Journal of Dental Research
2017, Vol. 96(5) 539–546

DOI: 10.1177/0022034517691507
journals.sagepub.com/home/jdr

Role of Bone Marrow Stromal Cells in Impaired Bone Repair from BRONJ Osseous Lesions

L.H. He[1,2], E. Xiao[1,2], J.G. An[1,2], Y. He[1,2], S. Chen[1,2], L. Zhao[1,2], T. Zhang[2,3], and Y. Zhang[1,2]

Abstract

Treatment of bisphosphonate-related osteonecrosis of the jaw (BRONJ) has posed significant challenges to maxillofacial surgeons because of the poor repair of BRONJ bone defects. Moreover, the pathological mechanisms remain unclear. Bone marrow stromal cells (BMSCs) play key roles during bone repair and bone regeneration. However, the activities of BMSCs derived from BRONJ lesions and the BRONJ lesion boundary, as well as the roles of BMSCs in BRONJ defect repair, are poorly defined. In this study, we found that BMSCs from the central area of the osteonecrotic BRONJ region (center-BRONJ BMSCs) and the peripheral area at the recommended debridement boundary (peri-BRONJ BMSCs) had decreased proliferative ability, self-renewal capacity, and multidifferentiation capacities compared with control BMSCs. Osteoclast-inducing ability was also impaired in BRONJ BMSCs. All of these results suggested that the decreased activities of BRONJ BMSCs, even the BMSCs derived from the BRONJ lesion boundary, might be an important factor leading to insufficient bone repair of BRONJ lesions. This study offers early stage evidence for the use of marrow stromal cells in the treatment of BRONJ.

Keywords: osteonecrosis, osteogenesis, bone resorption, osteoblast, osteoclast, cell differentiation

Introduction

The treatment of bisphosphonate-related osteonecrosis of the jaw (BRONJ) has posed significant challenges to maxillofacial surgeons (Ruggiero and Drew 2007). Clinically, jaw bone defects caused by either BRONJ lesions or surgical resection are difficult to repair regardless of the treatment approach; attempts at treatment have even resulted in further expanding the bone defects after recommended osseous sequestrectomy or debridement (Marx et al. 2005; Mücke et al. 2011). Furthermore, the margins of the affected area adjacent to normal-appearing bone are ill-defined (Fliefel et al. 2015), and the status of the cells at the necrotic bone boundary remains unknown.

Researchers have proposed several hypotheses, among which bone remodeling suppression and the antiangiogenic effect are the most popular theories; however, direct and sufficient data supporting these claims are limited (Allen and Burr 2009). Bone formation is an important process during bone repair. Bone marrow stromal cells (BMSCs), which are the progenitors of osteoblasts, are mostly responsible for bone formation and exert key roles during bone repair. Accumulated studies have proven that BMSC dysfunction or apoptosis will lead to poor bone repair and reduce bone regeneration (Gallagher and Sai 2010; Crane and Cao 2014). Li et al. (2013) recently found that BMSCs derived from the iliac crest in BRONJ-like lesions in minipigs exhibit low osteogenic abilities. However, the necrotic bone lesion is mainly confined to the jaw bone (especially the mandible), and no reports have shown the activity of BMSCs in osteonecrotic bone of BRONJ and the traditional boundary between dissected necrotic bone and normal healthy bone.

In this study, to explore the activity of BMSCs in BRONJ lesions and the peripheral regions at the recommended debridement boundary, we isolated BMSCs from the peripheral area (at the recommended debridement boundary) and the center area of the BRONJ lesion and assessed their proliferative capacity, ability to differentiate into multiple lineages, and osteoclastogenic induction ability by comparing these parameters with BMSCs extracted from normal mandible.

[1]Department of Oral and Maxillofacial Surgery, Peking University School and Hospital of Stomatology, Beijing, China
[2]National Engineering Laboratory for Digital and Material Technology of Stomatology, Beijing Key Laboratory of Digital Stomatology, Beijing, China
[3]Center for Craniofacial Stem Cell Research and Regeneration, Department of Orthodontics, Peking University School and Hospital of Stomatology, Beijing, China

A supplemental appendix to this article is available online.

Corresponding Author:
Y. Zhang, Department of Oral and Maxillofacial Surgery, Peking University School and Hospital of Stomatology, 22 Zhongguancun Nandajie, Haidian District, Beijing 100081, P.R. China.
Email: zhangyi2000@263.net

Methods and Materials

Patients

Three BRONJ patients (aged >50 y) without bone metabolism diseases, except cancer or osteoporosis, who presented with exposed necrotic bone with pain and purulent fistula in the maxillofacial region within 3 mo and underwent surgery at the Department of Oral and Maxillofacial Surgery, Peking University Hospital of Stomatology were included in this study. Surgery was conducted as previously described, and the debridement boundary was determined by the clinical appearance of bleeding bone (Wilde et al. 2011). The control group comprised patients who were older than 50 y, without bone metabolism diseases, except osteoporosis. These patients had tongue base or mucosal cancer and needed a mandibulotomy to broaden the scope of the operation during surgery. The control bone marrow tissues were collected at the mandibulotomy sites. Detailed patient information is listed in Appendix Table 1. This work was approved by the Ethics Committee of Peking University (approval number: IRB00001052–11002), and all participants gave their informed consent.

Isolation of BMSCs from BRONJ and Control Bones

BMSCs were isolated from the mandible bone as previously described (He et al. 2015). In brief, the bone tissues were thoroughly rinsed with phosphate-buffered saline (PBS), cut into small pieces, and vibrated for 30 min in a solution containing dispase II (0.4 mg/mL) and collagenase I (0.2 mg/mL). The suspension was passed through a 70-μm filter (BD Biosciences), collected by centrifugation at 1,200 r/min, and seeded in culture medium consisting of α-modified Eagle medium (Life Technologies Corporation) supplemented with 10% fetal bovine serum (FBS; Equitech-Bio, Inc.) and penicillin G and streptomycin sulfate (Life Technologies Corporation). The cells were cultured for 24 h at 37°C in a humidified atmosphere of 5% CO_2. After 24 h, the cells were rinsed 3 times with PBS and continuously cultured.

Colony-Forming Unit Assay

First-passage cells were seeded at a density of 200 cells in 60-mm culture dishes. The culture medium was changed every 3 d. On day 11, the cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet for 5 min, and colonies with diameters >1 mm were counted.

Flow Cytometry

The cells were washed 3 times with PBS and harvested by digestion and centrifugation. The cells were then incubated with the following labeled antibodies: CD90-FITC, CD44-PE, CD73-APC, CD105-CY5.5, and negative cocktail-PE. Control-conjugated immunoglobulin G (IgG) was used as the isotype control. All antibodies were purchased from BD Biosciences. Flow cytometry was performed with an Accuri C6 flow cytometer (BD Biosciences).

Cell Proliferation Assays

Third-passage BMSCs were seeded at a density of 2,000 cells per well in 96-well plates. The cell number was assessed on days 2, 4, 6, 8, and 10 using the Cell Counting Kit-8 (Dojindo Laboratories) assay according to the manufacturer's instructions.

Multidifferentiation Assays

For osteogenic differentiation, third-passage BMSCs were cultured in osteogenic medium supplemented with 10 nm dexamethasone, 0.1 mm L-ascorbic acid phosphate, and 10 mm β-glycerophosphate (Sigma-Aldrich). The osteogenic medium was changed every 3 d. After 7 d, messenger RNA (mRNA) and proteins were isolated for biological analysis. After 21 d, calcium nodes were stained with 2% Alizarin red (Sigma-Aldrich).

For adipogenic differentiation, third-passage BMSCs were cultured in adipogenic culture medium supplemented with 0.5 μm hydrocortisone, 0.5 mm 3-isobutyl-methylxanthine, 10 μg/mL insulin, 60 μm indomethacin (Sigma-Aldrich), and 10% FBS. After 7 d, mRNA and proteins were isolated for biological analysis. After 21 d, the cells were stained for cellular lipid droplets with Oil Red O (Sigma-Aldrich).

RNA Isolation and Real-Time Polymerase Chain Reaction

Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies). Complementary DNAs (cDNAs) were prepared using the GoScript Reverse Transcription System (Promega). Real-time polymerase chain reaction was performed with an ABI Prism 7500 (Applied Bioscience). β-Actin was used to normalize gene expression, and the relative mRNA expression levels were calculated. Primers used in this study are shown in Appendix Table 2.

Western Blot Assay

Proteins were extracted from BMSCs, and Western blot assays were performed as previously described (Yamada et al. 2013). Antibodies against β-actin, lipoprotein lipase (LPL), Runx2, Rankl, osteocalcin (OCN), and osteoprotegerin (OPG) were purchased from Biogot Technology Co. Ltd., and antibodies against peroxisome proliferator-activated receptor (PPAR)–γ2 were obtained from Santa Cruz Biotechnology.

In Vivo Bone Formation Assays

In vivo bone formation was assessed in the mouse model as previously described (Xiao et al. 2013). In brief, 2×10^6 third-passage cells were precultured with 40 mg spheroidal hydroxyapatite/tricalcium phosphate (Beijing YHJ Science and Trade Co. Ltd.) and transplanted into aseptically created subcutaneous pockets in 6-wk-old immunocompromised nude male mice (SCID Beige Mouse; Charles River Laboratories International) under anesthesia via 2% sodium pentobarbital. Transplants were harvested after 6 wk and assessed by histology.

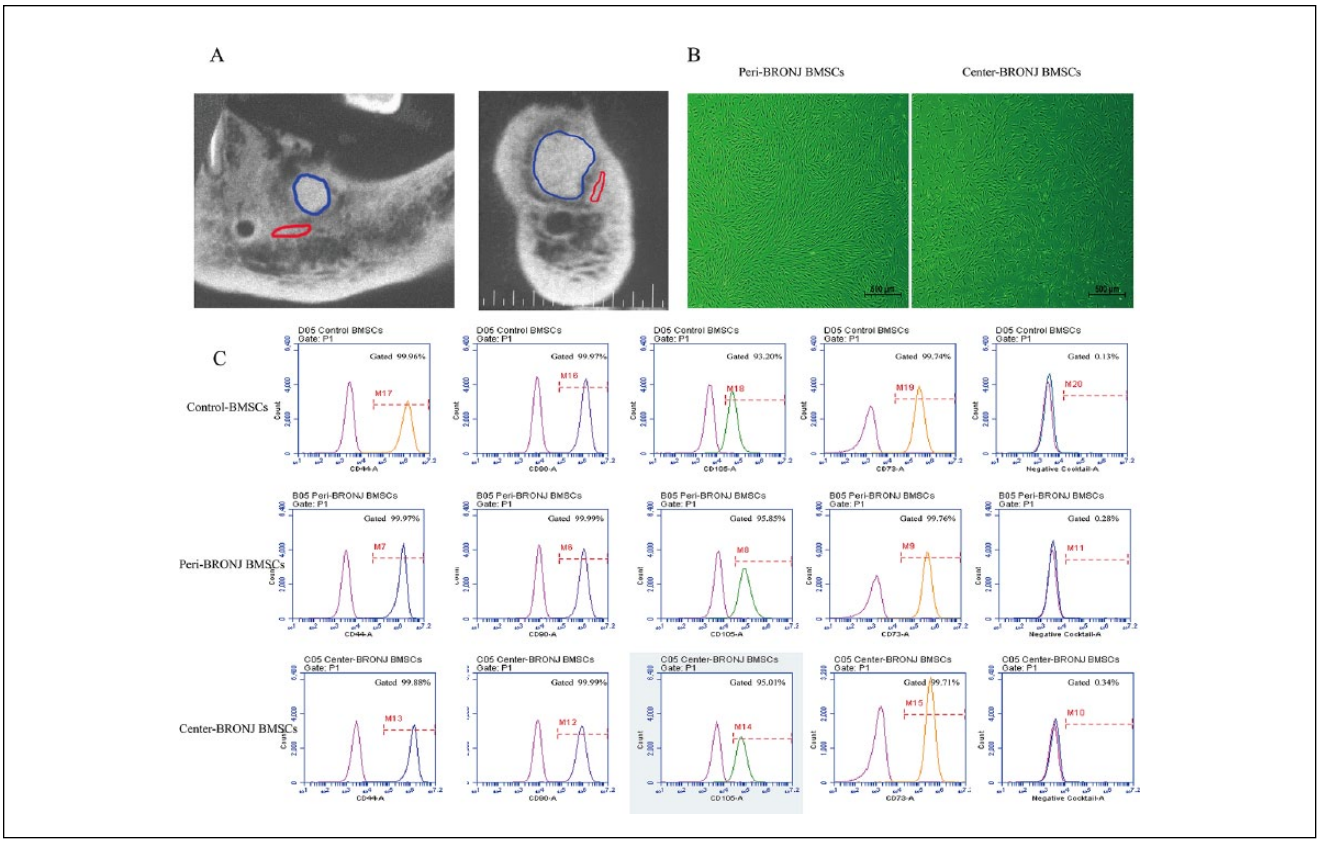

Figure 1. Isolation and identification of bone marrow stromal cells (BMSCs) from bisphosphonate-related osteonecrosis of the jaw (BRONJ) mandible bone. (**A**) Cone beam computed tomography images of BRONJ lesions. The left image shows the coronal plane, and the right image shows the sagittal plane. The blue circle denotes the central area of BRONJ, and the red circle denotes the peripheral area (standard debridement boundary) from where the BMSCs were isolated. (**B**) BMSCs derived from the BRONJ mandible bone were fibroblast-like cells. (**C**) BMSC identification by flow cytometry analysis.

Immunohistochemistry

Immunohistochemistry was conducted as previously reported (Zhu et al. 2014). In brief, transplants were fixed in 4% formalin, decalcified in 10% EDTA for 2 wk, and embedded in paraffin. Sections (5 μm) were deparaffinized, and peroxidase was eliminated. After antigen retrieval and antigen block, sections were incubated with OCN antibody at 4°C overnight, incubated with secondary antibody, and stained with DAB kit. The OCN-positive area was quantified by ImageJ software (National Institutes of Health).

Co-culture Assays

Co-culture assays were conducted as previously described (Zhao et al. 2009). Bone marrow macrophages (BMMs) were isolated using previously established methods (Zhao et al. 2009). In brief, the tibias and femurs were isolated under sterile conditions, and the bone marrows were flushed out and dispersed by a syringe. The suspension was passed through a 70-μm filter (BD Biosciences), collected by centrifugation at 1200 r/min, and cultured in medium consisting of α-modified Eagle medium (Life Technologies Corporation) supplemented with 20% FBS (Equitech-Bio, Inc.) and penicillin G and streptomycin sulfate (Life Technologies Corporation). The cells were cultured for 24 h. Subsequently, the nonadherent cells were harvested, cultured with 10 mL medium supplemented with 30 ng/mL macrophage colony-stimulating factor (M-CSF) (R&D Systems) for 3 d, and collected as BMMs. Third-passage BMSCs were seeded at a density of 5×10^3 per well in 96-well plates and precultured for 1 d. BMMs were then seeded at a density of 1×10^5 per well and co-cultured with the BMSCs in culture medium containing 10^{-8} M 1,25(OH)2D3 (Sigma-Aldrich) and 10^{-6} M prostaglandin E2 (Sigma-Aldrich) for 7 d. After co-culture, the cells were fixed and stained using tartrate resistant acid phosphatase (TRAP) (Sigma-Aldrich) according to the manufacturer's instructions, and over 3 nuclear osteoclasts were calculated per well.

Statistical Analyses

All statistical analyses were performed using GraphPad Prism 4 (GraphPad Software). Data are presented as the mean ± SD and assessed by 1-way analysis of variance (ANOVA) followed by a Tukey's honestly significant difference (HSD) post hoc test. Statistical significance was defined as $P < 0.05$.

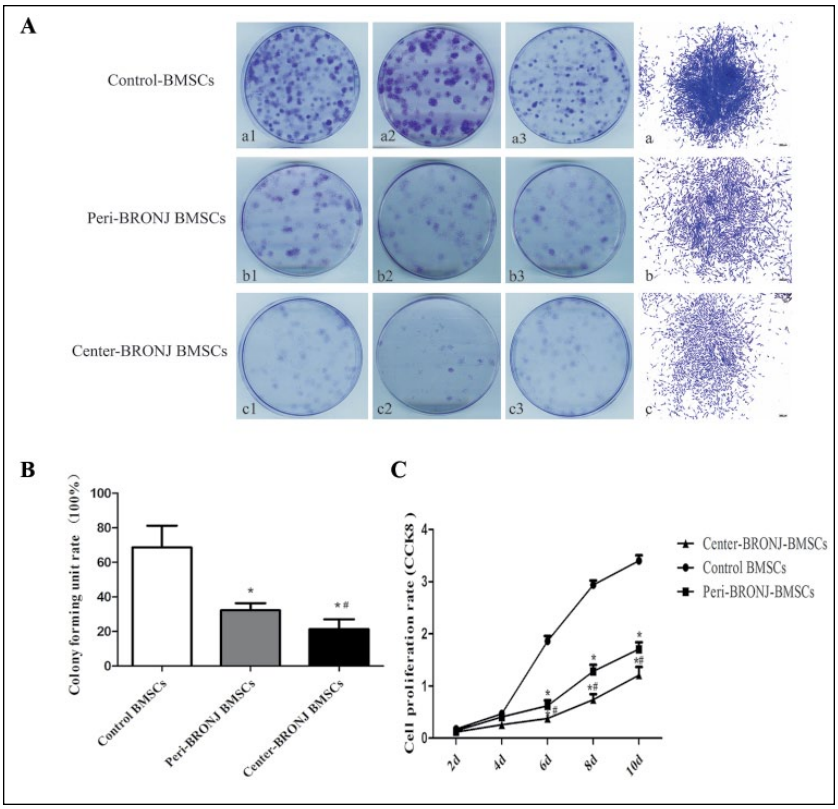

Figure 2. The proliferation ability assessment among bisphosphonate-related osteonecrosis of the jaw (BRONJ) bone marrow stromal cells (BMSCs) and control BMSCs. (**A**) The colony-forming unit assessment. a1, a2, and a3 represent 3 different control patients. b1 and c1, b2 and c2, and b3 and c3 represent 3 BRONJ patients. The a, b, c in the right panel represent the typical images of colony-forming units by microscope. (**B**) Quantification results of colony-forming units. (**C**) Comparison of proliferative rates of BMSCs. $^{*}P < 0.05$, BRONJ vs. control. $^{\#}P < 0.05$, center-BRONJ BMSCs vs. peri-BRONJ BMSCs.

Results

Patient Information and BMSCs Were Isolated from the Central and Peripheral BRONJ Areas

In total, 3 BRONJ patients were included in this study. These 3 patients underwent long-term zoledronic acid treatment to prevent either metastatic bone destruction or osteoporosis. All 3 patients also underwent recommended debridement of their BRONJ lesions. Normal mandible bone was collected from 3 age-matched patients who required mandible surgery and used as control samples. Detailed information is shown in Appendix Table 1. The computed tomography (CT) scans showed that the osteonecrotic bone in the mandible exhibited high bone density, and the peripheral area of the BRONJ lesions showed manifestations of osteomyelitis (Fig. 1A). The blue circle denotes the central region of the BRONJ, and the red circle denotes the peripheral area of BRONJ lesions at the recommended debridement boundary. The stromal cells isolated from the bone marrow in the central and peripheral BRONJ areas from all 3 BRONJ patients were mainly composed of fibroblast-like cells (Fig. 1B). The flow cytometry results showed that these cells were positive for mesenchymal stem cell (MSC)–related markers and negative for the negative markers (Fig. 1C).

BRONJ BMSCs Showed Lower Proliferation Capacity Than Control BMSCs

BMSCs are capable of self-renewal and rapid proliferation. Thus, we first assessed their colony-forming ability. The results showed that center-BRONJ BMSCs formed the fewest colony-forming units and peri-BRONJ BMSCs formed fewer colony-forming units than the control BMSCs after 10 d of culture (control BMSCs vs. peri-BRONJ BMSCs vs. center-BRONJ BMSCs, 68.67% ± 7.22% vs. 32.33% ± 2.33% vs. 21.33% ± 3.28%, respectively) (Fig. 2A, a1–c3 and B). In addition, the colonies formed by the control BMSCs were more compact than both types of BRONJ BMSCs (Fig. 2A, a–c). Moreover, the center-BRONJ BMSCs exhibited the slowest proliferation rate and peri-BRONJ BMSCs showed a lower proliferation rate than control BMSCs (Fig. 2C). These results suggested that the self-renewal ability and proliferative capacity of central and peripheral BRONJ BMSCs decreased.

BRONJ BMSCs Exhibited Impaired Multidifferentiation Ability

BMSCs can differentiate into osteoblasts, chondrocytes, and adipocytes. In all 3 patients, both peri-BRONJ BMSCs and center-BRONJ BMSCs formed fewer lipid droplets after culture in adipogenic medium for 21 d, whereas control BMSCs formed more lipid droplets (Fig. 3A). Consistently, control BMSCs expressed higher mRNA and protein levels of LPL (control BMSCs vs. peri-BRONJ BMSCs vs. center-BRONJ BMSCs, 1.02 ± 0.118 vs. 0.676 ± 0.077 vs. 0.483 ± 0.059, respectively) and PPAR-γ2 (control BMSCs vs. peri-BRONJ BMSCs vs. center-BRONJ BMSCs, 1.00 ± 0.012 vs. 0.475 ± 0.018 vs. 0.363 ± 0.053, respectively) during adipogenic differentiation (Fig. 3B, C), with center-BRONJ BMSCs showing the lowest levels of adipogenic differentiation. During osteogenic differentiation, the control BMSCs expressed higher levels of Runx2 (control BMSCs vs. peri-BRONJ BMSCs vs. center-BRONJ BMSCs, 1.157 ± 0.079 vs. 0.482 ± 0.061 vs. 0.296 ± 0.063, respectively), alkaline phosphatase (ALP) (control BMSCs vs. peri-BRONJ BMSCs vs. center-BRONJ BMSCs, 1.238 ± 0.083 vs. 0.314 ± 0.172 vs. 0.245 ± 0.134, respectively), and OCN (control BMSCs vs. peri-BRONJ BMSCs vs. center-BRONJ BMSCs, 1.00 ± 0.01 vs. 0.656 ± 0.047 vs. 0.749 ± 0.141, respectively) than either type of BRONJ BMSCs (Fig. 4A, C). In addition, the control BMSCs formed more calcium depositions than the BRONJ BMSCs in vitro (Fig. 4B). Center-BRONJ BMSCs showed reduced Runx2 expression levels and fewer calcium

depositions than peri-BRONJ BMSCs. The in vivo bone regeneration results suggested that the control BMSCs formed an extensive OCN-positive bone matrix in vivo, whereas both BRONJ BMSC groups showed no apparent OCN-positive bone matrix but exhibited OCN-positive bone forming cells in vivo (Fig. 4E, F). The quantification results showed that control BMSCs demonstrated a larger bone area than BRONJ BMSCs in vivo. Thus, BRONJ BMSCs exhibited impaired differentiation abilities.

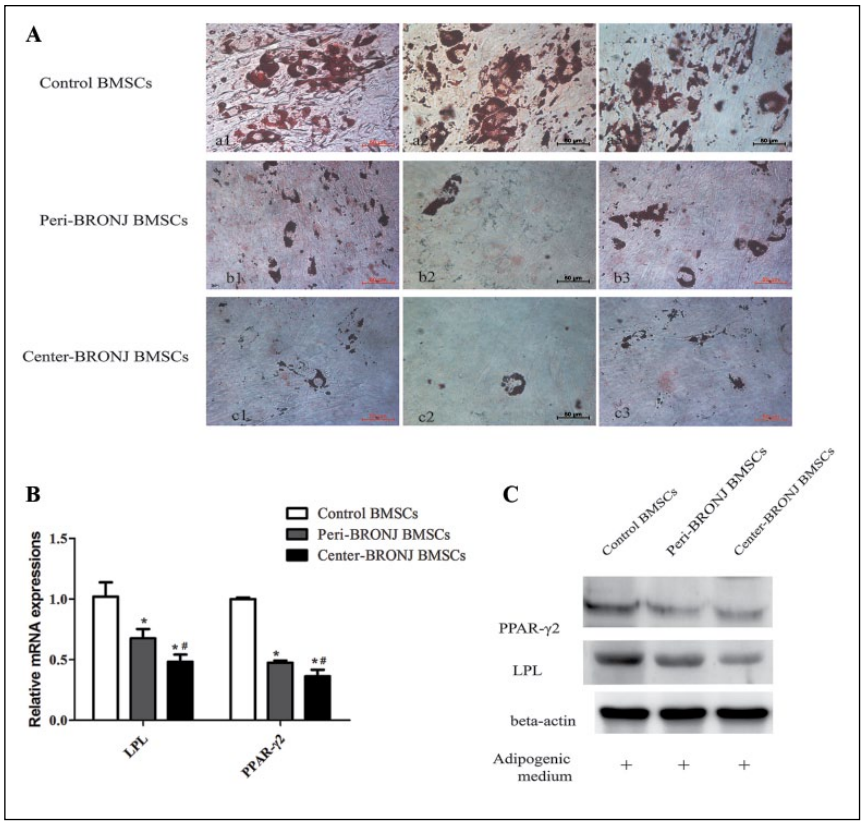

Figure 3. Adipogenic differentiation assessment of bone marrow stromal cells (BMSCs). (**A**) Adipogenic inducing for 21 d and staining by Oil Red O staining. a1, a2, and a3 represent 3 different control patients. b1 and c1, b2 and c2, and b3 and c3 represent 3 patients with bisphosphonate-related osteonecrosis of the jaw (BRONJ). (**B**) Messenger RNA (mRNA) expressions of peroxisome proliferator-activated receptor (PPAR)–γ2 and lipoprotein lipase (LPL) by real-time polymerase chain reaction (PCR) assay. (**C**) The PPAR-γ2 and LPL expressions by Western blot assay. $^{*}P < 0.05$, BRONJ vs. control. $^{\#}P < 0.05$, center-BRONJ BMSCs vs. peri-BRONJ BMSCs.

BRONJ BMSCs Induced Fewer TRAP-Positive Multinuclear Osteoclasts Than Control BMSCs

To assess the osteoclast differentiation-inducing capacity of BMSCs derived from BRONJ, we checked the expression levels of Rankl and OPG in BRONJ BMSCs. The mRNA (control BMSCs vs. peri-BRONJ BMSCs vs. center-BRONJ BMSCs, 1.347 ± 0.293 vs. 0.334 ± 0.149 vs. 0.062 ± 0.013, respectively) and protein levels of Rankl from BRONJ BMSCs were dramatically decreased compared with those from control BMSCs, whereas the expression of OPG (control BMSCs vs. peri-BRONJ BMSCs vs. center-BRONJ BMSCs, 1.059 ± 0.100 vs. 2.238 ± 0.310 vs. 3.182 ± 1.163, respectively) mildly increased. These shifts caused the Rankl/OPG ratio (control BMSCs vs. peri-BRONJ BMSCs vs. center-BRONJ BMSCs, 1.598 ± 0.439 vs. 0.131 ± 0.045 vs. 0.026 ± 0.004, respectively) to decrease, with the center-BRONJ BMSCs showing the smallest Rankl/OPG ratio (Fig. 5A, B). We further co-cultured BMSCs and BMMs for 7 d, and the results showed that the control BMSCs induced the differentiation of more multinuclear osteoclasts than BRONJ BMSCs (Fig. 5C, D). These results indicated that control BMSCs possessed higher osteoclast-inducing capacity than BRONJ BMSCs.

Discussion

In this study, we assessed the activities of BMSCs derived from the central area of BRONJ lesions and the peripheral area at the recommended debridement boundary. We found dramatic decreases in their self-renewal and proliferation abilities, multidifferentiation capacity, and osteoclast-inducing function, even in BMSCs derived from the peripheral area at the recommended debridement boundary. These results might help explain the mechanism underlying poor bone repair at BRONJ lesion sites, even after clinical debridement.

Different surgical procedures have been conducted for the management of BRONJ, but there is no general consensus regarding the mechanisms involved, poor bone repair at the sites of BRONJ lesions, and ill-defined affected margins (Fliefel et al. 2015). Many reports have pointed to bisphosphonate-induced remodeling suppression as a potent mechanism of BRONJ. No debate about this theory currently exists because it is the principal mechanism of bisphosphonates. Numerous researchers have also proposed that the antiangiogenic effects of bisphosphonates might contribute to bone necrosis by reducing the supply of oxygen, nutrition, and osteogenic progenitors to the lesion (Parfitt 2000; Allen and Burr 2009). There is evidence that has confirmed the antiangiogenic effects of bisphosphonates in vitro and in vivo by directly inhibiting endothelial cell migration, adhesion, proliferation, and function (Fournier et al. 2002; Hasmim et al. 2007; Walter et al. 2010). However, the changes in the activity of native BMSCs in and near the necrotic bone, which are responsible for bone repair, were ignored for many years. Previous studies suggested the

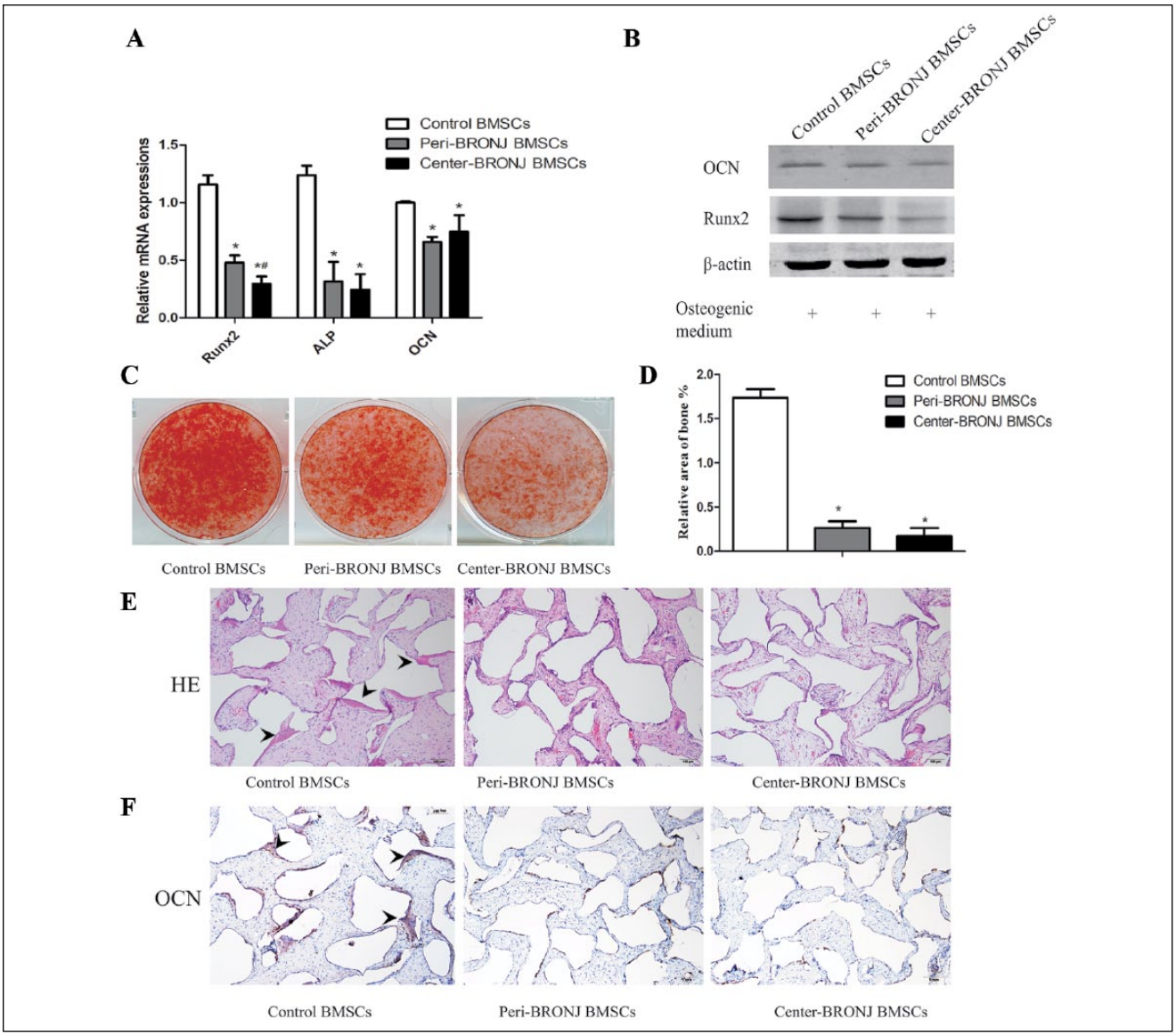

Figure 4. Assessment of the osteogenic differentiation assessment of bone marrow stromal cells (BMSCs). (**A**) Messenger RNA (mRNA) expression levels of Runx2, alkaline phosphatase (ALP), and osteocalcin (OCN) as determined by real-time polymerase chain reaction (PCR). (**B**) Runx2 and OCN expression levels as determined by Western blot. (**C**) Alizarin red staining for in vitro calcium deposition. (**D**) Quantification results of bone regeneration of BMSCs in vivo. (**E, F**) Hematoxylin and eosin staining and OCN immunohistochemistry show the bone formation in vivo. The black arrowheads showed the new bone formation. $^{*}P < 0.05$, bisphosphonate-related osteonecrosis of the jaw (BRONJ) vs. control. $^{\#}P < 0.05$, center-BRONJ BMSCs vs. peri-BRONJ BMSCs.

essential roles of BMSCs during bone healing (Granero-Molto et al. 2011; Xiao et al. 2011). Suh et al. (2005) and Houdek et al. (2016) found that either alcohol- or corticosteroid-induced femoral head osteonecrosis contains BMSCs with low osteogenic potential. However, no previous study has reported whether BMSCs are present in BRONJ lesions. In this article, we showed that the central and peripheral regions of BRONJ lesions contained BMSCs, and these cells were positive for MSC markers. In addition, BRONJ BMSCs exhibited self-renewal and colony-forming capacities. However, the proliferative rate of BRONJ BMSCs from both the central and peripheral regions dramatically decreased, and the colony-forming efficiency of these BMSCs was lower than that of control BMSCs. These results suggest that the pool of BMSCs is decreased in BRONJ bone, even at the recommended boundary of debridement, which could explain the decreases in the osteoblast counts at the sites of bisphosphonate-associated osteonecrotic lesions (Lesclous et al. 2009). Consistently, Hernigou et al. (1999) reported a decrease in the number of osteoprogenitors, and the proliferative rate of BMSCs in proximal femurs of corticosteroid-induced osteonecrotic bone is slow. The multipotential capacity of BMSCs, particularly

toward osteogenic differentiation, plays an important role in bone repair and bone regeneration. BRONJ BMSCs exhibited dramatically decreased osteogenic potential with lower expression levels of Runx2, ALP, and OCN, as well as reduced calcium depositions. In addition, the in vivo bone regenerative ability of BRONJ BMSCs was also impaired. These results suggested that the proliferative ability and multidifferentiation capacity were impaired in BRONJ BMSCs. Although BMSCs are present in osteonecrotic tissue at BRONJ sites and the debridement boundary, the functions of these BMSCs (even those from the peripheral area) were diminished, which might help elucidate why extended resection or debridement of BRONJ lesions still results in terrible bone repair or even further expansion of bone defects. Interestingly, Li et al. (2013) suggested that BMSCs derived from bisphosphonate-treated iliac crest have a lower osteogenic potential. We believe that most BMSCs within the body might also be affected in patients with BRONJ, and mandible BMSCs are the most seriously affected because of the high bisphosphonate concentration in the mandible (Allen and Burr 2009). Whether more extensive resections around BRONJ lesions can undergo successful bone repair is an interesting and controversial question. Recently, both BMSC transplantation and BMSC injection have been successfully used to treat BRONJ in animal studies and clinical settings (Kikuiri et al. 2010; Cella et al. 2011; Li et al. 2013). Although the function of BMSCs in these studies was attributed to immune modulation, these studies still could not exclude the possibility of whether these injected BMSCs repair osteonecrosis by restoring the osteogenic and proliferative ability of BMSCs, which is an interesting topic that should be explored further. Therefore, the decreased BMSC activities might be a potent mechanism of the poor reparative capacity in BRONJ lesions.

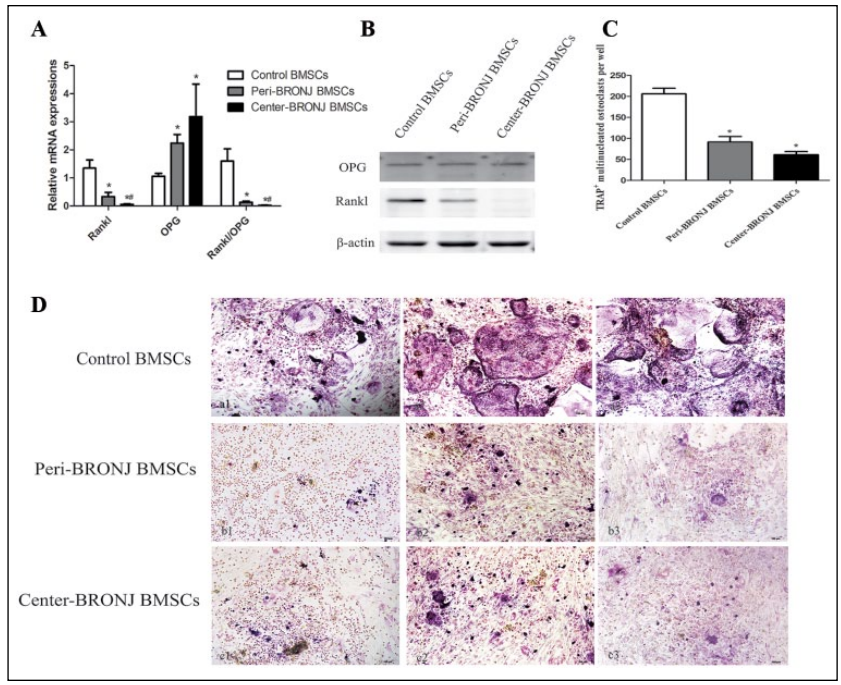

Figure 5. Assessment of the osteoclast-inducing capacity of bone marrow stromal cells (BMSCs). (**A**) Messenger RNA (mRNA) expression levels of Rankl and osteoprotegerin (OPG) as determined by real-time polymerase chain reaction (PCR). (**B**) Rankl and OPG expression levels as determined by Western blot. (**C**) The quantification results of relative osteoclasts numbers. (**D**) Results of the coculturing of BMSCs with bone marrow macrophages (BMMs). a1, a2, and a3 represent 3 different control patients, and b1 and c1, b2 and c2, and b3 and c3 represent 3 patients with bisphosphonate-related osteonecrosis of the jaw (BRONJ). $^{*}P < 0.05$, BRONJ vs. control. $^{\#}P < 0.05$, center-BRONJ BMSCs vs. peri-BRONJ BMSCs.

The decreased osteoclast-inducing capacity of BRONJ BMSCs contributed to the suppression of bone resorption. Bone resorption is an important process in angiogenesis that couples with bone formation (Cackowski et al. 2010), and the osteoclast-inducing capacity of BMSCs is also necessary for bone repair and bone remodeling. The Rank-Rankl-OPG system is the most important axis during osteoclast induction of BMSCs (Dougall 2012). Previous studies have shown that bisphosphonates can indirectly inhibit osteoclast activity and function of BMSCs by inhibiting Rankl expression (Nishida et al. 2005; Fernández et al. 2010). Consistent with those studies, our results showed that BRONJ BMSCs had a lower Rankl/OPG ratio, which indicated the decreased ability of osteoclast induction. The results of co-culture of BMSCs with BMMs further confirmed that the control BMSCs induced the differentiation of apparent multinuclear osteoclasts, whereas BRONJ BMSCs induced fewer osteoclasts. These results suggested that BRONJ BMSCs are deprived of their osteoclast-inducing ability, which might indirectly further aggravate the suppression of jaw bone resorption and reduce new angiogenic growth. This phenomenon may subsequently result in osteonecrotic bone and insufficient repair of bone defects.

In conclusion, this study demonstrated that BRONJ BMSCs from 2 different regions at the lesion site exhibited decreased activities, with impaired proliferative ability, reduced multidifferentiation potential (especially osteogenic potential), and lowered osteoclast-inducing capacity. These decreases were even observed at the peripheral area at the recommended debridement boundary, which might explain the poor bone repair at BRONJ lesions and help improve the treatment of patients with BRONJ. This study offers early stage evidence for the use of MSCs in the treatment of BRONJ.

Author Contributions

L.H. He, contributed to conception, design, and data acquisition, drafted the manuscript; E. Xiao, contributed to data analysis and interpretation, critically revised the manuscript; J.G. An, Y. He, contributed to the data analysis, critically revised the manuscript;

S. Chen, L. Zhao, T. Zhang, contributed to data acquisition, critically revised the manuscript; Y. Zhang, contributed to conception and design, critically revised the manuscript. All authors gave final approval and agreed to be accountable for all aspects of the work.

Acknowledgments

This work was supported by a grant from the National Natural Science Foundation of China (No. 81371117) and the Beijing Natural Science Foundation (No. 7152155). The funding agencies had no role in the project design, experimental execution, analysis of the results, or preparation of the manuscript. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

References

Allen MR, Burr DB. 2009. The pathogenesis of bisphosphonate-related osteonecrosis of the jaw: so many hypotheses, so few data. J Oral Maxil Surg. 67(Suppl 5):61–70.

Cackowski FC, Anderson JL, Patrene KD, Choksi RJ, Shapiro SD, Windle JJ, Blair HC, Roodman GD. 2010. Osteoclasts are important for bone angiogenesis. Blood. 115(1):140–149.

Cella L, Oppici A, Arbasi M, Moretto M, Piepoli M. 2011. Autologous bone marrow stem cell intralesional transplantation repairing bisphosphonate related osteonecrosis of the jaw. Head Face Med. 7(1):16–21.

Crane JL, Cao X. 2014. Bone marrow mesenchymal stem cells and TGF-beta signaling in bone remodeling. J Clin Invest. 124(2):466–472.

Dougall WC. 2012. Molecular pathways: osteoclast-dependent and osteoclast-independent roles of the RANKL/RANK/OPG pathway in tumorigenesis and metastasis. Clin Cancer Res. 18(2):326–335.

Fernández M, Pino AM, Figueroa P, Rodríguez JP. 2010. The increased expression of receptor activator of nuclear-κB ligand (RANKL) of multiple myeloma bone marrow stromal cells is inhibited by the bisphosphonate ibandronate. J Cell Biochem. 111(1):130–137.

Fliefel R, Troltzsch M, Kuhnisch J, Ehrenfeld M, Otto S. 2015. Treatment strategies and outcomes of bisphosphonate-related osteonecrosis of the jaw (BRONJ) with characterization of patients: a systematic review. Int J Oral Maxillofac Surg. 44(5):568–585.

Fournier P, Boissier S, Filleur S, Guglielmi J, Cabon F, Colombel M, Clezardin P. 2002. Bisphosphonates inhibit angiogenesis in vitro and testosterone-stimulated vascular regrowth in the ventral prostate in castrated rats. Cancer Res. 62(22):6538–6544.

Gallagher JC, Sai AJ. 2010. Molecular biology of bone remodeling: implications for new therapeutic targets for osteoporosis. Maturitas. 65(4):301–307.

Granero-Molto F, Myers TJ, Weis JA, Longobardi L, Li T, Yan Y, Case N, Rubin J, Spagnoli A. 2011. Mesenchymal stem cells expressing insulin-like growth factor-I (MSCIGF) promote fracture healing and restore new bone formation in Irs1 knockout mice: analyses of MSCIGF autocrine and paracrine regenerative effects. Stem Cells. 29(10):1537–1548.

Hasmim M, Bieler G, Rüegg C. 2007. Zoledronate inhibits endothelial cell adhesion, migration and survival through the suppression of multiple, prenylation-dependent signaling pathways. J Thromb Haemost. 5(1):166–173.

He LH, Xiao E, Duan DH, Gan YH, Zhang Y. 2015. Osteoclast deficiency contributes to temporomandibular joint ankylosed bone mass formation. J Dent Res. 94(10):1392–1400.

Hernigou P, Beaujean F, Lambotte JC. 1999. Decrease in the mesenchymal stem-cell pool in the proximal femur in corticosteroid-induced osteonecrosis. J Bone Joint Surg Br. 81(2):349–355.

Houdek MT, Wyles CC, Packard BD, Terzic A, Behfar A, Sierra RJ. 2016. Decreased osteogenic activity of mesenchymal stem cells in patients with corticosteroid-induced osteonecrosis of the femoral head. J Arthroplasty. 31(4):893–898.

Kikuiri T, Kim I, Yamaza T, Akiyama K, Zhang Q, Li Y, Chider C, Chen WJ, Wang SL, Le AD, et al. 2010. Cell-based immunotherapy with mesenchymal stem cells cures bisphosphonate-related osteonecrosis of the jaw-like disease in mice. J Bone Miner Res. 25(7):1668–1679.

Lesclous P, Abi NS, Carrel JP, Baroukh B, Lombardi T, Willi JP, Rizzoli R, Saffar J, Samson J. 2009. Bisphosphonate-associated osteonecrosis of the jaw: a key role of inflammation? Bone. 45(5):843–852.

Li Y, Xu J, Mao L, Liu Y, Gao R, Zheng Z, Chen W, Le A, Shi S, Wang S. 2013. Allogeneic mesenchymal stem cell therapy for bisphosphonate-related jaw osteonecrosis in swine. Stem Cells Dev. 22(14):2047–2056.

Marx RE, Sawatari Y, Fortin M, Broumand V. 2005. Bisphosphonate-induced exposed bone (osteonecrosis/osteopetrosis) of the jaws: risk factors, recognition, prevention, and treatment. J Oral Maxillofac Surg. 63(11):1567–1575.

Mücke T, Koschinski J, Deppe H, Wagenpfeil S, Pautke C, Mitchell DA, Wolff KD, Hölzle F. 2011. Outcome of treatment and parameters influencing recurrence in patients with bisphosphonate-related osteonecrosis of the jaws. J Cancer Res Clin Oncol. 137(5):907–913.

Nishida S, Tsubaki M, Hoshino M, Namimatsu A, Uji H, Yoshioka S, Tanimori Y, Yanae M, Iwaki M, Irimajiri K. 2005. Nitrogen-containing bisphosphonate, YM529/ONO-5920 (a novel minodronic acid), inhibits RANKL expression in a cultured bone marrow stromal cell line ST2. Biochem Biophys Res Commun. 328(1):91–97.

Parfitt AM. 2000. The mechanism of coupling: a role for the vasculature. Bone. 26(4):319–323.

Ruggiero SL, Drew SJ. 2007. Osteonecrosis of the jaws and bisphosphonate therapy. J Dent Res. 86(11):1013–1021.

Suh KT, Kim SW, Roh HL, Youn MS, Jung JS. 2005. Decreased osteogenic differentiation of mesenchymal stem cells in alcohol-induced osteonecrosis. Clin Orthop Relat Res. 431:220–225.

Walter C, Klein MO, Pabst A, Al-Nawas B, Duschner H, Ziebart T. 2010. Influence of bisphosphonates on endothelial cells, fibroblasts, and osteogenic cells. Clin Oral Investig. 14(1):35–41.

Wilde F, Heufelder M, Winter K, Hendricks J, Frerich B, Schramm A, Hemprich A. 2011. The role of surgical therapy in the management of intravenous bisphosphonates-related osteonecrosis of the jaw. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 111(2):153–163.

Xiao C, Zhou H, Liu G, Zhang P, Fu Y, Gu P, Hou H, Tang T, Fan X. 2011. Bone marrow stromal cells with a combined expression of BMP-2 and VEGF-165 enhanced bone regeneration. Biomed Mater. 6(1):015013.

Xiao E, Li JM, Yan YB, An JG, Duan DH, Gan YH, Zhang Y. 2013. Decreased osteogenesis in stromal cells from radiolucent zone of human TMJ ankylosis. J Dent Res. 92(5):450–455.

Yamada T, Yuasa M, Masaoka T, Taniyama T, Maehara H, Torigoe I, Yoshii T, Shinomiya K, Okawa A, Sotome S. 2013. After repeated division, bone marrow stromal cells express inhibitory factors with osteogenic capabilities, and EphA5 is a primary candidate. Bone. 57(2):343–354.

Zhao B, Takami M, Yamada A, Wang X, Koga T, Hu X, Tamura T, Ozato K, Choi Y, Ivashkiv LB, et al. 2009. Interferon regulatory factor-8 regulates bone metabolism by suppressing osteoclastogenesis. Nat Med. 15(9):1066–1071.

Zhu X, Wang Y, Liu Y, Huang GT, Zhang C. 2014. Immunohistochemical and histochemical analysis of newly formed tissues in root canal space transplanted with dental pulp stem cells plus platelet-rich plasma. J Endod. 40(10):1573–1578.