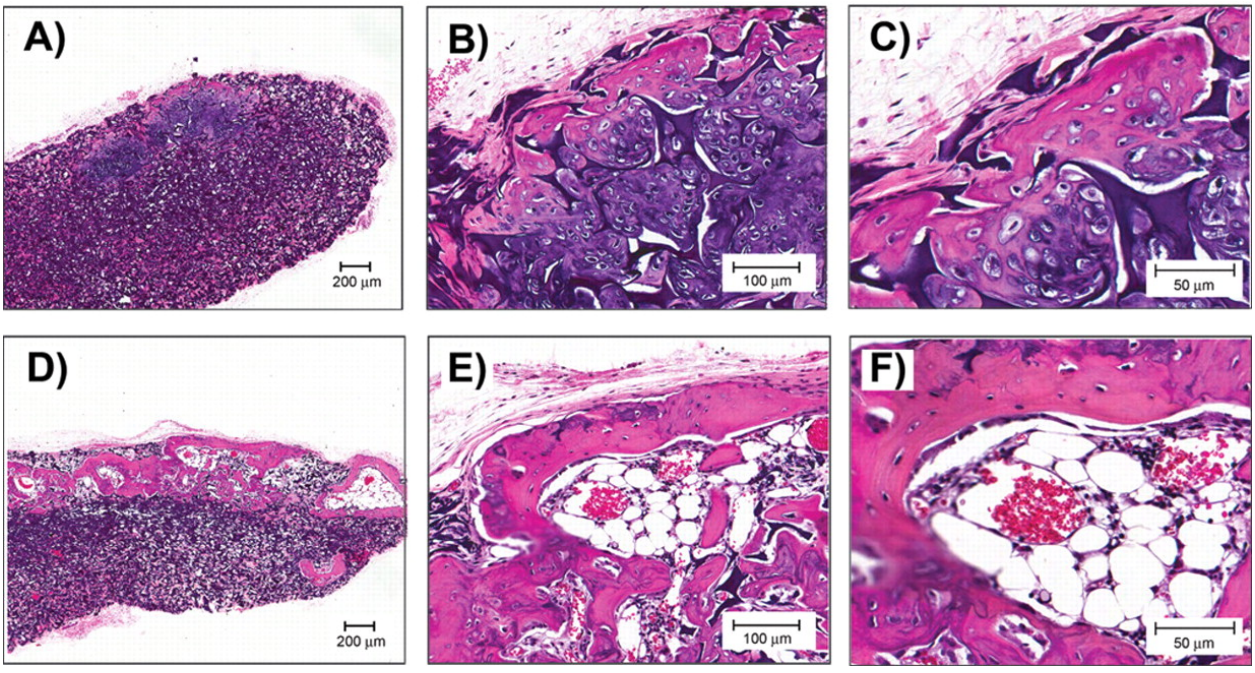


**Figure 3-4.** H&E staining of gelatin sponge seeded with long bone or mandible BMSCs. At 2X **(A,D)**, 10X **(B,E)**, and 20X **(C,F)** of sponges seeded with long-bone marrow cells **(A,B,C)** or sponges seeded with mandible marrow cells **(D,E,F)** and implanted at the intrascapular area of nude mice.

## DISCUSSION

Systemic diseases, such as osteoporosis, hyperparathyroidism, and Paget's disease, affect all bones, including the jaws (White and Pharoah 2004). Oral bone metabolism is affected in ovariectomized rodent models of osteoporosis and osteopenia, showing parallel responses in long bones and mandible (Hsieh et al. 1995; Miller et al. 1997). But in response to external stimuli, including ovariectomy and malnutrition, the mandible loses significantly less bone than the proximal tibia (Mavropoulos et al. 2007). In addition, cherubism (Ueki et al. 2001), hyperparathyroid jaw tumor syndrome (Simonds et al. 2002), and, more recently, bisphosphonate-related osteonecrosis of the jaws (BRONJ) (Ruggiero et al. 2004) affect only the maxilla and mandible, suggesting different homeostatic mechanisms between the jaws and long bones. This is further demonstrated by differences in mandibular mechanical loading during mastication (Mavropoulos et al. 2004). In fact, forces generated during walking are almost half those to which alveolar bone is exposed during mastication (Daegling and Hylander 1997; Knoell 1977).

Although craniofacial and long bones are similar histologically (Leucht et al. 2008), the craniofacial skeleton arises from neural crest cells that migrate ventrolaterally to the branchial arches (Chai et al. 2000). During neural crest cell migration, growth factors and their signaling pathways determine neural crest differentiation into functional cells forming craniofacial structures primarily *via* intramembranous ossification (Chai et al. 2000). In contrast, the axial and appendicular skeleton arises from mesenchymal condensations of mesoderm that undergo chondrocytic differentiation (Mackie et al. 2008). Taken together, *in vivo* evidence of varied responses to external stimuli, coupled with development of the mandible *vs.* long bones from

different germ layers, encourages study to evaluate differences between mandible and long bones.

A better understanding of the diseases and processes affecting the jaws would be significantly improved by an animal system to study cellular and molecular processes *in vitro.* Here, we established a protocol for isolation and culture of rat mandible BMSCs, to test our hypothesis that mandible *vs.* long-bone BMSCs possess distinct osteogenic differentiation potential. Isolated mandible BMSCs differentiate, under appropriate conditions, toward an osteoblastic phenotype, expressing osteoblastic markers and forming mineralized nodules. Interestingly, ALP staining and activity were stronger in mandible BMSCs compared with long-bone BMSCs. Von Kossa and [$^{45}$]Ca also showed increased mineral deposition in mandible *vs.* long-bone BMSCs. Furthermore, mandible BMSCs demonstrated significantly increased ALP and OCN mRNA expression. Analysis of these data, collectively, demonstrates that mandible BMSCs possess higher osteogenic potential compared with their long-bone counterparts. Our findings are consistent with those of studies utilizing trabecular bone from patient extraction sites, and showing that orofacial BMSCs demonstrate increased osteogenic differentiation compared with iliac-crest-derived BMSCs from the same individuals (Akintoye et al. 2006). Furthermore, a recent study with labeled neural-crest- and mesoderm-derived cells demonstrated bone defect healing through selective recruitment of cells from their specific embryonic origin (Leucht et al. 2008), reinforcing site-specific differences in BMSCs from the craniofacial *vs.* appendicular skeleton.

To investigate whether *in vitro*-observed differences between mandible *vs.* long-bone BMSCs osteogenic differentiation is reproducible *in vivo,* we used an ectopic bone formation

model (Akintoye et al. 2006; Krebsbach et al. 1997; Kuznetsov et al. 1997; Pettway et al. 2005). Differences between mandible and long-bone BMSCs *in vitro* were recapitulated *in vivo*. BMSCs implanted into nude mice underwent osteogenic differentiation and developed mineralized nodules. MicroCT qualitatively and quantitatively showed larger and more calcified structures from mandible *vs.* long-bone BMSCs. These data were confirmed by histology, which revealed increased and more mature lamellar bone derived from mandible BMSC sponges.

Analysis of our data strongly supports increased osteogenic potential of mandible *vs*. long-bone BMSCs both *in vitro* and *in vivo*. The diverse osteogenic potential between mandible- and long-bone-derived cells could be due to inherent differences of BMSCs between these two sites. However, marrow cells consist of a variety of cell lineages. Thus, some of the observed differences between these two cell populations could be due to the differential composition of marrow from mandible *vs*. long bone. Utilizing this *in vitro* mandible BMSC model system, we detected baseline differences and can now explore detailed characterization of mandible marrow composition, as well as evaluate responses to various external stimuli including hormones, growth factors, and signaling cascades as well as potential differences in bone remodeling and healing.

# CHAPTER 4

# OSTEOCLASTOGENIC POTENTIAL OF LONG BONE VERSUS MANDIBLE MARROW-DERIVED OSTEOCLASTS


## ABSTRACT

The mandible, part of the jaw bones, possesses unique metabolic and functional properties and demonstrates discrete responses to homeostatic, mechanical and developmental stimuli. Specific bone pathologies such as cherubism and osteonecrosis of the jaws only affect the jaws. Importantly, systemic diseases such as osteoporosis and malnutrition affect the jaws differently compared to other bones. Osteogenic potential of bone marrow stromal cells (BMSCs) differs between the mandible (MB) vs. long bones (LB). Furthermore, MB *vs.* LB derived osteoclasts (OCs) have different functional properties. Here, we hypothesized that MB *vs.* LB marrows have disparate osteoclastogenic potential at basal and stimulated conditions. To test our hypothesis, osteoclast precursors were differentiated with RANKL and M-CSF. The MB culture produced significantly higher numbers of TRAP+ multinucleated cells (MNCs) and greater resorption area compared to the LB culture. We then explored the ability of MB *vs.* LB BMSCs to support osteoclastogenesis at basal and stimulated conditions. Confluent BMSCs were cultured in osteogenic differentiation media in the presence of vehicle (veh), parathyroid hormone (PTH), or 1α,25-dihydroxyvitamin$D_3$ (1,25$D_3$), alone or in combination, and then the mRNA levels of RANKL and OPG were determined. No statistical difference in the gene expression was observed at baseline. However, LB BMSCs expressed significantly higher RANKL and lower OPG mRNA with a resultant increase in RANKL/OPG ratio in the presence

of $1,25D_3$ alone and PTH+$1,25D_3$. Thus, LB BMSCs appear to possess a significantly higher osteoclastogenic potential in stimulated conditions compared to MB BMSCs. Next, we cultured whole marrow, containing both BMSCs and osteoclast precursors, in the presence or absence of PTH and $1,25D_3$. No TRAP+ cells were observed at basal conditions. However, $TRAP^+$ MNCs were significantly increased in the LB *vs.* MB marrow. Ultimately, Sprague-Dawley rats were continuously infused with PTH+1,25D3 for their *in vivo* effect in osteoclastogenesis of mandible and tibia. Under this condition, the increase in osteoclast number was more pronounced in the LB in comparison to the MB. Collectively, our data demonstrate that although the MB marrow contains increased numbers of OC precursors, under PTH and 1,25D3 stimulation, LB marrow has higher osteoclastogenic potential. This appears to be, at least in part, due to the higher RANKL stimulation and OPG inhibition of LB *vs.* MB BMSCs by these hormones. These findings support an increased sensitivity in nature of the jaws to antiresorptive treatments that might explain, at least in part, the pathophysiology of osteonecrosis of the jaws (ONJs).

## INTRODUCTION

The mandible (MB), along with the maxilla, is part of the jaw bones. Rudimentarily, the MB demonstrates a unique embryological development, a process of formation, and employs a different homeostatic mechanism. Similar to craniofacial bones, the MB, however, is distinct from appendicular bones. In terms of the embryological development, the MB arises from neural crest cells of the neuroectodermal origin rather than mesoderm (Chai and Maxson 2006), and are formed primarily by intramembranous ossification as opposed to endochondral ossification (Ferguson et al. 1998). In response to systemic hormones important in bone metabolism,

mineralization of the MB is affected by 1,25-dihydroxyvitamin $D_3$ ($1,25D_3$) deficiency but unaltered by the abolishment of PTH in contrast to long bones (LB) that is affected by both hormones (Liu et al. 2009). Similarly, in ovariectomized and malnutrition rodent models, the MB loses significantly less bone than proximal tibia (Mavropoulos et al. 2007). Of further importance, there exist skeletal diseases manifesting only in the jaw bones, such as cherubism (Simonds et al. 2002), hyperparathyroid jaw tumor syndrome (Simonds et al. 2002), and recently revealed bisphosphonate-related osteonecrosis of the jaws (BRONJ) (Marx 2003), solidifying the distinction of the MB homeostasis.

The cellular heterogeneity exists between marrow-derived cells from the MB and other bones of the body. Our previous study uncovers an increased osteogenic potential of rodent MB *vs*. LB bone marrow stromal cells (BMSCs) both *in vitro* and *in vivo*, supporting other investigations that demonstrated enhanced response of human mandibular or maxillary BMSCs to osteogenic differentiation factor and bone morphogenetic protein 2 comparing to those derived from the iliac crests (Aghaloo et al. 2010; Akintoye et al. 2006; Osyczka et al. 2009). Marrow-derived osteoclasts (OCs) are multinucleated, bone-resorbing cells whose differentiation and maturation requires macrophage stimulating factor (M-CSF) and receptor of activator of NFκB ligand (RANKL), cytokines supplied among others by BMSCs (Boyle et al. 2003). Recently, phenotypic and functional differences of bone-site specific osteoclasts has been proposed (Everts et al. 2009). OCs from different bone sites (e.g. calvaria (CV) and LB) were shown to have differential usage of proteinases important for bone resorption (Everts et al. 1999a, 2006) and expression level of the enzyme tartrate resistant acid phosphatases (TRAP) (Perez-Amodio et al. 2006; Zenger et al. 2010). In addition, murine jaw and LB marrows have different osteoclastic

potential in the presence of M-CSF and RANKL stimulation and exhibit distinctive shape and response to culturing substrates (Azari et al. 2011; Souza Faloni et al. 2010).

In the bone marrow microenvironment, BMSCs have a crucial role in the regulation of osteoclastogenesis. Essential to the balance of this regulation, BMSCs produce osteoprotegerin (OPG), an osteoclast decoy receptor that competes with RANK for RANKL binding (Yasuda et al. 1998). Therefore, the RANKL/OPG ratio pivotally determines the direction of osteoclastogenesis. RANKL and OPG expression, hence the RANKL/OPG ratio, are modulated by systemic hormones such as PTH and $1,25D_3$ in order to maintain bone density and calcium homeostasis (Khosla 2001; Suda et al. 1995). In pathological conditions, notably hypocalcemia, these systemic hormones are of increased importance in BMSC-mediated osteoclastogenesis. However, differential ability of MB *vs.* LB BMSCs to support osteoclastogenesis in stimulated condition, mimicking disease stage, is not well understood.

Here, we compared *in vitro* osteoclastogenic potential of MB and LB marrow cells under basal and stimulated conditions at three different levels: osteoclast precursors, BMSCs as a master osteoclastogenic regulator, and whole marrow with intact local cell interaction. Ultimately, we evaluated the *in vivo* osteoclast formation in the MB *vs*. LB under continuous infusion of PTH and $1,25D_3$. We hypothesized that MB *vs*. LB marrows have disparate osteoclastogenic potential at basal and stimulated conditions.

## RESULTS

### Higher number of $TRAP^+$ MNCs formation and increased resorption of MB *vs.* LB Marrow-derived Osteoclast Precursors

To study the differences between MB and LB osteoclastogenic potential, osteoclast precursors were differentiated into mature OCs with M-CSF and RANKL. At the maximum potential (day 6), $TRAP^+$ MNCs were observed in both MB and LB cultures (white arrows, **Fig. 4-1 A, B**). However, with equal concentration of osteoclast differentiation factors, MB osteoclast precursors were capable of generating significantly greater numbers of TRAP+ MNCs in comparison to the LB culture (Fig.4-1 C). To verify that the observed $TRAP^+$ MNCs were functional osteoclasts, we differentiated the osteoclast precursors on calcium phosphate substrates. Visualized with von Kossa stain, the MB culture showed increased resorptive pit formation **(Fig.4-1 D, E)** with significantly higher total resorbed area **(Fig. 4-1 F)** than the LB culture.

**MB BMSCs Posses a Lower Osteoclastogenic Potential in Stimulated Conditions Compared to LB BMSCs**

BMSCs support osteoclastogenesis through modulation of the RANKL/OPG system. Hormones known to regulate bone homeostasis, such as PTH and $1,25D_3$, exert their effects on osteoclastogenesis by regulating osteoblastic/stromal cell production of these osteoclast regulatory cytokines (Nagai and Sato 1999; Suda et al. 1995). We then explored the osteoclastogenic ability of MB *vs.* LB BMSCs by determining RANKL and OPG expression at basal and hormone-stimulated conditions at 7 and 14 days. Basal RANKL and OPG mRNA expression was similar in unstimulated LB *vs*. MB BMSCs **(Fig. 4-2 A, B).** However, in the presence of $1,25D_3$ alone or in combination with PTH, RANKL expression was higher in LB *vs.* MB BMSCs at both 7 ($P<0.05$) and 14 days ($P<0.01$) **(Fig. 4-2 A).** Under the same treatments,

the inhibition of OPG expression was greater in LB *vs.* MB BMSCs with PTH+1,25$D_3$ (*P<0.01*) and 1,25$D_3$ alone (*P<0.05*) at day 14 **(Fig. 4-2 B).** Collectively, the RANKL/OPG ratio was substantially enhanced in LB *vs.* MB BMSCs under 1,25$D_3$ alone or in combination with PTH **(Fig. 4-2 C).** These findings suggest that LB BMSCs posses a more robust osteoclastogenic potential in stimulated conditions compared to the MB counterparts.

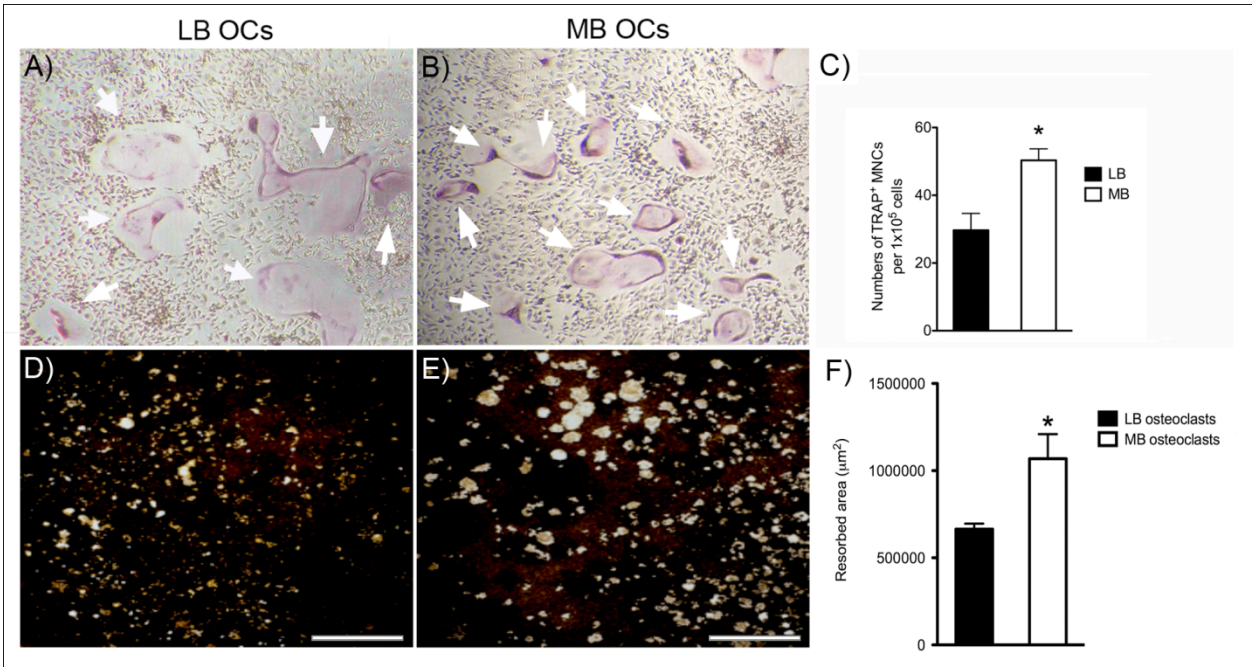


**Figure 4-1.** TRAP staining of osteoclast cultures from long bone (A) and mandible (B), after 6 days of differentiation with M-CSF and RANKL. $TRAP^+$ multinucleated cells are shown at 4X magnification (indicated by white arrows). C) Quantification of the total number of $TRAP^+$ multinucleated cells (>3 nuclei) formed by long bone and mandible osteoclast precursors after 6 days of differentiation. The number of $TRAP^+$ multinucleated cells in mandible culture is significantly higher than in the long bone culture. Von kossa stain reveals resorptive pits formed by osteoclast cultures from long bone (D) and mandible (E), after 10 days of differentiation in the presence of M-CSF and RANKL on calcium phosphate substrate. F) Quantification of total resorbed area formed by long bone and mandible osteoclasts. Mandible osteoclasts produced 1.6 fold greater resorbed area than long bone osteoclasts. Results from 3 independent experiments. * *P<0.05*; error bars represent standard error of the mean.

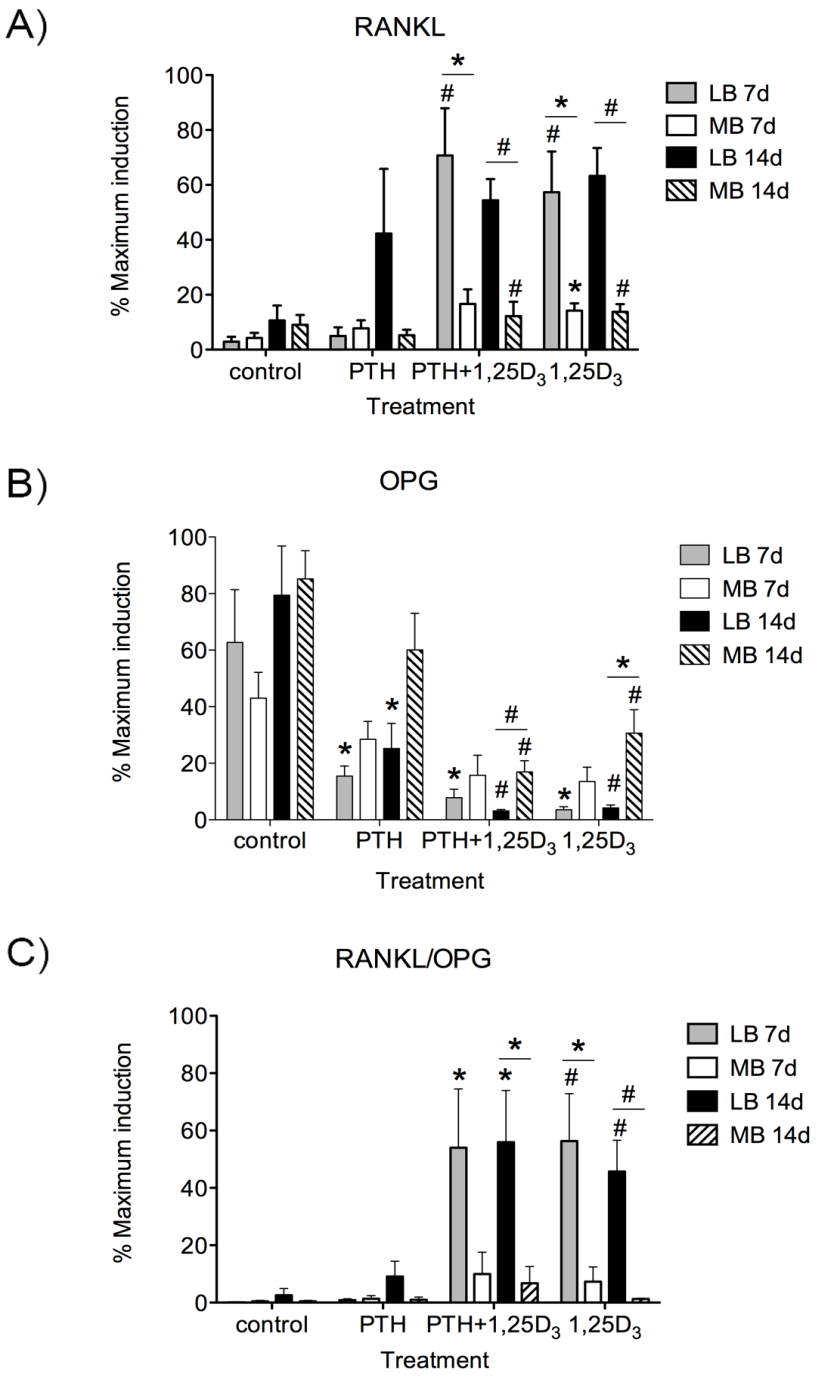


**Figure 4-2**. Effect of parathyroid hormone (PTH, 10 nM) and 1,25 dihydroxyvitamin$D_3$ ($1,25D_3$,10 nM), alone or in combination, on the expression of RANKL (A), OPG (B) and RANKL/OPG ratio (C) by long bone *vs.* mandible BMSCs in osteogenic media for 7 and 14 days. mRNA levels were normalized to GAPDH and expressed as a percentage of the maximum expression. For control, untreated BMSCs were cultured in osteogenic media for 7 and 14 days. Results from 3 independent experiments. * *P<0.05*; # *P< 0.01;* error bars represent standard error of the mean.

**LB-derived Whole Marrow Generates More TRAP$^+$ MNCs under Hormonal Induction *vs.* MB Marrow**

To evaluate the osteoclastogenic potential of MB *vs.* LB marrows, we cultured unseparated marrow, containing both osteoclast precursors and BMSCs, under basal and PTH, $1,25D_3$, or PTH+$1,25D_3$ stimulated conditions. At the end of the culture period (day7), TRAP staining revealed TRAP$^+$ MNCs in both LB and MB cultures under $1,25D_3$ alone or in combination with PTH but not in basal or PTH-stimulated conditions **(Fig. 4-3 A).** Quantitatively, LB marrow generated significantly greater TRAP$^+$ MNCs under a treatment with $1,25D_3$ alone or in combination with PTH **(Fig. 4-3 B).** Analysis of these data confirms the enhanced osteoclastogenic potential of LB BMSCs through their ability to differentiate osteoclast precursors obtained from the same local environment.

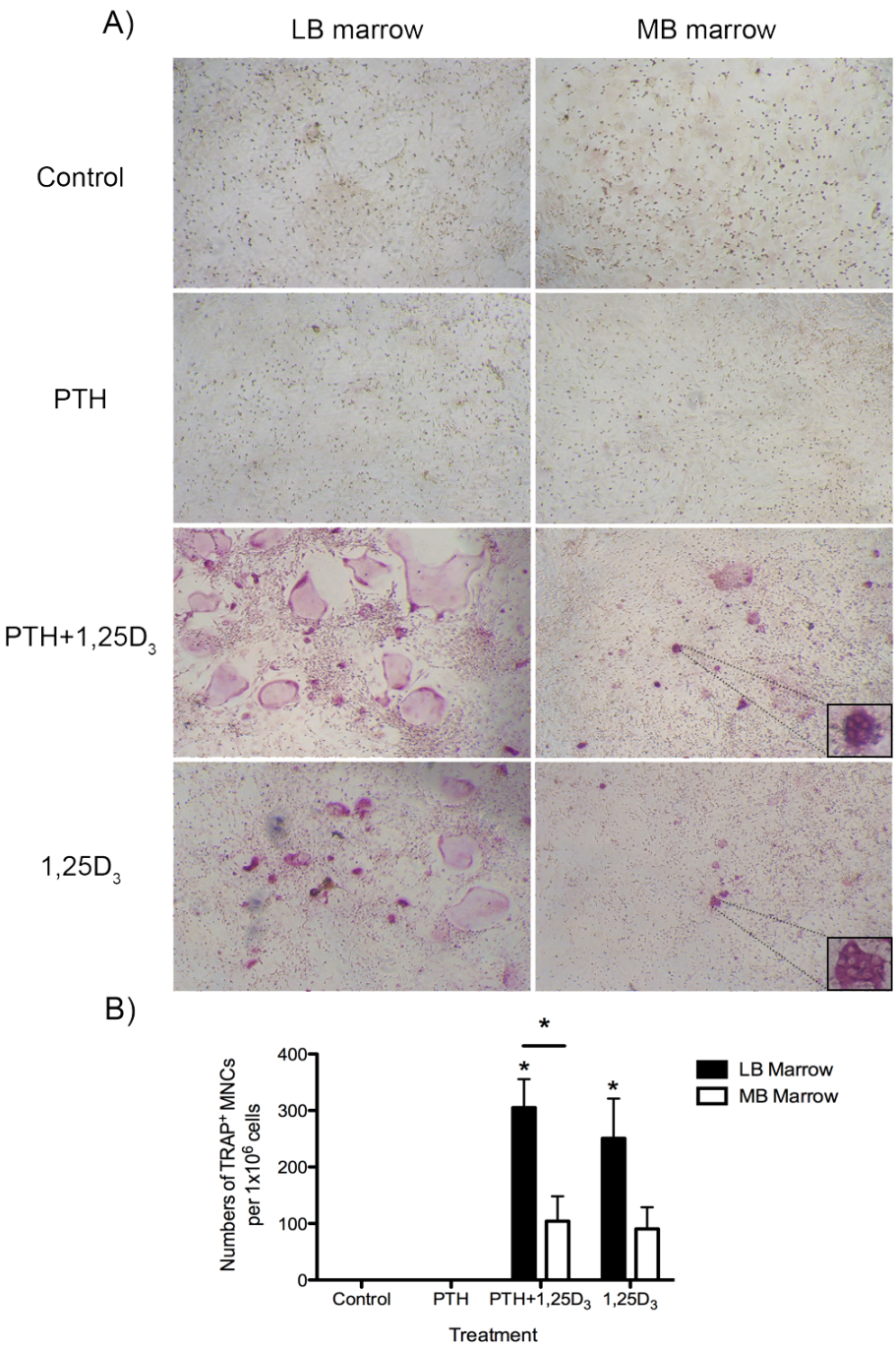


**Figure 4-3.** A) Osteoclastogenesis of long bone *vs.* mandible whole marrow in control or indicated hormonal stimulation after 7 days. $TRAP^+$ multinucleated cells were formed only in the presence of $1,25D_3$ alone or PTH+$1,25D_3$. B) Quantification of TRAP+ multinucleated cells in long bone or mandible marrow cultures under control and stimulated conditions. LB marrow generated greater numbers of TRAP+ multinucleated cells than the MB marrow when induced with $1,25D_3$. This pattern was significantly enhanced under the combination of PTH and $1,25D_3$. Results from 3 independent experiments. * $P<0.05$; error bars represent standard error of the mean.

***In vivo*-increased Osteoclast Formation in Rat Tibia *vs.* Mandible under Hormonal Stimulation**

The data described above were obtained from isolated primary cells. Ultimately, it is important to observe the overall effect of hormonal stimulation on the osteoclast formation in vivo. From the above *in vitro* data, the combination of PTH+1,25$D_3$ was shown to produce maximum osteoclastogenic potential in LB culture and, thus, was selected for continuous infusion. Mini-osmotic pumps containing either vehicle or treatment (PTH, 1,25D3) were subcutaneously implanted into three-month old male Spraque-Dawley rats for 3 days. At the end of the experiment, mandible and tibia bone samples were decalcified, and H&E stained sections were obtained from the alveolar bone region between the first and second molars of the mandible **(Fig. 4-4 A)** and the trabecular bone region of the proximal tibia **(Fig. 4-4 D).** A 3-day infusion with PTH+1,25$D_3$ induced greater numbers of osteoclasts in both tibial and mandibular sections (green arrows, **Fig. 4-4 C** and **Fig. 4-4 F**, respectively) in comparison to the controls (**Fig. 4-4 B**, and **Fig. 4-4 E,** respectively). Histomorphometric analysis was then performed to quantify the number of osteoclasts per bone perimeter (N.Oc/B.Pm). The quantification revealed a significantly increased osteoclast formation in treated animals compared to the control groups. Importantly, under PTH+1, 25$D_3$ continuous infusion, the increase in osteoclast number was more pronounced in the tibia in comparison to the mandible ($P<0.05$, **Fig. 4-4 G**). Analysis of these *in vivo* data recapitulated the *in vitro* findings demonstrating that LB has a higher osteoclastogenic potential under stimulated condition.

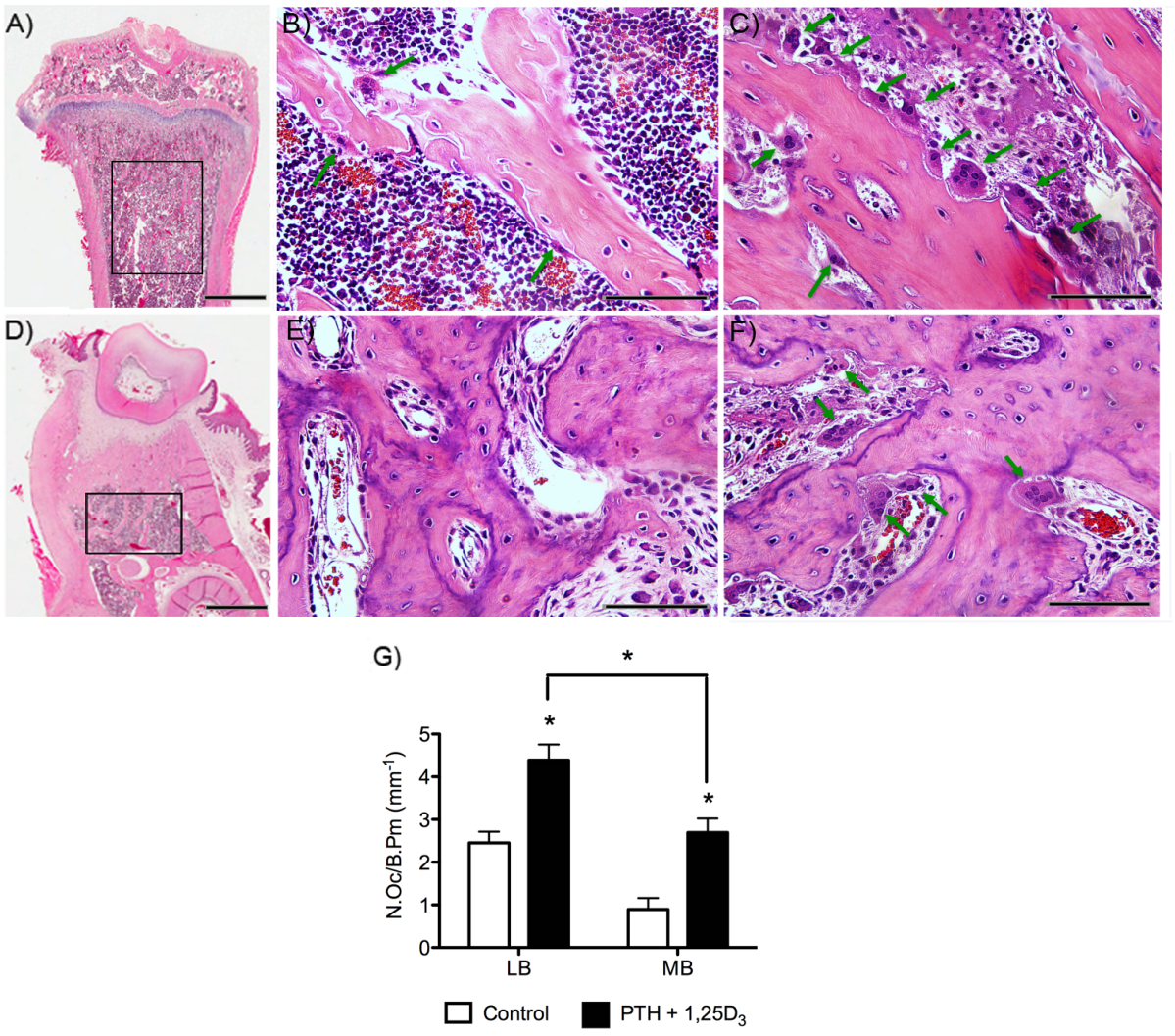


**Figure 4-4.** H&E sections at 2X (A, D), and 40X (B, C, E, F) of tibia (A, B, C) and mandible (D, E, F). Sprague-Dawley rats were infused with control vehicle (B and E) or continuous PTH+1,25$D_3$ (C and F) for 3days. G) Osteoclasts (indicated by green arrows) were quantified within the trabecular region of the tibia and alveolar bone of the mandible (rectangular insets, A and D). Continuous treatment of PTH+1,25$D_3$ induced significantly greater number of osteoclasts in the tibia in comparison to the mandible (n≥3). * *P<0.05*; error bars represent standard error of the mean.

## DISCUSSION

Mandible, as a part of the jaw bones, possesses unique metabolic and functional properties and demonstrates discrete responses to homeostatic, mechanical and developmental stimuli (Sodek and McKee 2000). Specific bone pathologies such as cherubism (Ueki et al.

2001), hyperparathyroid jaw tumor syndrome (Simonds et al. 2002), and recently revealed bisphosphonate-related osteonecrosis of the jaws (BRONJ) (Marx 2003) affect the jaws differently compared to other bones. We previously uncovered differences in osteogenic potential of LB *vs*. MB BMSCs that has led us to speculate BMSC response to other external stimuli and other potential differences in bone remodeling and healing. In this study, we report a disparity in osteoclastogenesis between mandible and long bone under basal and stimulated conditions.

We first investigated *in vitro* osteoclastogenic potential of MB *vs*. LB–derived osteoclast precursors in the presence of M-CSF and RANKL. We selected day 6 as the end of the culturing period to compare osteoclasts at their maximum maturation (Bradley and Oursler 2008). MB precursors significantly generated higher numbers of TRAP+ MNCs than the LB cultures. A previous study reported a faster osteoclastogenesis in the long bone precursors at an early time point (day 4), although, the difference was not observed at the end of the culture period (day 6). A difference in cellular composition of jaw and long bone osteoclast precursors, revealing monocytes and myeloid blasts as predominant cells in the jaw and long bones, respectively, accounted for the observed difference in the earlier time point (Souza Faloni et al. 2010). Since we compared osteoclasts at their maximum maturation, the difference we observed between the MB and LB was most likely not due to the difference in cellular composition.

Besides the difference in cellular composition, mineralized matrix such as bone and dentin could affect the differentiation and function of osteoclast precursors. More LB osteoclasts were formed on bone than on dentin, and this pattern was reversed with jaw-derived osteoclast cultures. However, there was no difference in the resorption between LB and jaw osteoclasts on

both substrates (Azari et al. 2011). We utilized a calcium phosphate substrate as a neutral culturing surface in order to confirm that the generated $TRAP^+$ MNCs were functional osteoclasts. The higher osteoclast formation ability of MB precursors was mirrored by the larger total resorbed area generated by MB osteoclasts in comparison to LB cultures. Our result was not in accordance with the above study, which could be explained partly by the difference in cell isolation methods and species-specific differences between rodent *vs*. murine. All in all, our findings suggested an increased osteoclastogenic potential of MB osteoclast precursors.

BMSCs have a crucial role in the regulation of osteoclastogenesis. Therefore, differences in osteoclastogenic potential of the MB *vs.* LB might result from the diverse BMSC function. Indeed, we have reported an increased osteogenic potential of rodent MB *vs*. LB BMSCs both *in vitro* and *in vivo* (Aghaloo et al. 2010). Recently, similar results from a murine study also demonstrated elevated osteogenic differentiation potential of mandibular BMSCs compared with BMSCs from long bone (Yamaza et al. 2011). As mentioned above, BMSCs mediate osteoclastogenesis through the production of RANKL and OPG. It is well established that systemic hormones such as PTH and $1,25D_3$ do not act directly on osteoclasts, but modulate osteoblasts/stromal cell activity, especially RANKL and OPG expression in order to maintain bone density and calcium homeostasis (Khosla 2001; Martin and Ng 1994; Suda et al. 1995). We, thus, studied the effect of these hormones on RANKL and OPG expression of BMSCs. In the presence of $1,25D_3$ alone or in combination with PTH, MB BMSCs expressed far less RANKL than that of LB BMSCs. Importantly, the inhibition of OPG expression in LB BMSCs was more pronounced, leading to an elevated RANKL/OPG ratio of LB BMSCs under

stimulated conditions. Such an elevated expression would cue LB BMSCs more toward osteoclastogenesis than the MB counterparts.

Next, we investigated the enhanced osteoclastogenic potential of LB BMSCs and their ability to differentiate osteoclast precursors obtained from the same local environment by means of unseparated marrow. Compared to the MB marrow, LB marrow generated significantly greater $TRAP^+$ MNCs, reinforcing the higher osteoclastogenic potential of LB marrow. Ultimately, we evaluated the overall effect of hormonal stimulation on osteoclast formation *in vivo*. Continuous infusion of PTH+1,25$D_3$ significantly induced greater osteoclast formation in rat tibial *vs.* mandibular bones. In this perspective, these systemic hormones act as a feedback mechanism to restore the balance of bone homeostasis especially in pathological conditions such as hypocalcaemia. Our results suggest that LB has a greater capacity to respond to bone homeostatic stimuli and an increased ability to support osteoclastogenesis.

In summary, our data demonstrate a diverse osteoclastogenic capacity of the MB *vs.* LB marrow. Although MB osteoclast precursors appeared to be more sensitive to RANKL and M-CSF stimulation, hormone-stimulated LB BMSC/LB marrow possesses an increased ability to support osteoclastogenesis. Bone-specific differences in osteoclasts may relate to osteoclast precursors and matrix compositions among others. Our study provides new evidence of bone-specific difference in BMSCs ability to support osteoclastogenesis under stimulated conditions both *in vitro* and *in vivo*. These findings demonstrate a reduced capacity of MB marrow cells to induce osteoclast formation and support an increased sensitivity of the jaws to antiresorptive treatments that might explain, at least in part, the pathophysiology of osteonecrosis of the jaws (ONJ) and other jaw-specific diseases resulting from the disturbance of bone homeostasis.

# CHAPTER 5

# LONG BONE VERSUS MANDIBLE BONE MARROW STROMAL CELL BONE REGENERATION POTENTIAL IN INTRAMEMBRANOUS VERSUS ENDOCHONDRAL CRITICAL-SIZED BONE DEFECTS


## ABSTRACT

Bone marrow stromal cells (BMSCs) are multipotential cells that are capable of forming mesenchymal tissues, including bone. The use of BMSCs in bone tissue engineering has emerged as a promising therapy for bone defect repair. It is well-known that mandible (MB) and long bone (LB) are different in their embryonic origin and bone development. Besides these differences, our previous study also uncovered an enhanced osteogenic potential of MB *vs.* LB BMSCs *in vitro* and *in vivo*. Recently, a study demonstrated that the bone defects heal through selective recruitment of cells from their own origin. Therefore, in the bone repairing process, for successful bone defect regeneration, the skeletal site where BMSCs are obtained and the anatomical site of the skeletal defect must be taken into consideration. In this study, we evaluated MB *vs.* LB BMSC bone regeneration potential in rat critical-sized calvarial *vs.* critical-sized femoral defects, representing intramembranous *vs.* endochondral bone defects, respectively. We found that MB and LB BMSCs had comparable bone regeneration potential in both types of defects. However, MB BMSCs generated a better bone quality in calvarial defects and were able repair bone defects in both skeletal sites. These results provide evidence that MB BMSCs are more advantageous in bone defect repair than their LB counterpart, if readily accessible.

## INTRODUCTION

Substantial loss or deformity of the bone tissue is physically and psychologically devastating, and can adversely affect the quality of life. Such large osseous defects resulting from severe trauma, infection, cancer, tumor resection or congenital malformations post a challenge for reconstructive surgeons. Among all treatment modalities, bone grafting is widely used clinically for bone defect regeneration which commonly include autogenous bone and allografts (Damien and Parsons 1991; De Long et al. 2007). Autogenous bone or autograft provides osteoinductive growth factors, osteogenic cells, and osteoconductive scaffold for the bone defect (Giannoudis et al. 2005). All of those features lead to a great osteogenic ability. Combining with the inherent compatibility with host tissues, autogenous bone is considered the gold standard treatment for the repair of bony defects. Although the benefits of autograft are paramount, there is a limited supply of host bone and a risk of donor site morbidity (Arrington et al. 1996; Damien and Parsons 1991; Ross et al. 2000). To avoid these problems, allografts have been used as an alternative. However, disease transmission and immunologic reaction are major disadvantages of the allografts. Though sterilization of the graft could circumvent these problems, osteogenic cells and osteoinductive property are greatly reduced (De Long et al. 2007; Giannoudis et al. 2005).

Recently, cell-based bone tissue engineering has grown recognition as a promising treatment modality for bone defect regeneration. Bone marrow stromal cells (BMSCs) are multipotential cells that are capable of differentiating into osteoblasts, chondrocytes, and adipocytes among others (Bianco et al. 2001; Pittenger et al. 1999). BMSCs are readily isolated from bone marrow, can be expanded for many passages, and are relatively unaffected by

cryopreservation and thawing process (Jafarian et al. 2008; Tsuchida et al. 2003). Additionally, they possess immunosuppressive and anti-inflammatory properties, and soon are recognized as an ideal alternative to autogenous bone graft (Kagami et al. 2011; Uccelli et al. 2008, 2007).

Critical-sized defects (CSDs) are intraosseous wounds that will not heal during the life time of the animal (Schmitz and Hollinger 1986). CSDs not only are suitable models used to evaluate the efficacy of materials used in bone regeneration, but they are also an appropriate representation of clinically observed large osseous defects in need of repairment. During the bone repairing process, BMSCs are recruited to the injury or defective sites. It has been demonstrated that the same molecular mechanism of fetal skeletogenesis continues through adulthood in the form of skeletal remodeling and repair (Ferguson et al. 1998). The mandible (MB) and long bone (LB) are different in their developmental origin and employ different molecular mechanisms of skeletogenesis. The MB originates from neural crest cells and is formed primarily by intramembranous ossification; on the other hand, LB arises from cells of mesodermal origin and is ossified via endochondral process (Chai and Maxson 2006; Karaplis 2008). A question has been raised whether skeletal sites, where BMSCs are obtained, would play a role in the healing of bone defects. Indeed, enhanced osteogenic potential of orofacial *vs.* LB BMSCs has been demonstrated in human, rodent and murine which leads us to investigate the MB *vs.* LB BMSC bone regeneration potential (Akintoye et al. 2006; Aghaloo et al. 2010; Yamaza et al. 2011). Reinforcing the basis of developmental origin, a study has demonstrated that skeletal defects heal through the recruitment of progenitor cells of their own origin (Leucht et al. 2008). Thus, in the bone repairing or bone regeneration process, molecular differences

between the grafted cells and the recipient defect sites must be taken into consideration for successful bone defect regeneration.

In this study, we validate and compare the multipotential of MB *vs.* LB BMSCs. The main objective of our study is to evaluate MB *vs.* LB BMSC bone regeneration potential in rat critical-sized calvarial *vs.* critical-sized femoral defects, representing intramembranous *vs.* endochondral bone defects, respectively. We hypothesize that MB BMSCs will induce greater and more mature bone regeneration than LB BMSCs with better bone regeneration outcome in the intramembranous calvarial defect.

## RESULTS

### LB BMSCs Have a Higher Adipogenic Potential, While MB BMSCs Have a Higher Osteogenic Potential.

We previously cultured and characterized rodent BMSCs in osteogenic differentiation media and found that MB BMSCs have a higher osteoblastic potential in comparison to LB BMSCs both *in vitro and in vivo,* demonstrated by higher alkaline phosphatase activity, increased osteogenic gene expression, and enhanced *in vivo* ectopic bone formation(Aghaloo et al. 2010). Under the adipogenic differentiating condition, the extent of adipocyte differentiation from each type of BMSCs was evaluated with oil red O staining, which revealed red colored lipid droplets accumulated in cytoplasmic vacuoles of differentiated cells. In LB BMSC culture, the majority of the cells were oil red O positive, and large lipid droplets were observed in some cells at day 7 **(Fig. 5-1 A)**. At day 14, intense red, multilocular lipid droplets were tightly distributed in cells, representing mature adipocytes **(Fig. 5-1 B).** In the MB BMSC culture,

adipocyte differentiation was observed at a lesser extent in comparison to the LB culture at both day 7 and day 14 with less intense staining, sparsely distributed lipid droplets, and several remaining undifferentiated BMSCs **(Fig. 5-1 C and D).** To further explore the adipogenic potential of MB *vs.* LB BMSCs, we analyzed the expression of adipogenic markers, including lipoprotein lipase (LPL), peroxisome proliferator-activated receptor-γ (PPARγ) and cluster differentiation 36 (CD36). Transcription factor PPARγ is a master regulator of adipogenesis and induces the expression of CD36, a scavenger receptor responsible for uptaking oxidized low-density lipoprotein (Febbraio et al. 2001; Rosen and MacDougald 2006). Adipogenic differentiated LB BMSCs expressed significantly higher mRNA level of PPARγ and CD36 than the MB BMSC culture. The expression of LPL, an early adipocyte differentiation marker, was also higher in LB BMSCs, but the difference was not statistically significant. In the MB BMSC culture, similar expression levels of PPARγ and CD36 were observed at both time points. These findings suggested that LB BMSCs have a higher adipogenic potential.

For chondrogenic differentiation condition, Alcian blue staining revealed that both LB and MB BMSCs were capable of forming cartilage matrix, but a larger size matrix was seen in LB BMSC culture at 7 and 14 days of differentiation **(Fig. 5-2 A and B)**. Further analysis of chondrogenic markers, however, showed comparable expression of Collagen X alpha 1 (Col Xa1) and SRY-box containing gene 9 (Sox 9) in both types of culture (**Fig. 5-2 F and G).** Though the trend of Collagen II alpha 1 (Col IIa1) expression was higher in LB BMSC culture, the difference was not statistically significant at 7 and 14 days **(Fig. 5-2 E).** These results suggest that the isolated LB and MB BMSCs are capable of multi-lineage differentiation.

Importantly, they have preferential differentiation paths with LB BMSCs favoring adipogenesis while MB BMSCs promotes osteogenesis.

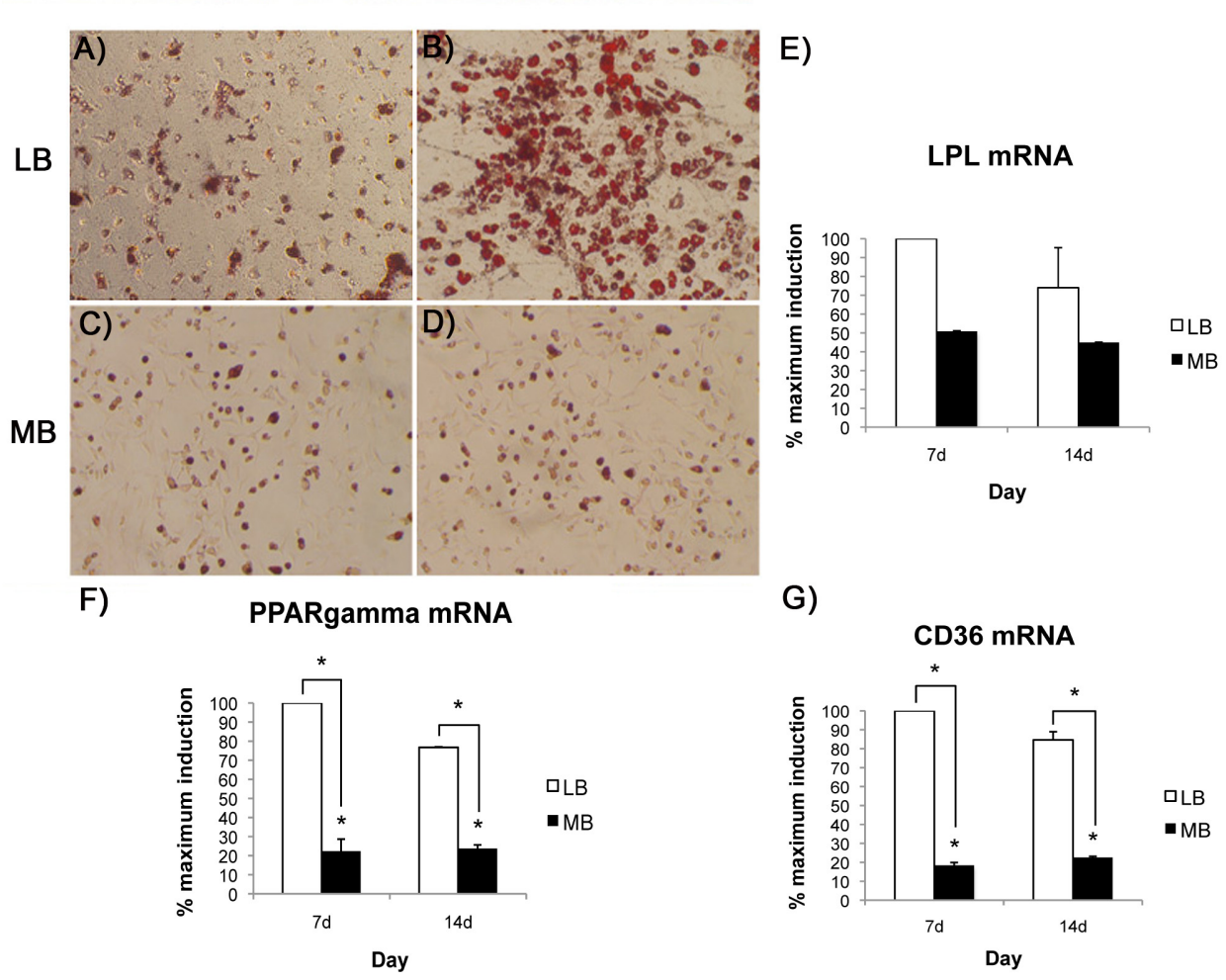


**Figure 5-1.** Analysis of adipogenic differentiation potential of LB *vs.* MB BMSCs. Oil-red-O staining at 10X of LB marrow cells (A, B) and MB marrow cells (C,D) cultured in adipogenic media for 7 (A, C) and 14 (B, D) days. E) LPL, F) CD36, and G) PPARgamma mRNA expression determined by qPCR of MB *vs*. LB BMSCs cultured in adipogenic media for 7 and 14 days.
*$p < 0.05$; error bars represent standard error of the mean.

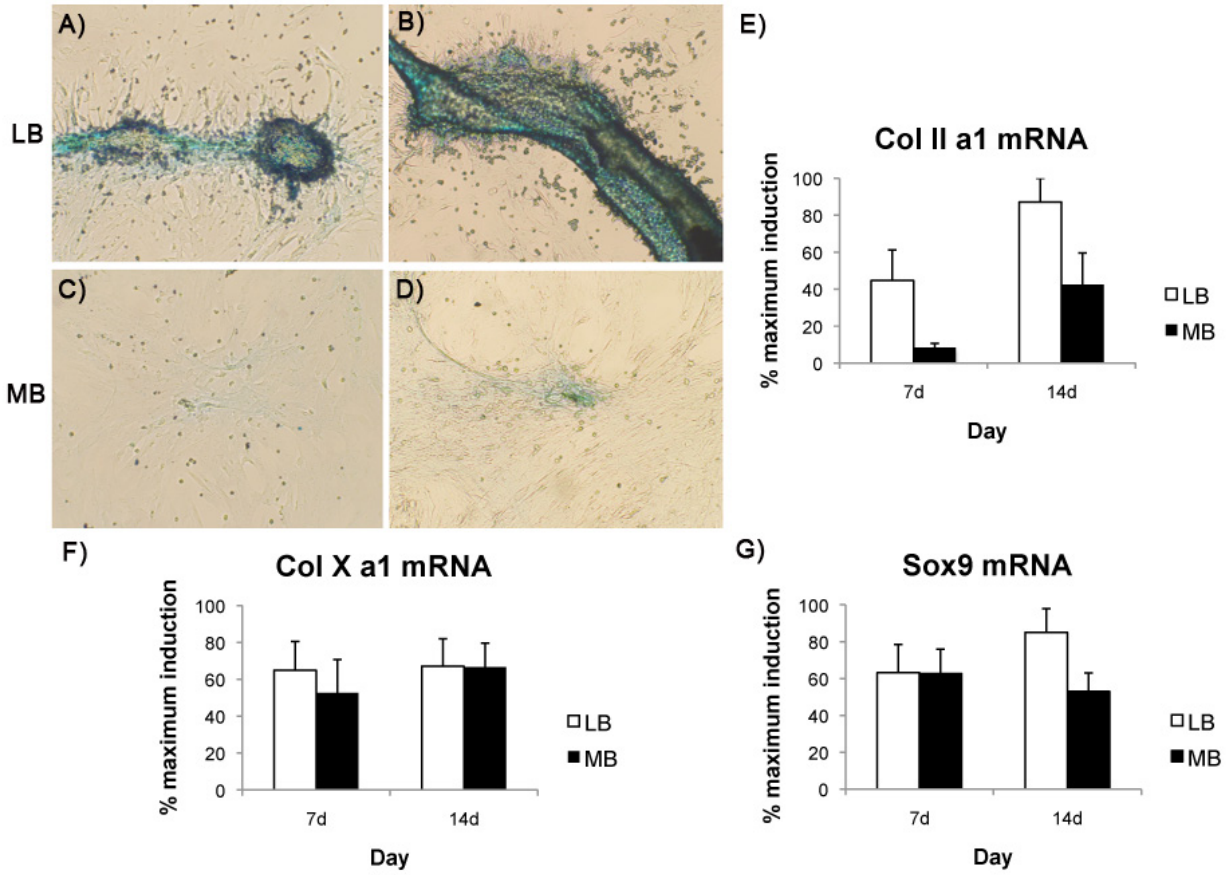


**Figure 5-2.** Analysis of chondrogenic differentiation potential of LB *vs.* MB BMSCs. Alcian blue staining of LB marrow cells (A, B) and MB marrow cells (C,D) cultured in chondrogenic media for 7 (A, C) and 14 (B, D) days at 10X (A, C) and 20X (B, D) magnification. E) Col IIa1, F) Col X, and G) Sox9 mRNA expression determined by qPCR of MB *vs*. LB MSCs cultured in chondrogenic media for 7 and 14 days. *$p < 0.05$; error bars represent standard error of the mean.

## High Resolution microCT Imaging and Bone Quantification of Bone Regeneration

To quantitate the extent of MB *vs.* LB BMSC induced bone regeneration in critical-sized calvarial defects, the new bone volume was investigated. After a 6-week healing period, high resolution microCT 3D reconstructed images of calvarial defects **(Fig. 5-3 A, D, E)** demonstrated minimal bone formation in calvarial defect implanted with the control sponge **(Fig. 5-3 A)**. Calvarial defects engrafted with LB and MB BMSCs demonstrated increased new bone

formation compared to the control, and MB BMSCs appeared to generate more bone than LB BMSCs **(Fig. 5-3 C and E)**. Using CT-based morphometric analysis, bone volume (BV) and tissue volume (TV) of 3D reconstructed microCT images only within the 8 mm round defects (**Figure 5-3 B, D and F)** were quantified. Quantitation of the percent BV/TV showed that only MB BMSCs generated significantly more bone than the control defects; however, there was no difference in new bone formation between MB and LB BMSC engrafted defects **(Fig. 5-3 G)**. To quantify the percentage of bone healing, the area within the defect covered by new bone formation was calculated. LB and MB BMSCs generated significantly higher percent bone healing, 40 and 55%, respectively, in comparison to gelatin sponge, 23% **(Fig. 5-3 H).** After 6 weeks of healing period, no complete healing of calvaria defects was observed.

For critical-sized femoral defect, we monitored the progress of bone regeneration within the defects with high resolution radiography. After an 8-week healing period, radiographs of the femoral defects engrafted with LB and MB BMSCs showed remarkable bone regeneration compared to the defects engrafted with gelatin sponge alone (**Fig. 5-4 A**, red box). Animals were sacrificed after 8 weeks, as this end point allowed comparison of bone regenerative ability of MB *vs.* LB BMSCs before defects are completely healed. Three dimensional reconstructed microCT images of the defects implanted with control sponge demonstrated minimal bone formation, mostly limited to the margins of the defect **(Fig. 5-4 B**, top panel**)**. Femoral defects engrafted with LB and MB BMSCs showed increased new bone formation compared to control, and the degree of bony bridging induced by MB BMSCs appeared to be more advanced than the defect implanted with LB BMSCs **(Fig. 5-4 B**, middle and bottom panels**)**. Quantitation of the percent BV/TV indicates that both LB and MB BMSCs generated significantly more bone than

the control sponge. Although MB BMSCs showed higher percentage of BV/TV, there was no statistical difference between a bone defect grafted with MB and LB BMSCs **(Fig. 5-4 C)**. The same trend was observed with the percent bone healing revealing approximately 20% for the defects with gelatin sponge alone compared to 80% and 82% for LB and MB BMSCs, respectively **(Fig. 5-4 D)**.

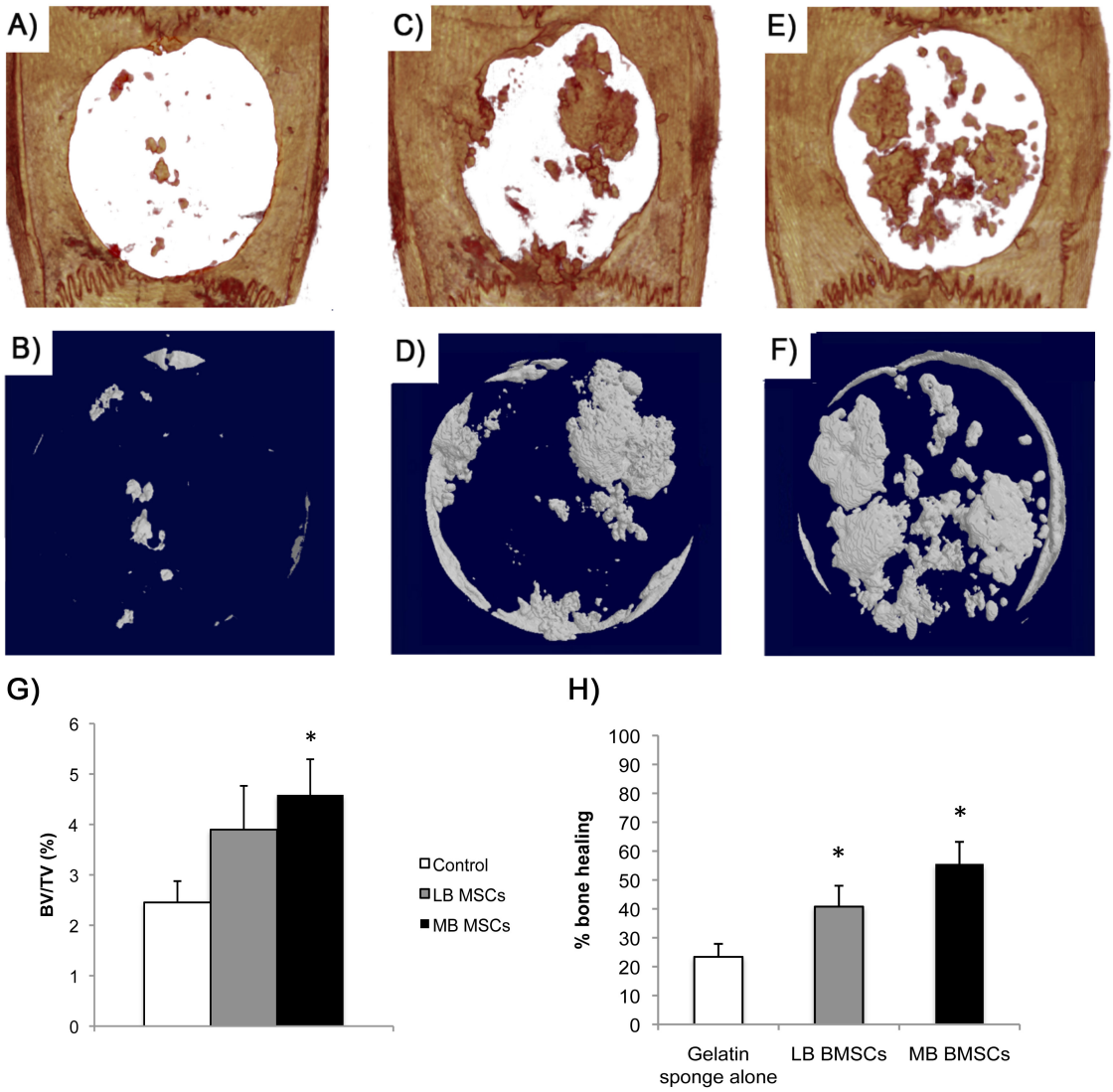


**Figure 5-3.** MicroCT analysis of rat calvarial defect engrafted with control sponge (A,B), MB BMSCs (C, D), and LB BMSCs (E, F). (A, C, E) 3D reconstruction on Dolphin software for representation. (B, D, F) 3D Reconstruction of new bone formation within 8mm diameter of defect. (G) Percent bone volume to tissue volume comparing bone regeneration in calvarial defects implanted with control sponge, MB BMSCs and LB BMSCs. Calvarial defects implanted with MB MSCs produced the highest bone regeneration and generated significantly ($p< 0.05$) more bone than control samples (n=9). *$p< 0.05$; error bars represent standard error of mean.

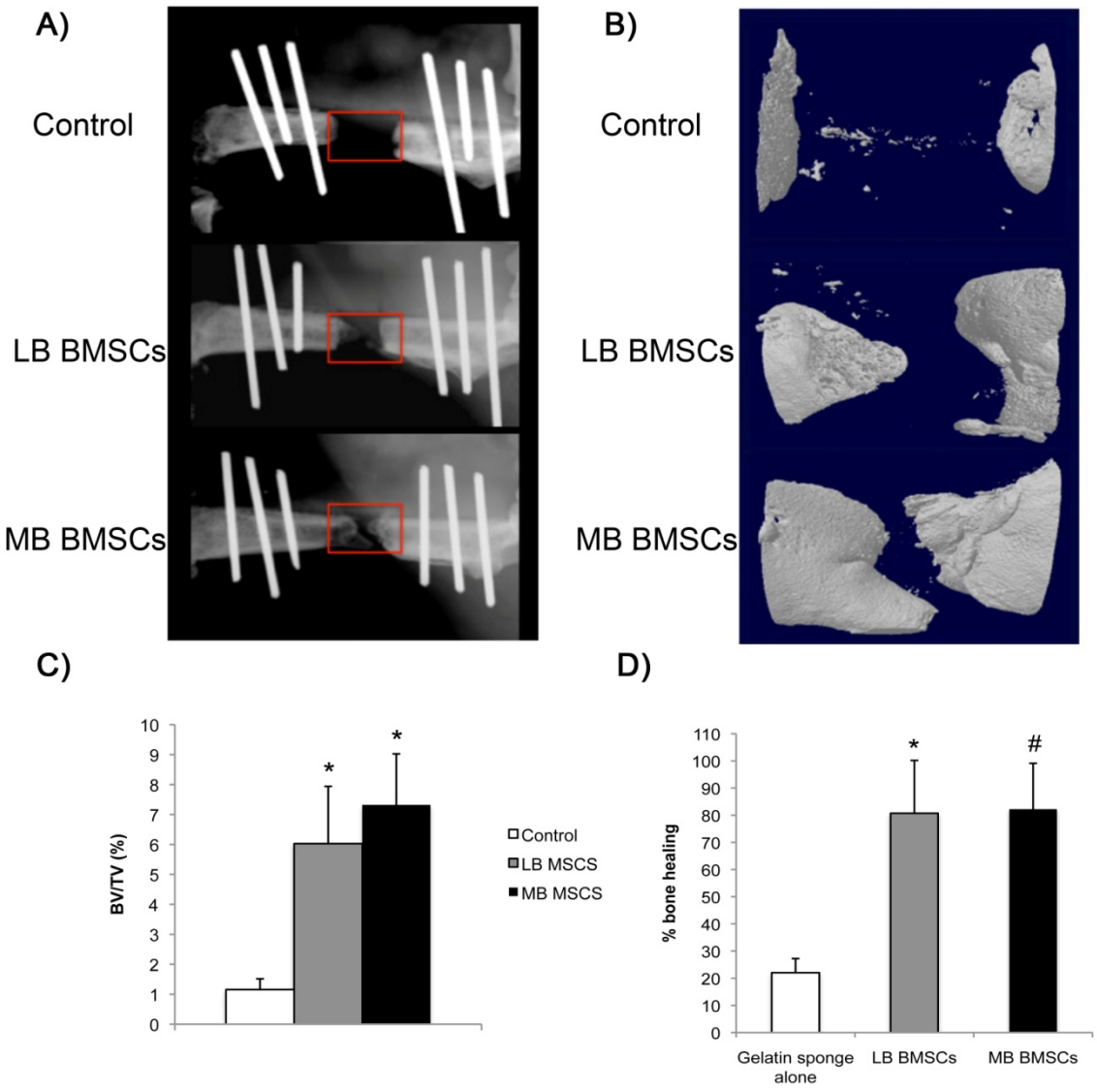


**Figure 5-4**. Radiograph of femoral defect grafted with the indicated grafts. B) 3D reconstruction of the new bone formation with the indicated grafts. C) Percent BV/TV quantitation of the new bone formation within the femoral defects with indicated grafts (n=6). *$p$< 0.05; error bars represent standard error of mean

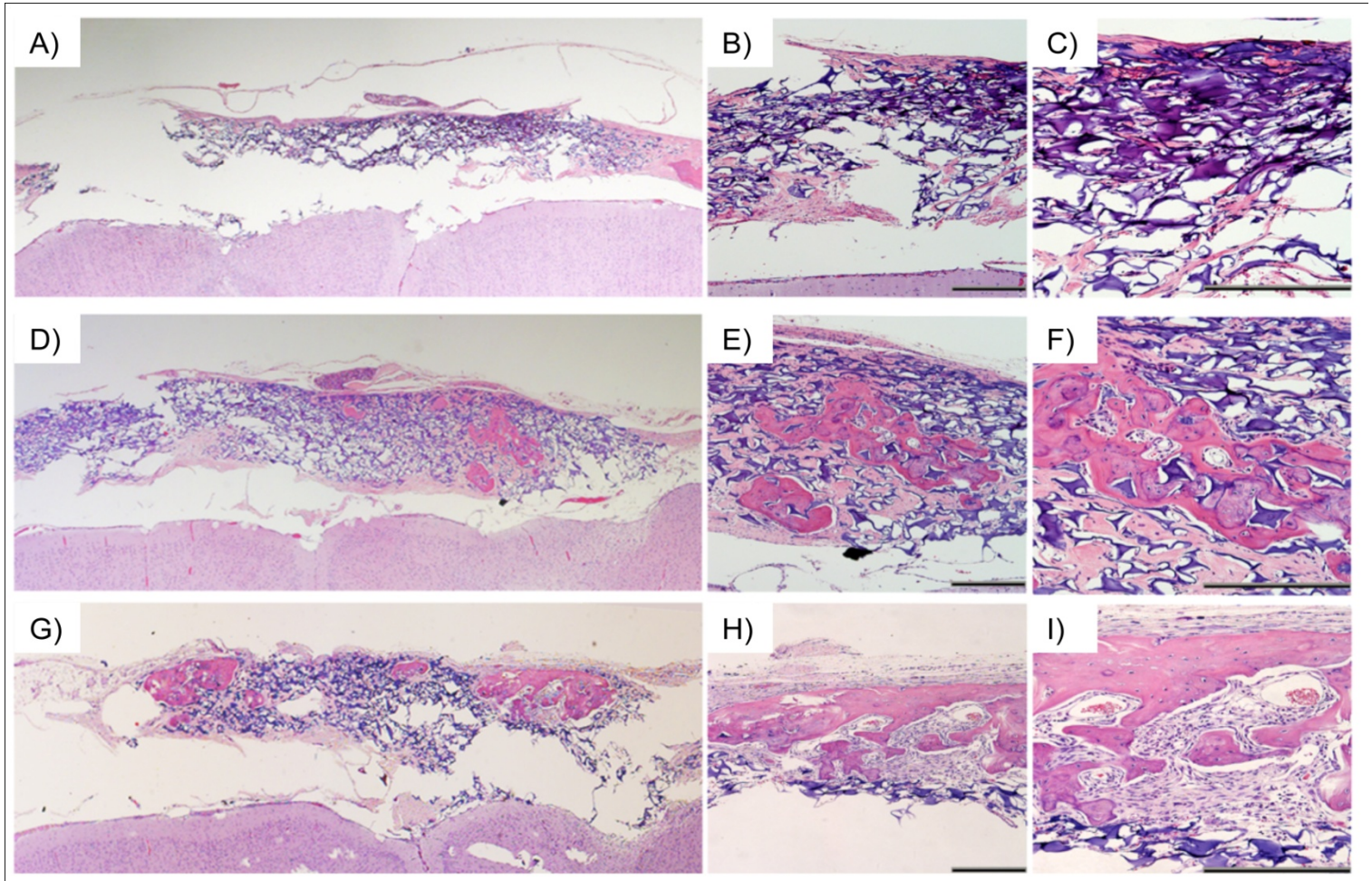


**Figure 5-5**. H&E-stained sections of calvarial defects implanted with control sponge (A-C), LB MSCs (D-F) and MB MSCs (G-I) at 2X magnification by photomerge (A,D,G), 10X magnification (B, E, H), and 20X magnification ( C, F, I).

**Histological Evaluation of Bone Regeneration**

H&E-stained of decalcified calvarial samples revealed mostly fibrous material within the defected grafted with gelatin sponge alone **(Fig. 5-5 A-C)** and confirmed the presence of new bone formation in both LB and MB engrafted defects **(Fig. 5-5 D-F and G-I)** at 6-week healing period. In LB BMSC implanted samples, immature woven bone and cartilaginous matrix were mostly observed surrounded by the remnants of resorbing gelatin sponge carrier **(Fig. 5-5 D-F).** On the other hand, in the MB BMSC engrafted samples, the regenerated bone was more organized and composed of mature lamellar bone with minimal residual gelatin scaffold in the bone regenerated area **(Fig. 5-5 G-I)**. Additionally, the presence of vessel-like structures indicated a vascularization within the grafted areas **(Fig. 5-5 F and I**).

Bone regenerated within the femoral defect was evaluated by histological analysis of H&E stained slides. In the defects grafted with gelatin sponge alone, minimal bone growth was generated, but it was limited to the margins of the defects only. A thick layer of connective tissue connecting the two bone segments and remnants of gelatin sponge scaffold could be observed within the defect **(Fig. 5-6 A-B, C** black oval**)**. For femoral defects engrafted with LB and MB BMSCs, bone regeneration was observed extending from the proximal and distal ends of the defects toward the middle of segmental defect **(Fig. 5-6 D-E and G-H)**. Newly formed bone was continuous with the host original bone and contained marrow space with little to no residual of gelatin sponge **(Fig, 5-6 F-H)**.

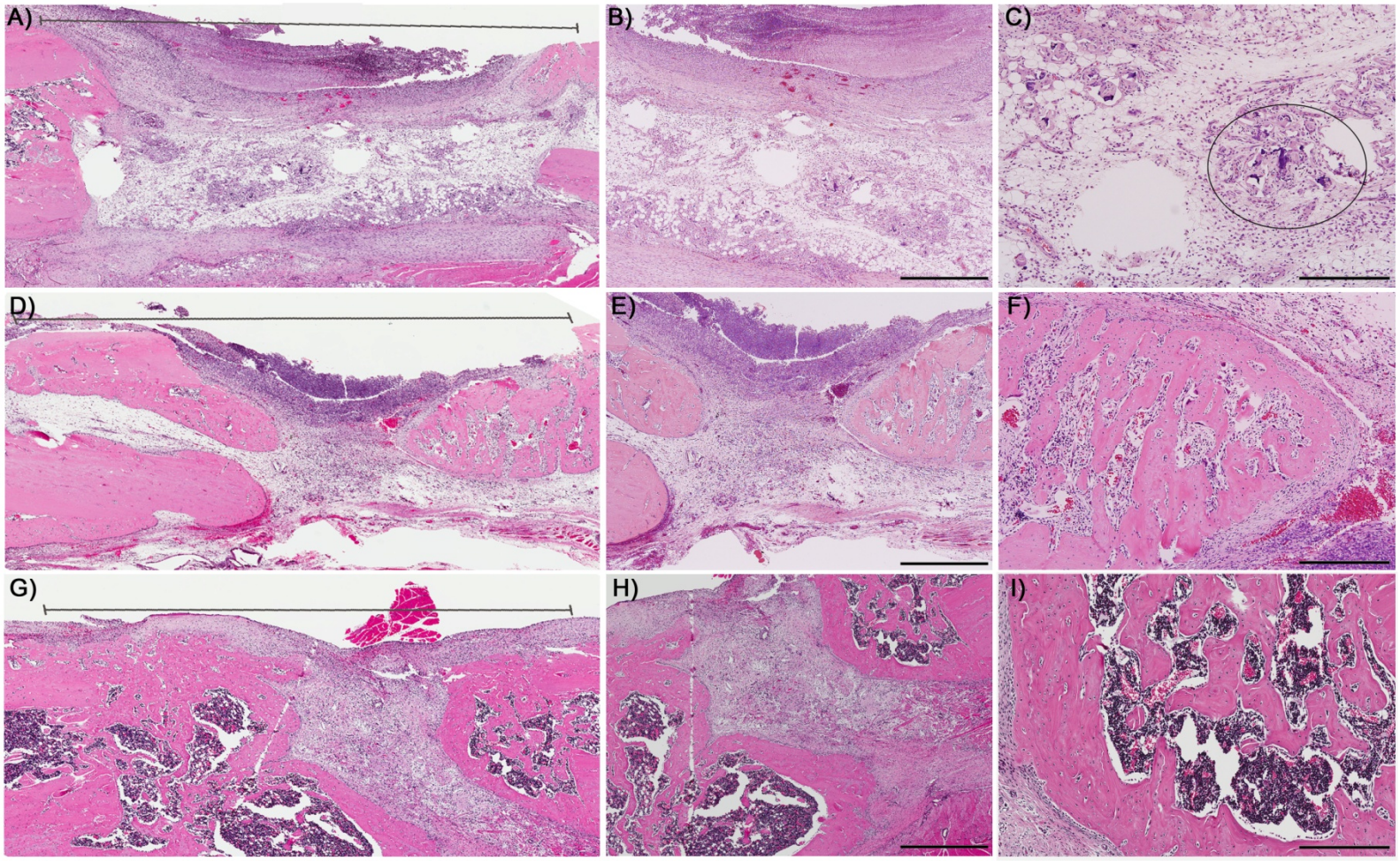


**Figure 5-6.** H&E-stained sections of femoral defect implanted with control sponge (A-C), LB MSCs (D-F) and MB MSCs (G-I) at 1X magnification by Aperio software imaging (A, D, G, line showing the defect length of 6 mm), at 4X magnification at the center of the defect (B, E, H) at 10X magnification (C, F,I, black oval indicated the remnants of gelatin sponge) by light microscope.

## DISCUSSION

Cell-based bone tissue engineering has emerged as a promising treatment for bone defect regeneration. BMSCs with many beneficial properties have been recognized as an ideal alternative to the gold standard autogenous bone grafts. BMSCs can be obtained from axial and appendicular bones such as ilium, femur, tibia and spine. However, marrow aspiration from these bones is an invasive procedure, compared to mandibular and maxillary BMSCs which can be obtained during dental surgical procedures (Matsubara et al. 2005). In our previous study, we demonstrated that MB BMSCs have enhanced osteogenic potential than those derived from long bone, prompting an investigation of MB *vs.* LB BMSC bone regeneration potential **(Chapter 3)** (Aghaloo et al. 2010).

We, first, verified the multipotency of the isolated BMSCs. Adipogenic and chondrogenic differentiation potential of MB *vs.* LB BMSCs were investigated in this study, since we extensively analyzed their osteogenic potential through a series of osteoblast phenotype assays. We found that MB BMSCs posses a much lower adipogenic potential than LB BMSCs, while the chondrogenic potential was comparable between the two cell types. The lower adipogenic potential of MB BMSCs was also found in a previous study with human alveolar BMSCs, and it was suggested that this lower potential might decrease unfavorable fat formation during tissue generation, an additional benefit of MB BMSCs (Matsubara et al. 2005). In any case, we confirmed the multi-lineage differentiation potential of the isolated MB and LB BMSCs.

Next we explored the clinical relevance potential of MB *vs.* LB BMSC ability to repair critical-sized calvarial and critical-sized segmental femoral defects. Quantitation of new bone

formation by microCT analysis revealed that overall MB and LB BMSCs, seeded onto the gelatin sponge, regenerated more bone than the gelatin sponge alone in both calvarial and femoral defects. Although MB BMSCs demonstrated higher bone regeneration compared with LB BMSCs, no statistical difference was found in both types of defects. It is interesting to note that, regardless of the skeletal sites where the defects were created, a gelatin sponge alone was capable of generating approximately 20% bone healing. The minimal bone formation was also observed when gelatin scaffold was implanted in the murine cranial defect model (Ben-David et al. 2011). This might be due to the intrinsic osteoconductive property of the gelatin sponge. Our study identified the baseline of bone regeneration potential of the gelatin sponge that could be expected when used as a carrier in tissue regeneration.

Focusing on the intramembranous calvarial bone defect, a study demonstrated that skeletal defects heal through the recruitment of progenitor cells of their own origin (Leucht et al. 2008). MB BMSCs, which were isolated from intramembranous mandibular bone marrow, were therefore expected to have a better healing potential than LB BMSCs. Though the difference in % BV/TV was statistically non-significant, histological analysis revealed that the *quality* of bone regenerated by MB BMSCs featured a more organized lamellar bone. This type of bone might be better than immature woven bone and cartilaginous matrix produced by LB BMSCs. Similar phenotypes of the regenerated bone were also observed in our previous study with the ectopic formation assay, indicating 'positional memory' of BMSCs (Aghaloo et al. 2010). Chondrogenic characteristic of the regenerated bone was also observed when murine tibular skeletal progenitors were implanted into an intramembranous mandibular bone defect. They

attributed this phenotype to the differences in embryonic origin and Hox status of the grafted cells and recipient site (Leucht et al. 2008).

Our study found that BMSC seeding onto gelatin sponge could heal about 40 – 55% of critical-sized calvarial defect. The success of bone defect healing depends on several factors including the defect size, carrier, number and condition of cells, and healing period. We used an 8-mm diameter calvarial defect model because it is a well established critical-sized calvarial defect that will not heal spontaneously for up to 12 weeks or 13 months depending on the species and age of the rat model (Hollinger and Kleinschmidt 1990; Takagi and Urist 1982). It was suggested that this defect size, expanding across the sagittal suture, might introduce the connective tissue of the suture and affect the overall bone regeneration potential of the defect (Bosch et al. 1998). We previously examined BMSC bone regeneration potential in bilateral 5-mm diameter calvarial defects. Similar results were observed after a 6-week healing period (data not shown). Therefore, the diameter of the critical-sized calvarial defect used in this experiment was optimal in our experiment.

Gelatin sponges were utilized as BMSC carriers in our study. They are biodegradable, biocompatible, porous and flexible in shape. In addition, they provide excellent cellular support, and have a high affinity to other matrix proteins and homeostatic properties. The porous structure of the gelatin sponge is known to facilitate *in vivo* infiltration by cells from surroundings sites, and cellular invasion was seen in the H&E-stained slides of the calvarial defect in our experiment (Takahashi et al. 2005). However, they have a high degree of biodegradation leading to low mechanical stability of the scaffold and an imbalance between new bone formation and scaffold degradation (Rohanizadeh et al. 2007). At the end of a 6-week

healing period, a substantial amount of gelatin sponge still remained in the control defect indicating that the scaffold degradation was not the main contributing factor of the healing outcomes.

The cell-based scaffold used in our experiment was accomplished by seeding $5x10^6$ of undifferentiated LB or MB BMSCs per gelatin sponge. Krebsbach et al. reported defect closure after 2 weeks following transplanting of 3 and 5 $x10^6$ mouse MSCs transplantation per gelatin sponge (Krebsbach et al. 1998). The discrepancy in the results might be due to the differences in the cell origin (rat *vs.* mouse). Recent studies demonstrated the ability of undifferentiated MSCs in scaffolds to repair bone defects *in vivo* (Korda et al. 2008; Niemeyer et al. 2010). We used undifferentiated BMSCs in our experiment with the intention to explore their native bone regeneration potential without any genetic manipulation or a use of growth factor. The latter two approaches could greatly enhance the osteogenic ability of BMSCs; however, there are several problems, such as uncontrolled osteogenic response, associated with the use of these approaches (Peng et al. 2002; Zhao et al. 2007). All in all, at the end of a 6 week-healing period, a considerable amount of gelatin sponge still remained in most of the defects in our experiment. The outcome of bone regeneration in calvarial defects could significantly improve with a longer healing time.

In endochondral femoral defect, MB and LB BMSCs generated approximately 80 % bone healing at an 8-week healing time point. We purposely did not allow the defects to completely heal in order to compare bone regeneration potential of the two cell types. Histological characteristics of regenerated bone from MB and LB BMSCs were indistinguishable. Despite the different embryonic origin of the grafted cells and the recipient site, MB and LB BMSCs resulted in

the same characteristics of regenerated bone possibly because they have the same Hox status (Leucht et al. 2008). Surprisingly, MB BMSCs regenerated higher % BV/TV and % bone healing than LB BMSCs, though the differences were not statistically significant.

In this study, we investigated *in vivo* bone regeneration of MB *vs*. LB BMSCs in repairing critical-sized calvarial and femoral defects. We found that the quality of bone generated in calvarial defect by MB BMSCs was better than those derived from LB BMSCs, although longer healing time would ultimately test their ability to completely repair such a defect. Repairment of critical-sized femoral defect by MB BMSCs was comparable if not better than LB BMSCs. These results confirmed an *in vivo* enhanced osteogenic ability of MB BMSCs, and suggested that MB BMSCs are capable of repairing both intramembranous and endochondral bone defects.

# CHAPTER 6

# CONCLUSION AND FUTURE DIRECTION

## CONCLUSION

Embryologic development and skeletogenesis of orofacial bones are different from axial and appendicular bones. The existence of jaw-specific diseases elucidates a unique homeostatic mechanism of the jaw bones. This dissertation contributes cellular evidence to the distinction of the two bone types. Mandible and long bone marrow cells were examined as they represent orofacial and appendicular bones, respectively. We established a protocol for rodent mandible marrow cell isolation that circumvents odontoblast contamination, an unavoidable problem in the murine model. As alluded to previously, bone homeostasis is achieved by the balance between bone formation and bone resorption. We then investigated the principal cells of bone formation, BMSCs, and discovered an enhanced ability of MB *vs.* LB BMSCs to induce bone formation both *in vitro* and *in vivo*. We tested this remarkable potential in a clinically relevant critical-sized bone defects and found that MB BMSCs could regenerate both intramembranous and endochondral bone defects. The clinical implication of this result is considerably significant as it suggests that mandible bone might be the preferred site for obtaining marrow aspiration for regenerative therapy. In addition, orofacial BMSCs are relatively accessible and could be obtained during dental surgical procedures as opposed to the traditional iliac crestal bone, which requires an invasive surgical procedure. Ultimately, we turned our focus on the bone resorption because an abnormality in this process often causes diseases associated with bone loss. We found that although the MB marrow contained higher numbers of osteoclast precursors, the MB marrow had a lower ability to induce osteoclast formation in hormone stimulated condition

compared to the LB marrow. The differences in the MB *vs.* LB at the cellular level could explain the existence of the jaw specific diseases, particularly bisphosphonate-related osteonecrosis of the jaw. Bisphosphonates systemically affect all bones of the body and function mainly as osteoclast inhibitors. We believe that the jaw bones intrinsically have a reduced osteoclast formation ability and cannot counteract the inhibitory effect of bisphosphonates to reestablish the normal bone resorption process, therefore, bone homeostasis. This could explain, at least in part, the pathophysiology of BRONJ and why clinical manifestations present exclusively in the jaw bones.

## FUTURE DIRECTION

### Homing and Fate of the Grafted BMSCs

In **Chapter 5**, the ability of MB *vs.* LB BMSCs to repair bone defects was investigated. A remaining question that has yet to be investigated is the contribution of the implanted BMSCs to the new bone formation. To accomplish this, the engrafted cells can be detected in the healed bone tissue with Y-chromosome fluorescence in situ hybridization with gender mismatch transplantation between male donor cells and female recipients. The findings of this experiment will verify the therapeutic effect of BMSC and demonstrate that the grafted cells reside within the implanted site.

**Long Bone versus Mandible Bone Marrow Stromal Cell Bone Regeneration Potential in Critical-sized Mandible Defects**

We evaluated the differences in MB *vs.* LB BMSCs and the ability of these cells to regenerate bone in calvarial and femoral defects. The results provided the evidence that molecular differences between the grafted cells and the recipient bone site are important determinants of successful bone repair and regeneration. Currently, most oral and maxillofacial defects have been treated with grafts composed of cells from mesodermal lineage, however better outcomes have been shown when using grafts composed of cells derived from the neural crest origin (D'Addona and Nowzari 2001). Up to date, several studies have investigated the bone healing of mandible defects, but have only utilized appendicular bone-derived BMSCs for the engraftment (Chung et al. 2011; Guo et al. 2012; Huang et al. 2010; Li et al. 2010; Park et al. 2003; Ren et al. 2007; Schliephake et al. 2009). Though most studies with the mismatch embryonic origin of grafted cells and defect site have demonstrated favorable bone healing, it often involves the use of gene therapy or growth factor to enhance the osteogenic ability of the grafted cells. Therefore, it is important to investigate the osseous repair of the skeletal defect with the corresponding BMSCs of the same origin. The mandible bone, a part of craniofacial bones, is derived from the same progenitor cells as the calvarial bone. Logically, the presumption is the ability of MB BMSCs to heal mandible defect would yield a similar result as shown in calvarial defect. Although the calvaria and MB share several commonalities, BRONJ complication has not been observed in skull bones, and the knowledge gained from calvarial studies might not equate to that of the mandible.

Thus, the investigation of the ability of LB *vs.* MB BMSCs to regenerate critical-sized mandibular bone defect will be investigated to identify an ideal cell source for oral and maxillofacial bone regeneration. The CSDs will be achieved by creating 6-mm round defect in the body of the mandible as previously described (Park et al. 2003). The defects will be implanted with gelatin sponge with and without LB or MB BMSCs. The appropriate healing time will be determined and at the end of the healing period, the degree of bone regeneration will be analyzed with high resolution microCT imaging and histological analyses.

**Gene Expression Profiling of Undifferentiated Long Bone versus Mandible Bone Marrow Stromal Cells**

Besides the distinction in embryonic origin and mechanism of bone development, we provided evidence that the MB and LB are also different at the cellular level. Cellular differences could be due to differential gene expression and molecular signaling. Using microarray technology, the fundamental differences in cellular growth, function and development between LB *vs.* MB BMSCs could be identified.

We cultured LB and MB BMSCs in complete media without differentiation factors for 7 and 14 days. Total RNA of the undifferentiated BMSCs was extracted and purified. A genome-wide gene expression analysis using Affymetrix GeneChip Rat gene 1.0 ST array was performed with the purified RNA. The microarray data were analyzed using dCHIP software (http://www.dCHIP.org) to identify differentially upregulated genes in LB *vs.* MB BMSCs at 7 and 14 days. Fold changes were determined from the ratio of LB *vs.* MB BMSC expression

signal, and upregulated genes were determined to have equal to or greater than 2 fold change in expression.

Lists of the upregulated genes in undifferentiated LB and MB BMSCs were shown in **Table 6-1** and **Table 6-2**, respectively. The expression of those genes was confirmed with qPCR **(Fig. 6-1 and 6-2).** LB BMSC upregulated genes (i.e. *Adipoq, Fabp4, and Pparg*) suggested a preference of LB BMSCs toward adipogenic differentiation **(Table 6-1)**. On the other hand, MB upregulated genes (i.e. *Wif1 and BMP7*) were suggestive of osteogenic commitment.

**Table 6-1. List of upregulated genes in undifferentiated LB BMSCs.**

| GENE NAME | GENE SYMBOL | MEAN FOLD CHANGE DAY 7 (N=2) | MEAN FOLD CHANGE DAY 14 (N=2) |
|---|---|---|---|
| Adiponectin, C1Q and collagen domain containing | *ADIPOQ* | -17.65 | -28.44 |
| Fatty acid binding protein 4 | *FABP4* | -10.48 | -10.34 |
| Chemokine (C-X3-C motif) ligand 1 | *CX3CL1* | -9.20 | -8.40 |
| Vascular cell adhesion molecule 1 | *VCAM1* | -8.83 | -5.18 |
| Peroxisome proliferator-activated receptor gamma | *PPARG* | -5.88 | -4.05 |
| Bone morphogenetic protein 5 | *BMP5* | -4.79 | -6.51 |
| Collagen, type XIV, alpha 1 | *COL14A1* | -4.71 | -4.19 |
| Chemokine (C-C motif) ligand 9 | *CCL9* | -4.02 | -3.94 |
| Chemokine (C-X-C motif) ligand 12 | *CXCL12* | -2.74 | -2.19 |

**Table 6-2 List of upregulated genes in undifferentiated MB BMSCs**

| GENE NAME | GENE SYMBOL | MEAN FOLD CHANGE 7 DAY (N=2) | MEAN FOLD CHANGE 14 DAY (N=2) |
|---|---|---|---|
| Wnt inhibitory factor 1 | *WIF1* | 20.20 | 14.05 |
| Bone morphogenetic protein 7 | *BMP7* | 11.32 | 8.68 |
| Gremlin 1 | *GREM1* | 5.52 | 3.50 |
| Gremlin 2 | *GREM2* | 5.49 | 3.34 |
| Chemokine (C-X-C motif) ligand 5 | *CXCL5* | 4.27 | 4.20 |
| Collagen, type XII, alpha 1 | *COL12A1* | 3.25 | 2.43 |

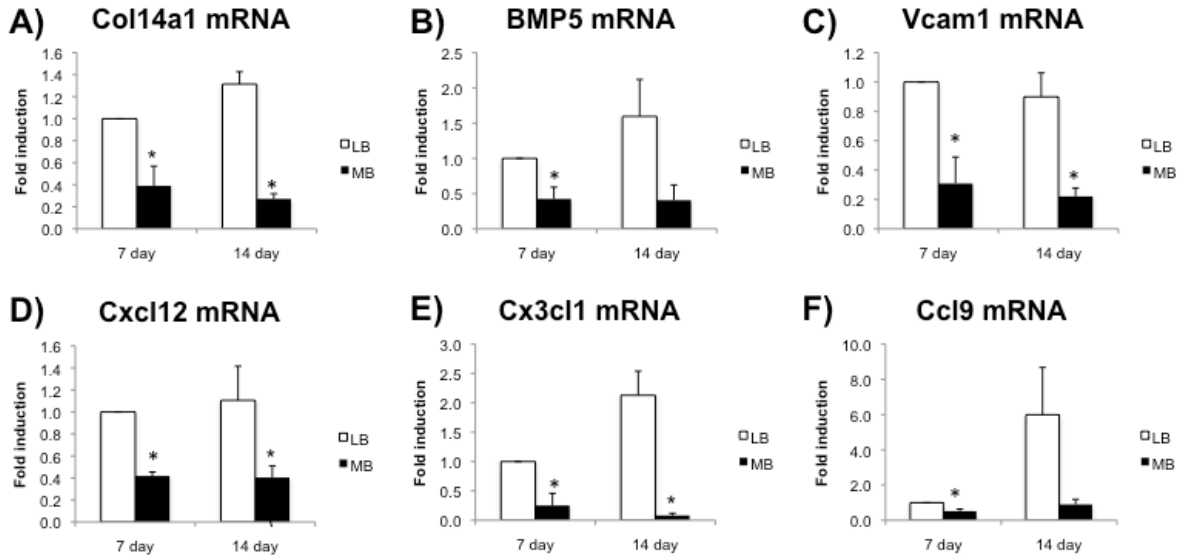


**Figure 6-1.** . qPCR confirmation of LB BMSC upregulated genes A) Col14a1, B) BMP5, C) Vcam11, D) Cxcl12, E) Cx3cl1, and F) Ccl9 mRNA expression determined by qPCR of undifferentiated MB vs. LB MSCs at 7 and 14 days. (average of 3 independent experiments). *p < 0.05; error bars represent standard error of the mean.

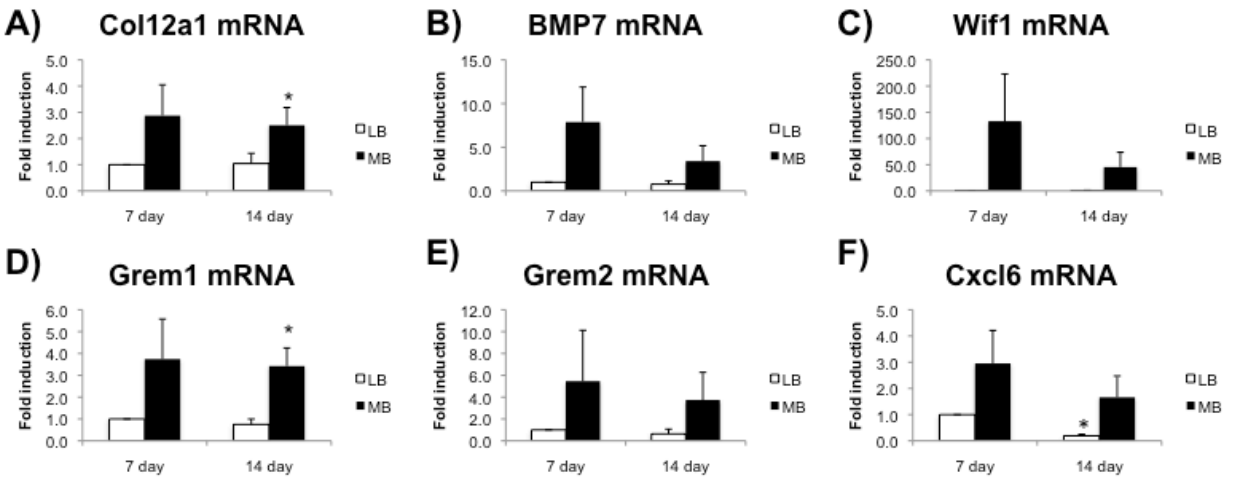


**Figure 6-2**. qPCR confirmation of MB BMSC upregulated genes. A) Col12a1, B) BMP7, C) Wif1, D) Grem1, E) Grem2, and F) Cxcl6 mRNA expression determined by qPCR of undifferentiated MB vs. LB MSCs at 7 and 14 days. (average of 3 independent experiments). *$p < 0.05$; error bars represent standard error of the mean.

In the evaluation process of the upregulated genes, we focused on mesenchymal tissue-related gene expression. Surprisingly, high expressions of epithelial cell-related genes (i.e. Keratin 14 (K14) and Keratin 17) were observed in MB BMSC samples. A stringent and careful examination of MB BMSC cultures showed, occasionally, the presence of epithelial-like cells in a few wells **(Fig. 6-3)**. These epithelial-like cells could possibly be cells from dental enamel epithelium origin. To further investigate the source of keratin expression, we performed K14 immunofluorescence staining in the culture with the presence of epithelial-like cells and confirmed that these cells were responsible for the upregulated keratin expression seen in microarray data **(Fig. 6-3)**. It is important to note that this phenomenon only occurs in undifferentiated cultures.

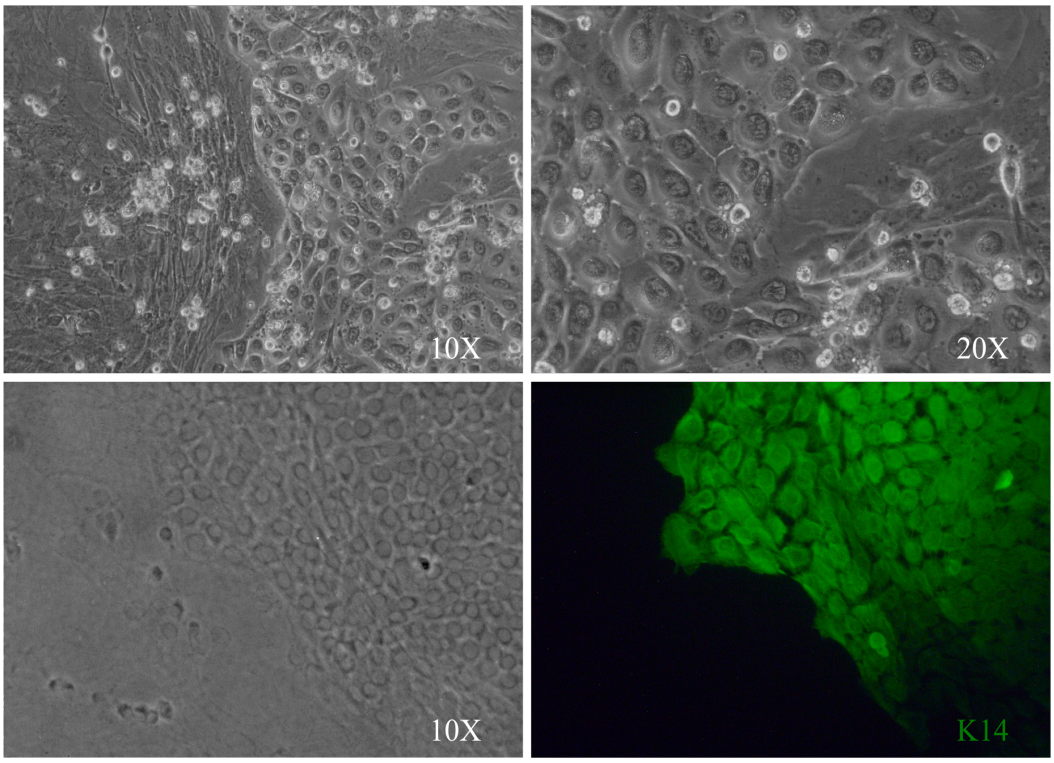


**Figure 6-3.** Morphology and keratin 14 immunofluorescence staining of epithelial-like cells in undifferentiated MB BMSC cultures.

The $K14^+$ cells have a distinct cellular appearance and can be distinguished by the morphology alone or in combination with immunofluorescence staining. We propose the utilization of laser capture microdissection to isolate pure MB BMSCs, free of epithelial-like cells, and perform microarray analysis to obtain true upregulated genes in MB BMSCs **(Fig. 6-4)**. Alternatively, microarray analysis could be performed on the epithelial-like cells. The gene expression of the epithelial-like cells can be compared and subtracted from the original MB BMSC expression to get the true upregulated genes in MB BMSCs as well.

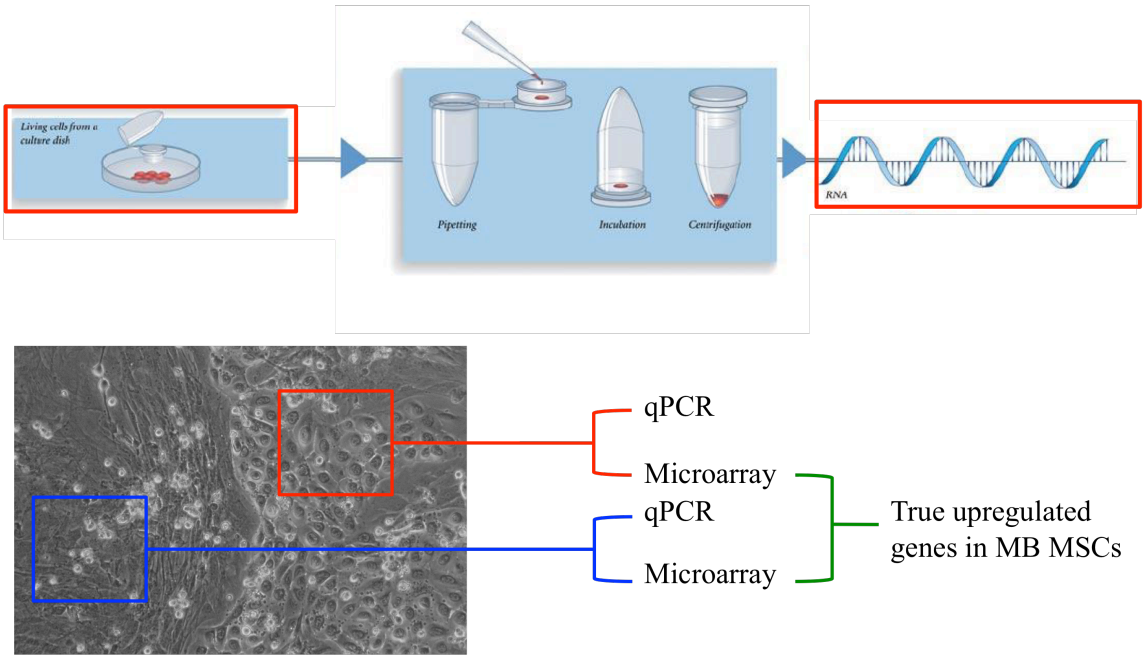


**Figure 6-4.** Laser capture microdisserction scheme to obtain true MB BMSC upregulated gene expression. Adapted from PALM MicroBean manufacturer's manual
Carl Zeiss MicroImaging GmbH
www.zeiss.de/microdissection

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