Y. Zhang^{1,2}, L. Wei¹, R.J. Miron¹,
Q. Zhang¹, and Z. Bian^{1*}

¹State Key Laboratory Breeding Base of Basic Science of Stomatology (Hubei-MOST) and Key Laboratory of Oral Biomedicine, Ministry of Education, School and Hospital of Stomatology, Wuhan University, People's Republic of China; and ²Department of Dental Implantology, School and Hospital of Stomatology, Wuhan University, People's Republic of China; *corresponding author, bianzhuang@whu.edu.cn

J Dent Res 93(11):1095-1100, 2014

ABSTRACT

Semaphorin 4d (Sema4d) has been proposed as a novel target gene for the treatment of osteoporosis. Recently, we fabricated a site-specific bone-targeting system from polymeric nanoparticles that demonstrates an ability to prevent bone loss in an osteoporotic model by interfering with Sema4d gene expression using small interference RNA (siRNA) molecules. The aim of the present investigation was to determine the effects of this targeting system on the periodontium, an area of high bone turnover. We demonstrated, by single photon emission computed tomography, that intravenous injection of this molecule in ovariectomized Balb/C mice is able to target alveolar bone peaking 4 hr post-injection. We then compared, by histological analysis, the bone volume/total volume (BV/TV), alveolar bone height loss, immunohistochemical expression of Sema4d, and total number of osteoclasts in mandibular alveolar bone. Four treatment modalities were compared as follows: (1) sham-operated, (2) OVX-operated, (3) OVX+estrogen replacement therapy, and (4) OVX+siRNA-Sema4d animals. The results from the present study demonstrate that an osteoporotic condition significantly increases alveolar bone height loss, and that the therapeutic effects *via* bone-targeting systems featuring interference of Sema4d are able to partly counteract alveolar bone loss caused by osteoporosis. While the future therapeutic demand for the large number of patients suffering from osteoporosis faces many challenges, we demonstrate within the present study an effective drug-delivery moiety with anabolic effects on the bone remodeling cycle able to locate and target alveolar bone regeneration.

KEY WORDS: anabolic bone formation, osteoporosis, osteopenic, bone remodeling or bone metabolism, age-related disorder, bisphosphonates.

DOI: 10.1177/0022034514552676

Received May 22, 2014; Last revision September 1, 2014; Accepted September 1, 2014

A supplemental appendix to this article is published electronically only at <http://jdr.sagepub.com/supplemental>.

© International & American Associations for Dental Research

Prevention of Alveolar Bone Loss in an Osteoporotic Animal Model *via* Interference of Semaphorin 4d

INTRODUCTION

Osteoporosis is a chronic disease affecting over 200 million people worldwide and characterized by low bone mass, poor bone strength, and micro-architectural deterioration of bone tissues (Genant *et al.*, 1999; Roush, 2011). It is an age-related disease commonly resulting from post-menopausal estrogen deficiency caused by a misbalance between bone-forming osteoblasts and osteoclasts (Gruber *et al.*, 1985; Kanis *et al.*, 1994; Rodan and Martin, 2000; Namkung-Matthai *et al.*, 2001; Moazzaz *et al.*, 2005; Hao *et al.*, 2007). In relation to periodontal treatment, osteoporosis is believed to affect periodontal tissues by preventing proper bone remodeling and ultimately causing severity in pre-existing periodontal disease (von Wörm *et al.*, 1994; Rawlinson *et al.*, 2009; Passos Jde *et al.*, 2010; Sultan and Rao, 2011).

At present, the two major pharmacological approaches for the treatment of osteoporosis are anabolic agents such as parathyroid hormone (PTH), which act by stimulating the bone formation process, and anti-resorptive agents, including bisphosphonates, calcitonin, raloxifene, receptor activator of nuclear factor kappa-B ligand (RANKL) antibodies, and estrogen replacement therapy, which act by inhibiting osteoclastic bone resorption (Silva and Bilezikian, 2011). While the abovementioned agents have demonstrated clinical improvements in fracture prevention, the disruption of the bone remodeling cycle by primarily halting bone resorption of osteoclasts has led to a number of undesirable side-effects.

Recently, semaphorins have been targeted as new molecules directly implicated in cell-cell communication pathways that occur between osteoclasts and osteoblasts (Delorme *et al.*, 2005; Takegahara *et al.*, 2006; Zhao *et al.*, 2006; Sutton *et al.*, 2008; Irie *et al.*, 2009; Leah, 2011; Negishi-Koga *et al.*, 2011; Hayashi *et al.*, 2012; Fukuda *et al.*, 2013; Ohlsson, 2013). Originally depicted as axon-guidance molecules, recent studies have demonstrated their involvement outside of the nervous system, where they play key roles in cell migration, tissue development, and angiogenesis (Tamagnone and Comoglio, 2000; Dickson, 2002; Huber *et al.*, 2003; Tran *et al.*, 2007; Suzuki *et al.*, 2008; Larrivee *et al.*, 2009). Genetic evidence from knockout animals has demonstrated a specific role for Sema4d derived from osteoclasts as a key regulator of bone formation through its receptor Plexin-B1 expressed by osteoblasts (Janssen *et al.*, 2010; Negishi-Koga *et al.*, 2011). It has previously been demonstrated that osteoclasts repel osteoblast activity through Sema4d, and it was demonstrated in a gene knockdown animal model that while Sema4d does not affect osteoclast behavior, it has a pronounced effect on osteoblast behavior by increasing osteoblast numbers on bone surfaces and

improving their mineralization potential (Negishi-Koga *et al.*, 2011).

Of primary importance for the therapeutic healing of human disease is the development of effective administration of pharmacological agents delivered specifically to target tissues (Wang *et al.*, 2010). Recently we have fabricated a specific bone-targeting drug delivery system from polymeric nanoparticles including the incorporation of an interference molecule for *Sema4d* by small interference RNA (siRNA) (Zhang *et al.*, 2014). The aim of the present study was to determine the delivery of this molecule to periodontal tissues following intravenous injection and to monitor the effect of siRNA-*Sema4d* on alveolar bone loss in an osteopenic animal model induced by ovariectomy (OVX). Four weeks post-OVX surgery to induce an osteopenic phenotype, animals were compared by μ CT and histological analysis after receiving injections with the following 4 treatment modalities: (1) sham-operated, (2) OVX-operated, (3) OVX+estrogen, and (4) OVX+siRNA-*Sema4d* animals.

MATERIALS & METHODS

Fabrication of Asp8-(STR-R8)-*Sema4d* siRNA

The Asp8 bone-affinity polymer system was utilized according to a previously published study (Wang *et al.*, 2006). The packaging of siRNA was performed according to a protocol adapted from DNA transfection experiments (Tonges *et al.*, 2006). A 1.5- μ L quantity of Stearyl-R8 was diluted in 50 μ L of neurobasal medium without supplements and combined with 10 pmol of siRNA for *Sema4d* (produced by GenePharma, Shanghai, China) in 50 μ L of neurobasal medium without supplements after 5-minute incubation at room temperature. Incubation was continued for 20 min at room temperature, and the mixture was then delivered to animals for *in vivo* study.

Animals and Surgical Procedures

Thirty-six mature female Balb/c mice (eight-week-old, mean body weight 25 g) were used in this study (Table 1), with all protocols being in accordance with the policies of the Ethics Committee for Animal Research, Wuhan University, China. All animal handling was in compliance with the ARRIVE guidelines. Animals had access to food and water *ad libitum*.

To obtain an osteoporotic model, we performed bilateral ovariectomy (OVX) by a minimally invasive surgical technique as previously described (Zhang *et al.*, 2012; Cheng *et al.*, 2013). Briefly, with the animals under general anesthesia induced by intraperitoneal injection of chloral hydrate (10%, 4 mL/kg body weight), 10-mm lateral lumbar skin incisions were made bilaterally. When the enterocele was exposed by blunt dissection of muscle and peritoneum, the ovarian artery and vein were ligated, and the ovaries were removed. The mice in the blank group were sham-operated. Finally, the muscle and skin were sutured stratified. Post-operatively, penicillin (40,000 IU/mL, 1 mL/kg) was injected for 3 days for prevention of infection.

μ CT Analysis

For confirmation of the establishment of an osteoporotic model, the femoral samples of the sham and OVX groups at 4 wk

post-operation were fixed in 4% formaldehyde for 12 hr at 4°C. A μ CT imaging system (μ CT50, Scanco Medical, Bassersdorf, Switzerland) was used to reveal new bone formation within the defect region. Scanning parameters set at 70 kV and 114 μ A with a thickness of 0.015 mm *per slice* in medium-resolution mode. For 3D reconstruction, the mineralized bone tissue was differentially segmented with a fixed low threshold (value = 212). Representative images were cut from the axial section after 3D reconstruction with the built-in software of the μ CT.

Bone-selective Delivery

To examine whether Asp8 could selectively target alveolar bone, we divided an additional 42 Balb/c mice into 2 groups that received rhodamine-labeled Asp8 (27 μ mol/kg, 0.2 mL) or rhodamine alone *via* tail vein injection. After 0 min, 30 min, 1 hr, 2 hr, 4 hr, 24 hr, and 48 hr, 3 mice from each group were euthanized in batches, and the mandibular regions were collected and processed for detection of the fluorescence signal by means of a Xenogen IVIS imaging system (Alameda, CA, USA).

Animal Experiments

For treatment (intravenous injection began 4 wk after the osteopenic model was confirmed) of osteoporosis, mice were divided into Sham+PBS, OVX+PBS, OVX+estrogen, and OVX+(Asp8-(STR-R8)-*Sema4d* siRNA) groups ($n = 9$ for each group). A total amount of siRNA (20 OD/kg) was given by intravenous injection every wk, and 17 β -estradiol (Sigma Chemical Co., St. Louis, MO, USA) was injected subcutaneously at a dose of 10 μ g/kg 3 times *per wk*. Four wk later (8 wk after the original OVX surgery), the mice were euthanized and subjected to bone analysis.

Histological and Immunohistochemical Analysis

After decalcification of samples, the mandibular samples were gradient-dehydrated and embedded in paraffin with sections parallel to the long axis of the molar roots. Serial sections of 5 μ m were cut and mounted on polylysine-coated slides, then stained with H&E and tartrate-resistant acid phosphatase (TRAP) (Sigma #387A; Sigma-Aldrich) in accordance with the manufacturer's protocol.

For immunohistochemical assessment, the expression of *Sema4d* was detected according to the following procedure. Following the process of deparaffination, rehydration, and washing, the sections were antigen-retrieved by trypsin and then incubated with 0.3% hydrogen peroxide for 20 min, followed by incubation with goat serum. The sections were then incubated with primary antibody for *Sema4d* (1:250; 14422-1-AP, Proteintech, Inc., Chicago, IL, USA) and osteocalcin (OCN, 1:500; LS-C124318-100, LifeSpan BioSciences, Seattle, WA, USA) for 2 hr at 37°C. In accordance with the manufacturer's protocol, the sections were incubated with an SP 9000 immunohistochemical kit (Zhongshan Biotechnology Co., Ltd, China) and visualized by 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Zhongshan Biotechnology Co., Ltd). Last, the sections were counterstained with hematoxylin.

For histometric measurement, we adapted bone density, alveolar bone height loss, immunohistochemical intensity, number of TRAP-positive cells (osteoclasts), and number of

Table 1. Weight of Mice at (1) Experimental Start, (2) Following Sham+OVX (Ovariectomy), and (3) at the End of the Experiment

	8w (start of experiment)	12w (Sham+OVX)	16w (end point)
Sham+vehicle	24.68 ± 1.56	28.89 ± 1.46	31.23 ± 1.25
OVX+vehicle	24.75 ± 1.64	31.01 ± 1.62*	35.09 ± 1.30*
OVX+estrogen	24.49 ± 1.32	31.76 ± 1.73*	33.02 ± 1.23*
OVX+Asp8-(STR-R8)-Sema4d siRNA	24.97 ± 1.51	31.15 ± 1.57*	35.42 ± 1.31*

*indicates significant difference between group and Sham+PBS, $p < .05$.

Figure 1. Establishment of a mouse osteoporotic model as confirmed in the femur head. Micro-computed tomography representation in 2D of normal bone (A-C) and osteoporotic bone (D-F). 3D visualization of subchondral trabecular regions of the normal bone (B) in (A) and the osteoporotic bone (E) in (D). Representative H&E staining for the normal (C) and osteoporotic (F) femurs (magnification 40x).

OCN-positive cells (osteoblasts) by processing the images, which were captured with an Olympus DP72 microscope, according to the literature as previously reported (Tuominen *et al.*, 2010; Luo *et al.*, 2012; Yuan *et al.*, 2013; Yang *et al.*, 2014).

Statistical Analysis

Statistical analyses were performed by one-way ANOVA and the Student-Newman-Keuls test, and statistical significance was considered at $p < .05$. All data are expressed as the mean ± SD.

RESULTS

Establishment of an Osteoporotic Model

Micro-computed tomography (μ CT) images and histological sections were used to visualize the establishment of an osteoporotic model (Fig. 1). The 3D μ CT images of the OVX mouse femurs demonstrated a striking decrease in the subchondral trabecular bone volume, thickness, and density (Figs. 1C, 1D) when compared with sham-operated animals (Figs. 1A, 1B). Analysis of the μ CT data confirmed an increase in trabecular separation, reduced cortical thickness, and enlarged marrow cavities, as compared with those in sham-operated mice (Table 2). The H&E staining is consistent with the differences revealed by μ CT, demonstrating abundant trabecular bone with healthy marrow-like tissue in sham-operated animals and a small quantity of trabecular bone with inflammatory cells in ovariectomized mice (Figs. 1C, 1F).

Table 2. Results from μ CT of the Condylar Femur for Bone Volume over Total Volume (BV/TV), Cortical Bone Thickness, and Trabecular Separation in Sham and Ovariectomized (OVX) Animals.

	BV/TV (%)	Cortical Bone Thickness (μ m)	Trabecular Separation (μ m)
Sham	24.68 ± 2.56	268.89 ± 5.46	211.28 ± 9.25
OVX	13.21 ± 1.38**	232.68 ± 8.60**	324.38 ± 12.05**

Confirmation of a Bone-targeting Drug Delivery System

Planar and tomographic images of Balb/C mice were viewed with a Xenogen IVIS imaging system at 24 hr after i.v. administration of rhodamine either alone or as a label of an HPMa copolymer-D-Asp8 conjugate (Appendix Fig., A, B). Then, the pharmacokinetics and biodistribution were analyzed in the HPMa copolymer group with D-Asp8 (Appendix Fig., C). Analysis of the data revealed strong labeling at high bone turnover sites, including the mandibular region, with staining intensity peaking at 4 hr post-injection of our bone-targeting moiety (Appendix Fig., C).

Sema4d Delivered via a Bone-targeting Moiety Prevents Bone Loss in an Osteoporotic Model in the Mandibular Region

Following four-week establishment of an osteopenic animal model, animals were euthanized, and histological analysis was performed. The average weight of the animal groups is reported in Table 1. H&E staining demonstrated the highest BV/TV in the furcation area of first molars in mice that were sham-operated (Fig. 2A). While the OVX group showed significantly lower levels of BV/TV, the additional use of the bone-targeting moiety containing Sema4d-siRNA recovered the lost bone induced by OVX to levels similar to those of the sham group (Figs. 2A-2J). Then, alveolar bone height loss was calculated in the 4 treatment modalities (Figs. 2F-2J). These results demonstrated that the OVX group demonstrated significantly greater alveolar height loss when compared with all other treatment modalities (Fig. 2). The use of siRNA-Sema4d as well as estrogen replacement therapy significantly prevented bone loss in an OVX mice model 4 wk post-injection when compared with the OVX group (Fig. 2J). Immunohistological analysis was utilized to visualize the staining intensity of Sema4d in the various treatment groups (Figs. 3A-3E). The use of siRNA for Sema4d

Figure 2. Histological observation (H&E stain) of the bone volume/total volume (BV/TV) (A-E) and mandibular inter-molar alveolar bone height loss (F-J) of the first molar in various groups (20x magnification). The Sema4d-siRNA improved BV/TV and decreased inter-molar height loss to levels similar to those in the OVX group. The detected differences were statistically significant at $p < .05$.

Figure 3. Representative immunohistological detection of Sema4d (A-E) and numbers of multinucleated cells (MNCs) as depicted by TRAP staining (F-J) around the inter-molar regions (40x magnification). (K) Number of osteoblasts. The Sema4d-siRNA group significantly reduced the expression of Sema4d when compared with all other modalities. The detected differences were statistically significant at $p < .05$.

significantly decreased the intracellular expression of Sema4d in cells by three-fold when compared with OVX animals (Fig. 3E). Last, TRAP staining was used to quantify the number of osteoclasts (Figs. 3F-3J). While OVX significantly increased the number of osteoclasts, a significant increase was also observed in the group treated with our bone-targeting moiety, delivering siRNA-Sema4d, when compared with sham animals (Fig. 3I). The use of estrogen replacement therapy decreased osteoclast numbers when compared with OVX animals receiving no therapy (Fig. 3H). The number of osteoblasts was significantly up-regulated in the bone-targeting moiety group when compared with the OVX+estrogen group, and an increase of over three-fold was seen when compared with the OVX group (Fig. 3K).

DISCUSSION

The aim of the present study was to characterize the effects of a new, innovative drug-targeting moiety including siRNA for Sema4d and to determine its effects on alveolar bone change in

an osteoporotic animal model induced by OVX. Currently, there are no clinically available bone-specific delivery systems for the treatment of osteoporosis, a disease that now affects over 200 million people worldwide. Given the progressively aging population, there is a major need to develop safe anabolic agents that increase bone formation to compensate for the increase in bone resorption caused by post-menopausal estrogen deficiencies. We have recently developed a targeting system capable of re-establishing bone remodeling in an osteopenic animal model by anabolic means (Zhang *et al.*, 2014). In this study, the effects of the siRNA for Sema4d were demonstrated to have little effect on *in vitro* osteoclast function and number; however, the effect on osteoblast function was greatly enhanced following transfection. It was shown that, in a co-culture system of osteoclasts and osteoblasts, alkaline phosphatase activity was increased over two-fold along with an increase in osteoblast differentiation markers including collagen I and osteocalcin as assessed by real-time PCR (Zhang *et al.*, 2014). The aim of the present study was to characterize the effects this system may have on periodontal tissues by determining (a) what effect an osteoporotic model might have on periodontal tissues, (b) the delivery of our bone-targeting moiety specifically to alveolar bone, and (c) the effects of siRNA-Sema4D on bone remodeling in alveolar bone in a compromised OVX animal model.

We first observed that the effect of OVX on our animal model led to a decrease in BV/TV, mainly in the furcation areas of mouse mandibular first molars (Fig. 2). In this group, a higher number of multinucleated osteoclasts (Fig. 3) as well as greater alveolar bone height loss (Fig. 3) were observed, demonstrating and confirming previous reports that osteoporosis has negative effects on alveolar bone remodeling, an area known to display high bone turnover (von Wörmann *et al.*, 1994; Passos Jde *et al.*, 2010; Sultan and Rao, 2011).

Following confirmation of our osteopenic animal model, we then sought to characterize the ability of the drug-targeting moiety to specifically target and bind to alveolar bone. As depicted in the Appendix Figure, the targeting system was able to target alveolar bone specifically in the mandibular regions, peaking at 4 hr post-injection. We also confirmed, *via* immunohistochemical analysis, that Sema4d expression in these tissues was markedly decreased up to three-fold when compared with that in OVX animals (Fig. 3). The targeted delivery of siRNA-Sema4d to alveolar tissues is important. Since Sema4d is highly

implicated in the development of neuronal and other tissues, its specific delivery is critical for the safety of this moiety. Future research aimed at elucidating any possible side-effects will be necessary in large-animal models prior to any clinical testing, and the bone-targeting system needs to be further characterized in large animals.

Once it was observed that the expression of Sema4d was decreased over three-fold in alveolar tissues, the effects on the surrounding alveolar tissues demonstrated a significant rise in BV/TV as well as decreased bone loss height in alveolar tissues in OVX animals (Fig. 2). One of the significant findings from the present investigation was that the drug-targeting moiety containing siRNA-Sema4d did not change the number of osteoclasts in OVX animals (Fig. 3). There have been numerous reports on the effects of principally decreasing osteoclast number/activity in the treatment of osteoporosis. Although bisphosphonates have been the treatment of choice in osteoporotic patients for a number of years for fracture prevention, their main side-effect includes halting the bone remodeling cycle, thus indirectly resulting in no new bone formation. We demonstrate in the present study that interference of Sema4D acts primarily in an anabolic fashion by increasing osteoblast activity and preventing bone loss mainly by increasing osteoblast function. While other investigators have previously suggested that Sema4d acts as a guidance molecule for bone-cell positioning, analogous to the function of semaphorins in axon guidance, in this study, we show that interference of its expression in osteoclasts further confirms its evident role in the cross-talk between osteoclasts and osteoblasts.

While the future field of osteoporosis therapy faces many challenges from the growing numbers of patients who suffer from post-menopausal bone loss, here, we demonstrate a solution by selectively targeting Sema4d in osteoclasts via a bone-specific delivery system. We also confirm its positive effects on bone remodeling within alveolar tissues, an area of high bone turnover where an osteoporotic phenotype can largely contribute to faster periodontal breakdown. In conclusion, the results from the present study further demonstrate that preventive measures by interfering with Sema4d expression increase BV/TV in mandibular bone and prevent alveolar bone loss height in an animal model induced by OVX.

ACKNOWLEDGMENTS

This work was supported by the Program for New Century Excellent Talents in University (NCET-11-0414), by funds from the National Natural Science Foundation of China [81271108 to Y.F. Zhang, and 81120108010 (international cooperation and exchange projects) to Z. Bian], and by the Pre-National Basic Research Program of China (973 Plan) (2012CB722404 to Z. Bian). The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

REFERENCES

- Cheng N, Dai J, Cheng X, Li S, Miron RJ, Wu T, *et al.* (2013). Porous CaP/silk composite scaffolds to repair femur defects in an osteoporotic model. *J Mater Sci Mater Med* 24:1963-1975.
- Delorme G, Saltel F, Bonnelye E, Jurdic P, Machuca-Gayet I (2005). Expression and function of semaphorin 7A in bone cells. *Biol Cell* 97:589-597.
- Dickson BJ (2002). Molecular mechanisms of axon guidance. *Science* 298:1959-1964.
- Fukuda T, Takeda S, Xu R, Ochi H, Sunamura S, Sato T, *et al.* (2013). Sema3A regulates bone-mass accrual through sensory innervations. *Nature* 497:490-493.
- Genant HK, Cooper C, Poor G, Reid I, Ehrlich G, Kanis J, *et al.* (1999). Interim report and recommendations of the World Health Organization Task-Force for Osteoporosis. *Osteoporos Int* 10:259-264.
- Gruber HE, Ivey JL, Thompson ER, Chesnut CH 3rd, Baylink DJ (1985). Osteoblast and osteoclast cell number and cell activity in postmenopausal osteoporosis. *Miner Electrolyte Metab* 12:246-254.
- Hao YJ, Zhang G, Wang YS, Qin L, Hung WY, Leung K, *et al.* (2007). Changes of microstructure and mineralized tissue in the middle and late phase of osteoporotic fracture healing in rats. *Bone* 41:631-638.
- Hayashi M, Nakashima T, Taniguchi M, Kodama T, Kumanogoh A, Takayanagi H (2012). Osteoprotection by semaphorin 3A. *Nature* 485:69-74.
- Huber AB, Kolodkin AL, Ginty DD, Cloutier JF (2003). Signaling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance. *Annu Rev Neurosci* 26:509-563.
- Irie N, Takada Y, Watanabe Y, Matsuzaki Y, Naruse C, Asano M, *et al.* (2009). Bidirectional signaling through ephrinA2-EphA2 enhances osteoclastogenesis and suppresses osteoblastogenesis. *J Biol Chem* 284:14637-14644.
- Janssen BJ, Robinson RA, Perez-Branguli F, Bell CH, Mitchell KJ, Siebold C, *et al.* (2010). Structural basis of semaphorin-plexin signalling. *Nature* 467:1118-1122.
- Kanis JA, Melton LJ, Christiansen C, Johnston CC, Khaltaev N (1994). The diagnosis of osteoporosis. *J Bone Miner Res* 9:1137-1141.
- Larrivee B, Freitas C, Suchting S, Brunet I, Eichmann A (2009). Guidance of vascular development: lessons from the nervous system. *Circ Res* 104:428-441.
- Leah E (2011). Bone: finding that osteoclasts repel osteoblast activity through Sema4D reveals novel target for bone-boosting therapies. *Nat Rev Rheumatol* 7:681.
- Luo T, Wu C, Zhang Y (2012). The in vivo osteogenesis of Mg or Zr-modified silicate-based bioceramic spheres. *J Biomed Mater Res A* 100:2269-2277.
- Moazzaz P, Gupta MC, Gilotra MM, Gilotra MN, Maitra S, Theerajunyaporn T, *et al.* (2005). Estrogen-dependent actions of bone morphogenetic protein-7 on spine fusion in rats. *Spine (Phila Pa 1976)* 30:1706-1711.
- Namkung-Matthai H, Appleyard R, Jansen J, Hao Lin J, Maastricht S, Swain M, *et al.* (2001). Osteoporosis influences the early period of fracture healing in a rat osteoporotic model. *Bone* 28:80-86.
- Negishi-Koga T, Shinohara M, Komatsu N, Bito H, Kodama T, Friedel RH, *et al.* (2011). Suppression of bone formation by osteoclastic expression of semaphorin 4D. *Nat Med* 17:1473-1480.
- Ohlsson C (2013). Bone metabolism in 2012: novel osteoporosis targets. *Nat Rev Endocrinol* 9:72-74.
- Passos Jde S, Gomes-Filho IS, Vianna MI, da Cruz SS, Barreto ML, Oliveira TJ, *et al.* (2010). Outcome measurements in studies on the association between osteoporosis and periodontal disease. *J Periodontol* 81:1773-1780.
- Rawlinson SC, Boyde A, Davis GR, Howell PG, Hughes FJ, Kingsmill VJ (2009). Ovariectomy vs. hypofunction: their effects on rat mandibular bone. *J Dent Res* 88:615-620.
- Rodan GA, Martin TJ (2000). Therapeutic approaches to bone diseases. *Science* 289:1508-1514.
- Roush K (2011). Prevention and treatment of osteoporosis in postmenopausal women: a review. *Am J Nurs* 111:26-35.
- Silva BC, Bilezikian JP (2011). New approaches to the treatment of osteoporosis. *Annu Rev Med* 62:307-322.
- Sultan N, Rao J (2011). Association between periodontal disease and bone mineral density in postmenopausal women: a cross sectional study. *Med Oral Patol Oral Cir Bucal* 16:e440-e447.
- Sutton AL, Zhang X, Dowd DR, Kharode YP, Komm BS, Macdonald PN (2008). Semaphorin 3B is a 1,25-dihydroxyvitamin D3-induced gene in

- osteoblasts that promotes osteoclastogenesis and induces osteopenia in mice. *Mol Endocrinol* 22:1370-1381.
- Suzuki K, Kumanogoh A, Kikutani H (2008). Semaphorins and their receptors in immune cell interactions. *Nat Immunol* 9:17-23.
- Takegahara N, Takamatsu H, Toyofuku T, Tsujimura T, Okuno T, Yukawa K, *et al.* (2006). Plexin-A1 and its interaction with DAP12 in immune responses and bone homeostasis. *Nat Cell Biol* 8:615-622.
- Tamagnone L, Comoglio PM (2000). Signalling by semaphorin receptors: cell guidance and beyond. *Trends Cell Biol* 10:377-383.
- Tonges L, Lingor P, Egle R, Dietz GP, Fahr A, Bahr M (2006). Stearylated octaarginine and artificial virus-like particles for transfection of siRNA into primary rat neurons. *RNA (New York, NY)* 12:1431-1438.
- Tran TS, Kolodkin AL, Bharadwaj R (2007). Semaphorin regulation of cellular morphology. *Annu Rev Cell Dev Biol* 23:263-292.
- Tuominen VJ, Ruotoistenmaki S, Viitanen A, Jumppanen M, Isola J (2010). ImmunoRatio: a publicly available Web application for quantitative image analysis of estrogen receptor (ER), progesterone receptor (PR), and Ki-67. *Breast Cancer Res* 12:R56.
- von Wowern N, Klausen B, Kollerup G (1994). Osteoporosis: a risk factor in periodontal disease. *J Periodontol* 65:1134-1138.
- Wang D, Sima M, Mosley RL, Davda JP, Tietze N, Miller SC, *et al.* (2006). Pharmacokinetic and biodistribution studies of a bone-targeting drug delivery system based on N-(2-hydroxypropyl)methacrylamide copolymers. *Mol Pharm* 3:717-725.
- Wang Y, Li Z, Han Y, Liang LH, Ji A (2010). Nanoparticle-based delivery system for application of siRNA in vivo. *Curr Drug Metab* 11:182-196.
- Yang S, Lan L, Miron RJ, Wei L, Zhang M, Zhang Y (2014). Variability in particle degradation of four commonly employed dental bone grafts. *Clin Implant Dent Relat Res* [Epub ahead of print 1/3/2014] (in press).
- Yuan H, Zelka S, Burkatovskaya M, Gupte R, Leeman SE, Amar S (2013). Pivotal role of NOD2 in inflammatory processes affecting atherosclerosis and periodontal bone loss. *Proc Natl Acad Sci USA* 110:E5059-E5068.
- Zhang Y, Cheng N, Miron R, Shi B, Cheng X (2012). Delivery of PDGF-B and BMP-7 by mesoporous bioglass/silk fibrin scaffolds for the repair of osteoporotic defects. *Biomaterials* 33:6698-6708.
- Zhang Y, Wei L, Miron RJ, Shi B, Bian Z (2014). Anabolic bone formation via a site specific bone targeting delivery system by interfering with Semaphorin 4D expression. *J Bone Miner Res* [Epub ahead of print 8/4/2014] (in press).
- Zhao C, Irie N, Takada Y, Shimoda K, Miyamoto T, Nishiwaki T, *et al.* (2006). Bidirectional ephrinB2-EphB4 signaling controls bone homeostasis. *Cell Metab* 4:111-121.