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Gingival MSCs Improve Bone Phenotype in Ovariectomy-Induced Osteoporosis via Programmed Cell Death Pathway

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Abstract

Estrogen deficiency-related osteoporosis is a skeletal system disorder that affects women after menopause taking a toll on financial and health institutions. Gingival Mesenchymal Stem cells (GMSCs) are a distinctive population of dental tissue-derived stem cells that possess uniquely high proliferation abilities and are capable of self-maintenance and multipotent differentiation. Their use has been investigated in different disease model applications, including cutaneous wound healing models, colitis, and allergy-related inflammatory disease models, but their effect on the bone phenotype of ovariectomy-induced osteoporosis hasn't been explored. In our study, we show that a single systemic infusion of GMSCs elevated the bone mass reduction caused by Ovariectomy (OVX)-induced osteoporosis in both the femurs and mandibles of OVX mice, they also successfully rescued the function of the defective endogenous population of Bone Marrow Mesenchymal Stem Cells (BMMSCs). We saw that the systemic infusion of GMSCs exerted an immunomodulatory effect on the host, leading to the elevation of T-regulatory lymphocytes (T-regs) and the downregulation of T helper type 1 lymphocytes (Th1) levels in recipient OVX mice. Mechanistically, PD-1/PD-L1 is a popular cellular death pathway being heavily investigated in multiple research fields, In our study, we found that GMSC expresses PD-L1 and that the improved bone phenotype resulting from the GMSC infusion was via the programmed cell death (PD-1/PD-L1) pathway triggering activated T-cell apoptosis in the OVX mice, eventually resulting in an improvement of the bone phenotype.

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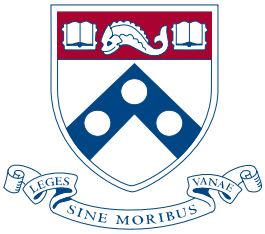

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Gingival MSCs Improve Bone Phenotype in Ovariectomy-Induced Osteoporosis via Programmed Cell Death Pathway

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Abstract

Menopause, an event that occurs in all women's life cycle, attributes physiologically to a decreased ovarian function, and an associated decline in the female reproductive hormones. Manifestations include hot flashes, sleep disturbances, anxiety, depression, and other changes. A common disease affecting post-menopausal women is osteoporosis or "estrogen-deficiency related osteoporosis." Osteoporosis is a skeletal system disorder where bone mineral density progressively decreases, which in turn increases the risk of fragility related fractures. The underlying mechanism behind the estrogen-related osteoporosis, in general, is through estrogen being a key regulator of bone remodeling and regulating cytokines. When that balance is disrupted due to the lack of estrogen in menopause, an intensified bone resorption rate takes place without the adequate formation of new bone, correlated with the inflammatory nature of the post-menopausal osteoporosis disorder. Multiple treatment modalities are utilized for the treatment and management of osteoporosis, but there are drawbacks that confound their use. Among several efforts and reports on alternative methods and approaches for better treatment options in osteoporosis and bone diseases, cellular therapy, a promising method which was profoundly investigated, has evolved noticeably in recent years as a powerful investigation tool in the field of bone biology, and related bone disorders.

Mesenchymal Stem Cells (MSCs) are adult stem cells that act as a viable source of cell for cell replacement to treat bone disease due to their inherent properties of self-renewal and plasticity. Another benefit they have is their potent immunomodulatory ability to induce immunological tolerance in the recipient host, which makes them a desired tool for investigations in developing new treatment modalities for immune-related diseases. Dental tissue-derived stem cells are stem cells harvested from the oral cavity, with several promising ongoing investigated applications including bio-root engineering, regeneration of periodontal defects, pulpal tissue engineering, and calvarial defects regeneration and they show promising outcomes. In this thesis project, we investigate the application and properties of Gingival Mesenchymal Stem Cells (GMSCs). These cells are adult, tissue-specific stem cells with several reported clinical applications in multiple murine disease models, including wound healing models, colitis models, and allergy-related inflammatory disease models, and the results were promising. The availability of GMSCs is also considerably high, and there is reduced morbidity associated with their harvesting procedures in comparison to other types of stem

cells. GMSCs possess immunomodulatory properties and can modulate the microenvironment of the recipient host and are capable of multipotent differentiation into multiple cell types. These benefits of GMSCs prompted us to further want to explore their therapeutic effect in a chronic inflammatory disease model, the estrogen deficiency-induced osteoporosis model.

In this dissertation, we investigated the application of GMSCs as a novel cellular therapeutic approach in an attempt to ameliorate the bone phenotype of estrogen deficiency-induced osteoporosis in the OVX mouse model. Our findings demonstrate that the single transplantation of GMSCs was able to markedly improve the bone phenotype, as seen in both the femurs and mandibles of OVX mice. In addition to that, our investigations also showed that the GMSCs transplantation was able to rescue the osteogenic functions of the endogenous population of Bone Marrow Mesenchymal Stem Cells (BMMSCs) in the recipient ovariectomized mice. Furthermore, to explore the mechanisms underlying the improved bone phenotype and inflammatory state associated with the estrogen-induced osteoporosis model, we examined possible pathways that may be involved. We showed that GMSCs exerted an immunoregulatory effect on the recipient host via the PD-L1/PD-1 pathway. Using the siRNA approach to knockdown PD-L1 in GMSCs (siPdl1 GMSCs) or an anti-PDL1 drug (Atezolizumab) treatment, we showed a diminished effect of the GMSCs infusion on the OVX bone phenotype, suggesting the role of PD-L1 as an immune checkpoint in the GMSCs-mediated effect in the ovariectomized mouse model. Furthermore, at the cellular level, GMSCs-treated mice showed a decreased expression of Th1, Th17, and an increase in the expression of T-reg as compared to OVX mice. On the other hand, siPdl1 GMSCs-treated mice displayed similar expression of Th1, T-reg, and a decreased expression of Th17 as compared to OVX mice.

In conclusion, cell-based therapy using GMSCs can improve the osteoporotic bone phenotype, as well as rescue the function of the endogenous populations of BMMSCs in the estrogen deficiency OVX mouse model of osteoporosis, and the PD-L1/PD-1 pathway plays a role in the GMSCs mediated effects seen in the OVX mice.

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List of Abbreviations

- MSCs: Mesenchymal stem cells
- BMMSCs: Bone marrow mesenchymal stem cells
- GMSCs: Gingival mesenchymal stem cells
- DPSCs: Dental pulp stem cells
- SHED: Stem cells from human exfoliated deciduous teeth
- PDLSc: Periodontal ligament stem cells
- DFPCs: Dental follicle precursor cells
- SCAP: Stem cells from apical papilla
- OVX: Ovariectomized mice model
- BRONJ: Bisphosphonate related osteonecrosis of the jaw
- DDS: Dextran sulfate sodium
- TNF-A: tumor necrosis factor alpha
- IL-6: Interleukin 6
- IL-1: Interleukin 1

- IFN-γ: Interferon gamma
- M-CSF: Macrophage colony-stimulating factor
- GM-CSF: Granulocyte-macrophage colony-stimulating factor
- RANKL: Receptor activator of nuclear factor kappa-B ligand
- TGF-B: Transforming growth factor Beta
- NIH: National institute of health
- BMD: Bone mineral density
- DXA: Dual-energy x-ray absorptiometry
- WHO: World health organization
- SERMs: Selective estrogen receptor modulators
- PTH: Parathyroid hormone
- ASGCT: American society of gene & cell therapy
- CDs: Cluster differentiation molecules
- OCT-4: Octamer-4
- SSEA-4: Stage-specific embryonic antigen 4
- PBS: Phosphate buffered saline

- µCT: X-ray microtomography
- TRAP: Tartrate-resistant acid phosphatase
- H&E: Hematoxylin and eosin
- EDTA: Ethylenediaminetetraacetic acid
- ELISA: Enzyme-linked Immunosorbent assay
- PFA: Paraformaldehyde
- BV/TV: Bone volume density
- Conn.D: Connectivity density
- SMI: Structure model index
- BRDU: Bromodeoxyuridine
- TNF-R: Tumor necrosis factor receptor
- DISC: Death inducing signaling complex
- WT: Wild type
- IBD: Irritable bowel disease
- SLE: Systemic lupus erythematosus
- DSS: Dextran sulfate sodium

- SS: Systemic sclerosis
- GVHD: Graft versus host disease
- CFU-F: Colony forming units – fibroblast
- LPL: Lipoprotein lipase
- PPAR-γ: Peroxisome proliferator-activated receptor gamma
- RUNX2: Runt-related transcription factor 2
- ALP: Alkaline phosphatase
- WT: Wild type
- PBS: phosphorus buffered saline
- BRONJ: Bisphosphonate related osteonecrosis of the jaw
- MAR: Mineral apposition rate
- FDA: Food and drug administration

List of Illustrations

Introduction

Osteoporosis:

The national institute of health (NIH) describes osteoporosis as a skeletal system disorder occurring due to the reduction of bone density, which in turn leads to an increased risk of bone fractures (1). The disease resembles a growing worldwide concern, and it is estimated that today, over 200 million people worldwide are diagnosed with the disease (2). From a physiological point of view, the disease of osteoporosis occurs when there is an imbalance between bone formation and bone resorption rates, this could happen by either a decrease in the bone formation rates or an increase in bone resorption rates. The disease is also a chronic and silent disease in nature, up until it reaches the point of clinical detection or manifestation or until an incident of bone fracture occurs (3).

The diagnosis of the disease is a process that mainly involves an overall assessment of the subject's bone mineral density value (BMD), which is measured by different available diagnostic means, such as the dual-energy x-ray absorptiometry (DXA) scan. Then if the value obtained is equal to or more than 2.5 standard deviations less than the reference young adult mean value, they would be diagnosed with osteoporosis (4). Several risk factors for fractures are also taken into consideration when diagnosing patients, these multiple known risk factors for osteoporosis have been long investigated as well as reported in the literature over the years. Some of these factors leading to low bone mineral density and

osteoporosis are age, sex, race, estrogen levels, previous fragility-related fractures, and others (Fig.1). These investigations and researches aim to progressively better understand the relation of these risk factors to osteoporosis and fractures because of the considerable magnitude of the disease and impact of disability on societies as well as the significant toll of the increased financial weight for the costs associated with the treatment of osteoporosis-related fractures (5-9).

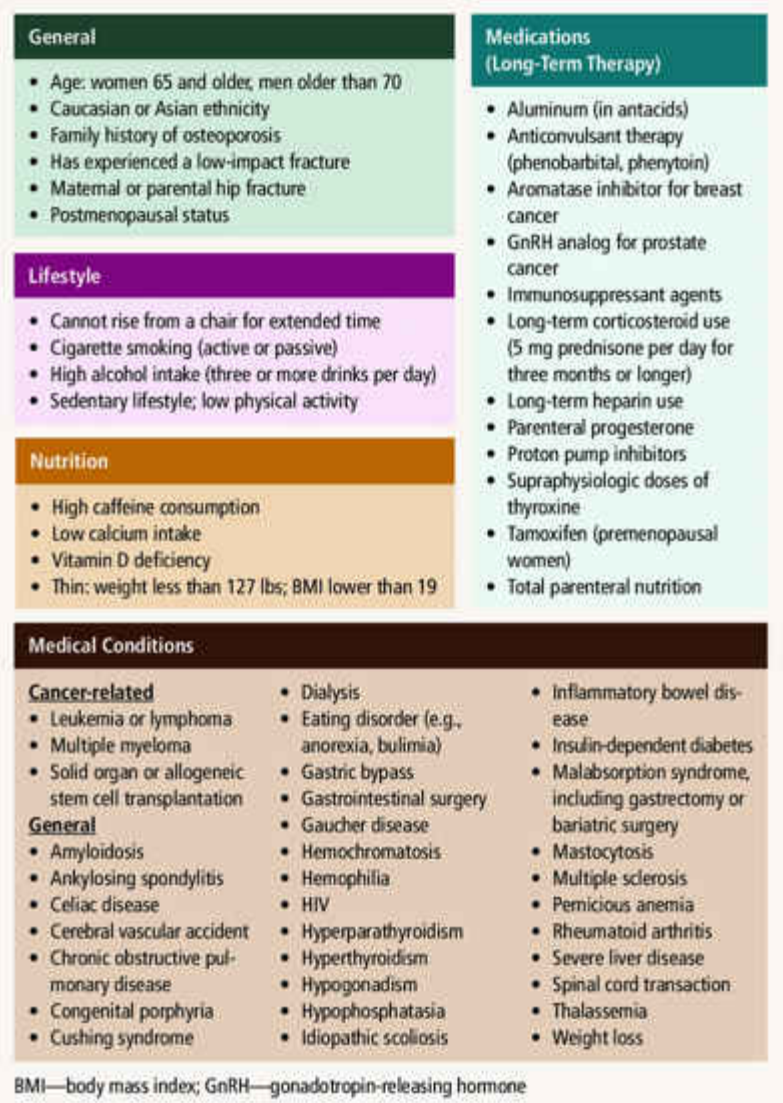


Figure 1. Risk factors for osteoporosis. Risk factors for osteoporosis and low bone mineral density. (Figure adapted from Wickham 2011(9))

Menopause and postmenopausal osteoporosis:

Menopause is a risk factor for developing osteoporosis. The world health organization (WHO) defines menopause as "the permanent cessation of menstruation resulting from the loss of ovarian follicular activity." Moreover, it is estimated that in the united states, the yearly number of women reaching menopause is around 1.5 million, while menopause occurs in every woman's life cycle, it is marked beginning with the first missed period of 12 consecutive months of menstrual cycle termination. The average age for which menopause occurs is around 50 years, and it is characterized by multisymptomatic changes, mainly vasomotor symptoms such as hot flashes, night sweats, and flushes. It is also accompanied by a decrease in ovarian hormones, mainly Estrogen, this decline reaches its maximum level of decrease 3 to 4 years from the last occurring menstrual cycle (10-12). The effects of this decrease also involve the skeletal system and are linked to osteoporosis in post-menopausal women leading to a disruption of the existing balance between bone formation and bone resorption rates. Furthermore, even though the mechanism behind it is yet to be fully understood, the effect of Estrogen on the bone is known to be primarily by its modulation capabilities of cytokine production and blocking osteoclastogenesis, as well as indirectly increasing the absorption of calcium from the intestines, and decreasing the renal output of calcium (13,14).

In addition to the effect of Estrogen on the decrease in bone resorption rates, it is further known to promote osteoclast apoptosis directly by the increase in TGF-β production (15). In menopause, when the levels of estrogen decrease,

an increase in some pro-inflammatory cytokines has been described, which in turn, increases osteoclast formation and inhibits their apoptosis; it also influences other cytokines affecting osteoclast differentiation such as IL-6, Macrophage Colony-Stimulating Factor (M-CSF), and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) (16,17). In addition to the effect on cytokines, the immune system's T-lymphocytes are recognized too as key players in modulating the adverse effects on the bone from Estrogen deficiency and the noted increase in the inflammatory cytokines by the production of IFN-γ, and TNF-α which in turn intensifies the effect of M-CSF and RANKL induced osteoclastogenesis (18,19). Overall, estrogen has an anti-inflammatory effect and it has been shown that OVX mice exhibit an increase in inflammation and a shortened life span. (20)

Current treatment modalities:

Multiple approaches to the intervention of osteoporosis have been reported in the literature. Those interventions could be classified as either pharmacological or non-pharmacological based interventions. Non-pharmacological interventions incorporate dietary and lifestyle recommendations that include calcium or vitamin D intake and exercise routines (1). On the other hand, pharmacologic based interventions like antiresorptive agents include the widely-used bisphosphonates class of medications, a class of drugs that reduces bone resorption by increasing the apoptosis of osteoclasts (21). Some of the different bisphosphonates class drugs used for the treatment of osteoporosis are alendronate (22), Risedronate (23), and Ibandronate (24). These drugs have been evaluated for their use in post-

menopausal osteoporosis and reported to be efficient. However, although their efficiency in treatment, their use has been connected to complications such as osteonecrosis of the jaw and atypical femur fractures (25,26). Another class of antiresorptive agents that have been used in osteoporosis treatment includes Estrogens and selective estrogen receptor modulators (SERMs) (27,28). In addition to the reported drawbacks that are linked to bisphosphonates, with hormonal replacement therapy, there have been reports of their association with breast cancer and some cardiovascular events (29,30).

Moreover, an additional pharmacological class of drugs available are the anabolic agents, these act on the enhancement of bone formation rates rather than decreasing the bone resorption rates, an excellent example for anabolic agents is the parathyroid hormone (PTH). It is a well-known potent inducer of bone matrix synthesis, and it increases osteoblast activation while decreasing their apoptosis (31). Its use has been investigated in the treatment of osteoporosis, and its use in both the animal and human models and the animal model shows improvement in the bone mass and strength, those in human trials involving both genders show a pronounced increase in the spinal bone mineral density in contrast to those reported with antiresorptive agents (32). However, a reported setback of PTH includes the fact that an incidence of osteosarcoma was reported when studied in animals for two years period of use in a different variety of doses of PTH. Thus, recommendations have been made against its use for prolonged periods of time (33). Considering these setbacks that might be a limitation to the utilization of these medications, there is a constant need for the development of novel treatment

approaches as further viable options in the management and treatment of osteoporosis. Moreover, with the available current day advancements revolving around cell therapy applications in the field of research and bone diseases, a cell therapy-based approach without adverse effects or detrimental drawbacks would be a promising alternative method to be utilized as a novel treatment in the management and alleviation of osteoporosis and potentially other chronic illnesses.

Cell therapy and stem cells:

The term cell therapy is prescribed by the American Society of Gene & Cell Therapy (ASGCT) as "the administration of live whole cells or maturation of a specific cell population in a patient for the treatment of a disease" (34). Their application is continuously investigated in disease applications and stem cells are believed to be a valuable source in cell therapy of bone diseases due to their ability to directly replace damaged tissues as well as their influence on cellular mechanisms of recovery (35). Mesenchymal stem cells (MSCs) were first reported around the 1960s by A.J. Friedenstein; it was when the observation he made was that bone marrow cells contain a population of cells that were capable of self-maintenance and to differentiate into multiple mesenchymal cell lineages (36). In addition to self-maintenance, these stem cells are cells that are available after birth; they are multipotent, and capable of giving rise to various cell types, including those of mesodermal (i.e., bone or cartilage cells), endodermal (i.e., muscle cells), and ectodermal (i.e., epithelial cells) lineages (Fig.2) (37,38). Cluster

Differentiation molecules (CD) are amongst the methods used in the process of identifying stem cells. Some of the markers that are commonly used to identify MSCs include, CD105, CD73, CD44, CD90, CD71, CD271, and the STRO-1 antibody also recognizes them (38). As for their sources, MSCs have been isolated and mainly named after their origin, they have been harvested from multiple parts in the body, primarily from bone marrow and it is the most common source of MSCs, other sources are the adipose tissues, placenta, umbilical cord, Wharton's jelly, and dental tissues (39). Both MSCs isolated from bone marrow and dental-tissues fit the criteria set by the international society for cellular therapy to define multipotent mesenchymal stem cells (40). Those isolated from bone marrow are reported to be able of giving rise to different cell types such as osteocytes, chondrocytes, adipocytes, hepatocytes, neuronal cells, cardiomyocytes, and pancreatic cells. Additionally, those derived from dental-tissues are reported to have the capacity to differentiate into osteocytes, chondrocytes, adipocytes, pancreatic cells, melanocytes, and neuronal cells (39,41). These multipotential differentiation characteristics made stem cells an attractive treatment option for investigations in disease research fields. The National Institute of Health (NIH) currently reports on clinicaltrials.gov over a thousand clinical trials in different investigation phases ranging from phase I to phase IV investigating and related to MSC cell therapy. Trials reported include researches in the fields of bone defects in different regions, cutaneous wounds, urinary system cartilaginous defects, cardiovascular disorders, autoimmune diseases, liver diseases, and many other chronic illnesses (42).

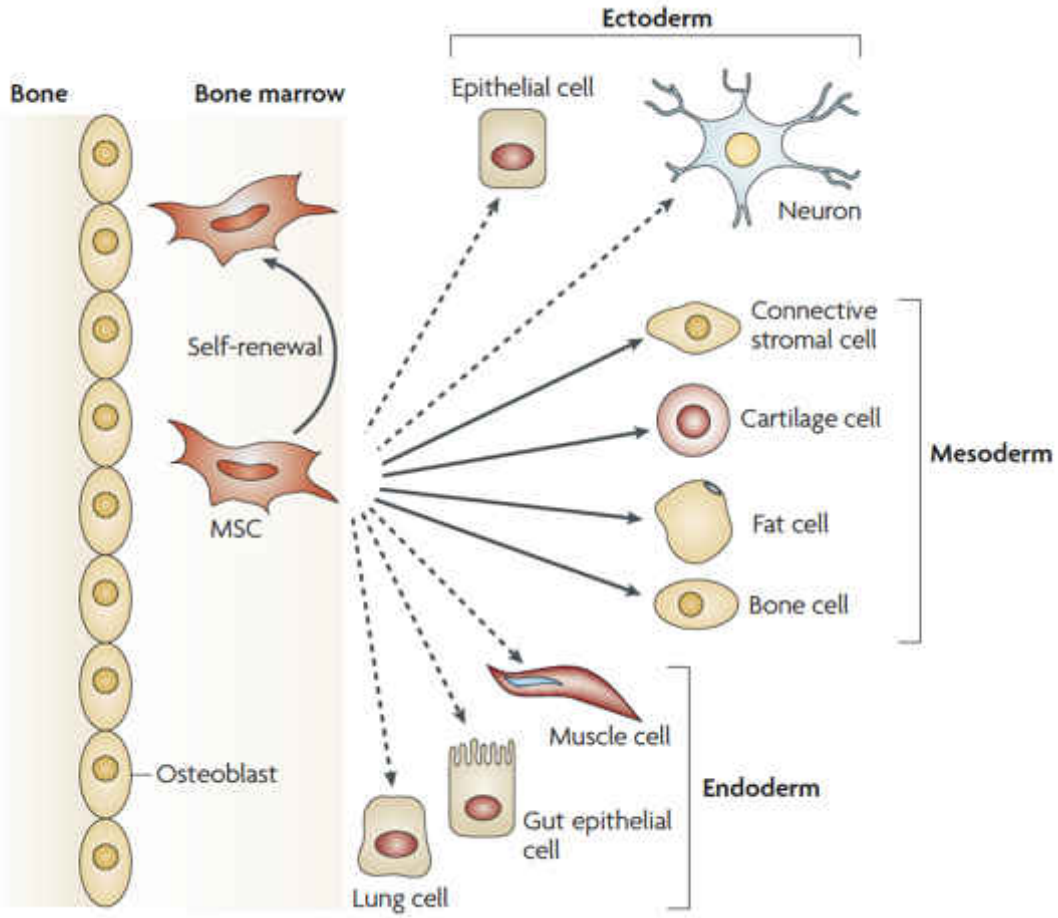


Figure 2. Different potential cell lineages of MSCs. Potential for differentiation of BMMSCs to give rise to different cell types. (Diagram adapted from Uccelli 2008 (38))

Dental tissue derived stem cells:

In addition to the previously mentioned different sources for isolation of stem cells, it is reported in the literature that stem cells derived from the oral cavity include several types of cells including Dental Pulp Stem Cells (DPSCs) (43), Stem Cells from Human Exfoliated Deciduous Teeth (SHED) (44), Periodontal Ligament

Stem Cells (PDLSCs) (45), Stem Cells from Apical Papilla (SCAP) (46), Dental Follicle Precursor Cells (DFPCs) (47), Gingival Mesenchymal Stem Cells (GMSCs) (48), and a more recently reported type that was discovered in 2013 by Marrelli et al and is considered an additional source of stem cells which is human periapical cysts and it was named Human Periapical Cyst-Mesenchymal Stem Cells (hPCy-MSCs) (49).

These dental tissues derived stem cells can give rise to lineages like those of BMMSCs, and they are shown to be neural crest-derived in origin, their application has been investigated in both oral and non-oral applications. Some of their oral applications investigated include bio-root engineering, regeneration of periodontal defects, pulp tissue engineering, and regeneration (41). They have also been evaluated in non-oral cell therapy applications. Some of these regenerative investigations include using scaffolds seeded with DPSCs to accelerate defect healing and bone regeneration in calvaria of rats. (50), DPSCs were also reported to differentiate into corneal epithelial progenitors when seeded in contact lenses and delivered to human corneas indicating their potential application in eye diseases (51).

Moreover, SHED's application was investigated in Systemic Lupus Erythematosus (SLE) mouse model and showed a successful reversal of the disorders associated with the disease as well as elevating the levels of T-regulatory cells (52). SHED's application was also investigated in multiple disease models, including Parkinson's rat models, Liver disease mice models, as well as

Diabetes mellitus mice models, and researchers report promising outcomes (53-55).

In the field of bone disease research, amongst the multiple animal models available for the investigation of osteoporosis, a popular well-established model is the ovariectomy model (OVX). It is known to be a reliable model for research. The ovaries are surgically removed, which induces estrogen deficiency, and the animal loses 50% of their cancellous bone because of the procedure resembling an osteoporotic phenotype, one other model available is the glucocorticoid treated model where the animal is treated with glucocorticoids for seven days to induce bone loss. Investigators have widely utilized These types of small animals in the research of bone diseases (56). Up to date, the use of stem cells has been investigated for osteoporosis treatment from multiple sources, such as umbilical cord blood-derived stem cells, bone marrow mesenchymal stem cells, and adipose tissues derived stem cells (57). Several investigations utilized the use of BMMSC in the treatment of osteoporosis by using the OVX animal model as well. Hsiao et al. reported the use of systemic injection of labeled mice bone marrow MSCs and that they have been shown to home the bone marrow of the host two months post-injection and improve the bone density and volume in the OVX mouse model (58). Liu et al. also reported the use of human BMMSCs in the OVX mouse model and showed that single transplantation of stem cells successfully prevented the early phases of bone loss as well as significantly downregulated the levels of Th1, T17 and upregulated T-reg cells (59). These results verify the familiar role of BMMSCS in immunomodulation and their ability to suppress the proliferation of T-cells (60).

In addition to that, the ability of BMMSCs to inhibit proinflammatory cytokines such as TNF-α and IFN-γ and increase the expression of anti-inflammatory cytokines such as IL-10 has also been reported (61). These characteristics of BMMSCs capability of improving the osteoporotic bone phenotype of OVX animals made them an attractive tool for further investigation and development in cell therapy. However, although BMMSCs are the most commonly researched source of stem cells, the morbidity associated with their collection is high, and the accessibility to them and their harvesting procedure is of a complex nature, as well as the number of cells able to be collected from the tissues is considered low (62). These reasons, amongst others, led investigators to explore further and research for alternative sources to use in cell therapy disease research.

Gingival mesenchymal stem cells:

On the other hand, Gingival Mesenchymal Stem Cells (GMSCs) are also dental tissues derived stem cells isolated from the gingiva, and they have been characterized by self-renewal properties, multipotent differentiation abilities, and immunomodulatory effects and their availability is of abundance as they are easily obtained from donor gingival tissue (48,63,64). Their application has been investigated in different experimental disease models. Some of their uses include their application in the experimental colitis model, as reported by Zhang et al. In an induced dextran sulfate sodium (DSS) model of colitis representing an inflammatory bowel disease (IBD). A single infusion of GMSCs ameliorated the disease, lowered the rate of T-helper lymphocytes recruitment, increased the

levels of T-regulatory lymphocytes. Additionally, in chemotherapy-induced oral mucositis model, alleviation of the disease was evident by the reversal of body weight loss and the restoration of the broken down epithelial lining (65). In another disease model, a wound healing model that utilizes excisional cutaneous wounds was studied, accelerated wound closure was achieved, also a reduction in the local inflammatory cells and pro-inflammatory cytokines (64). GMSCs were also reported to successfully regenerated boney defects in the Mandible and Calvaria of rats by their direct participation in bone formation, and the recruitment of bone progenitor cells (66). The hallmark of stem cells from of gingiva in different disease models is that those Gingival Stem Cells achieved promising outcomes mainly through the ability to decrease the levels of pro-inflammatory cytokines, and T-helper lymphocytes as well as the increase of T-regulatory lymphocytes (48,64). And, even though their use has been evaluated in the utilization of the mentioned above disease models, the therapeutic effect of their use as a potential approach for the amelioration and improvement of boney effects of osteoporosis has not been assessed in the Ovariectomy model.

In this dissertation, we hypothesized that the systemic infusion of GMSCs would improve the osteoporotic bone phenotype of the estrogen deficiency related inflammatory mouse model, rescue the overall functions of the endogenous populations of BMMSCs of the study animals, and that the GMSCs transplantation would exert an immunoregulatory effect on T-cells and would induce immunological tolerance leading to the improvement of the bone phenotype, we also hypothesized the involvement of a cell death pathway as mechanism of action

behind the GMSCs-mediated influence on T-cells. To test our hypotheses and explore and understand the effects of the GMSCs treatment on the osteoporotic mouse model, we proposed three specific aims as elaborated next.

Specific Aims

1- **Specific Aim #1:** To investigate whether stem cell-based therapy using a single systemic transplantation of GMSC can improve the bone phenotype in the estrogen deficiency, ovariectomy (OVX)-induced osteoporosis mouse model.

2- Specific Aim #2: To explore the therapeutic effects of GMSCs in the OVX mouse model and to test the ability of GMSCs to rescue the biological functions of the defective endogenous BMMSC populations in the osteoporotic OVX mice.

3- Specific Aim #3: To delineate the mechanisms underlying the rescue of bone phenotype in osteoporotic OVX mice, specifically the immunomodulatory effect of GMSCs.

Materials and Methods

Animals and the induction of estrogen-deficiency related osteoporosis:

The Ovariectomized mouse model is a well-established model for bone disease research representing estrogen deficiency-related osteoporosis as a result of the loss of ovaries. Bone disease research utilizes C3H/HeJ mice due to their increase trabeculation in the bone. All animal experiments described in this dissertation were performed under institutionally approved protocols for the use of animal research under protocol #805478 approved by the University of Pennsylvania Institutional Animal Care and Use Committee (ICAUC). In order to explore the effect of GMSCs on the bone phenotype of ovariectomized mice, 8-week-old female C3H/HeJ mice were purchased from Jackson Labs (Bar Harbor, ME, USA). Mice were then divided into three age-matched groups as illustrated in (Fig.3)

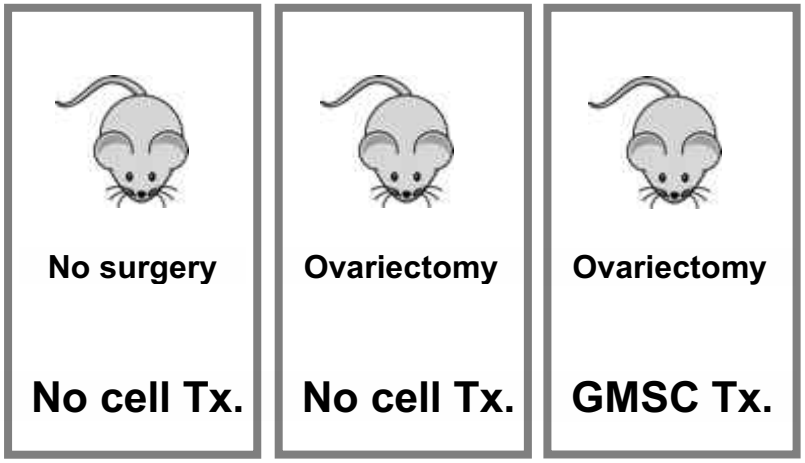


Figure 3. Study groups. Illustration of study groups assignment.

First group was the negative control group where the mice were not subjected to any surgical procedure nor treatment serving as a negative control. The second group was the OVX group, this group of mice received surgical ovariectomy procedure of both ovaries but did not receive any cellular infusion serving as the positive control group. The third group was the GMSC- treatment group, these mice received surgical ovariectomy of both ovaries then a single infusion of GMSCs as outlined in the following experimental timeline described in (Fig 4). The GMSC-treated mice group was systemically infused with 2×10^5 mouse GMSCs.

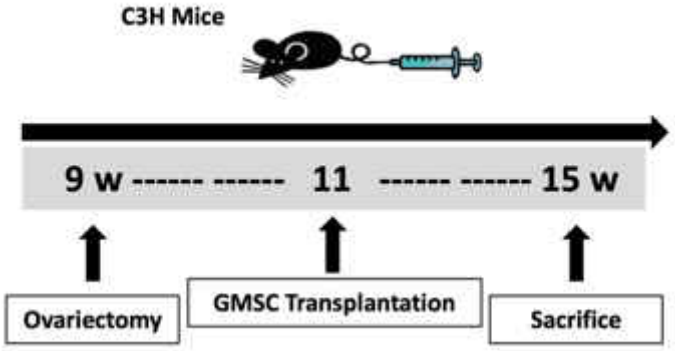


Figure. 4 Experimental Timeline. Ovariectomy procedure done at 9 weeks to designated groups, GMSCs were injected into designated group intravenously via the tail vein.

The ovariectomy procedure was performed on C3H/HeJ mice using an electrocautery machine for maximum hemostasis achievement. A Two-weeks period was allowed for the OVX phenotype to be properly established; all mice were sacrificed at 4 weeks post-injection for subsequent examination. The sample size of the animals used in the experiments were at least an n = 5 in each group.

Additional *Prkdc*^{scid} mice (females, six weeks old) were purchased from Jackson Labs (Bar Harbor, ME, USA) for the experiment of testing BMMSC transplantation into immunocompromised mice.

For testing of drug Atezolizumab, 8-week-old female C3H/HeJ mice were purchased from Jackson Labs (Bar Harbor, ME, USA). After dividing mice to two groups of n=5. The ovariectomy procedure was performed mice also using an electrocautery machine. A Two-weeks period was allowed for the OVX phenotype to be properly established, after two weeks the injection of GMSCs was infused into both groups, The drug group further received a systemic injection of Atezolizumab anti-PDL1 drug obtained from Invivogen USA (#hpdl1-mab9) 80mcg of drug was administered, and the placebo received PBS. Two weeks after the drug group had an additional 40mcg of drug administered to insure the maintenance of the effect. all mice were sacrificed at 4 weeks post-injection for subsequent examination.

Isolation and preparation of mouse GMSCs:

Adult mice were used to harvest gingival tissue. A single suspension of gingival tissue was processed for all nuclear cells (ANCs, 15×10^6) from mice gingiva was seeded in 10 cm culture dishes (Genesee) then incubated at 37 ℃ with 5% CO_2. After 48 hours, non-adherent cells were removed, and adherent cells were cultured for additional 14 days in alpha minimum essential medium (α-MEM, Invitrogen)

supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine (Invitrogen), 55 μM 2-mercaptoethanol (Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Passage one and two mouse GMSCs were used in for cell infusion. GMSCs were tested for common MSC markers CD90 CD105, and SCA-1 as shown in Fig.5. Cells were prepared for infusion and ($2X10^5$) cells were prepared in sterile 200 μl phosphorus buffered saline (PBS) for each mouse and injected as an infusion systemically via the tail vein.

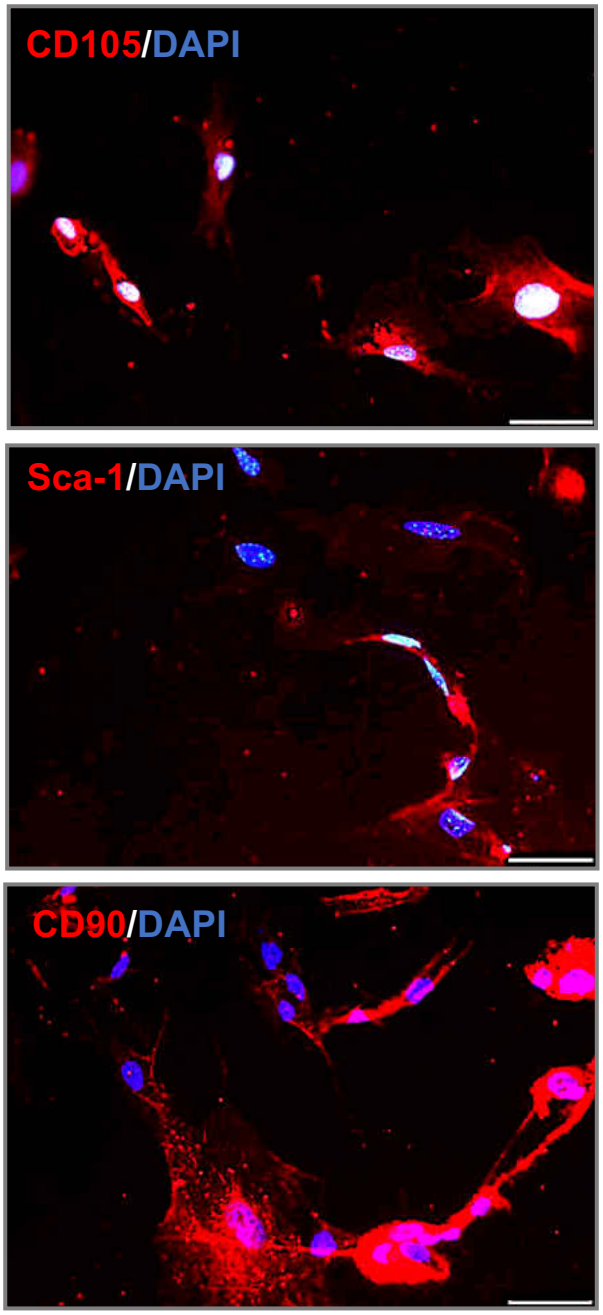


Figure 5. GMSC's expression of common MSC markers. Immunostaining images showing the expression of common MSC markers CD90, CD105, and SCA-1 by GMSCs, these markers are amongst methods used to identify Mesenchymal stem cells. Scale bar, 50 μm.

Computed Microtomography and analysis of datasets:

To analyze the Bone Mineral Density (BMD) and Total Bone Volume (BV/TV). The femurs and mandibles of mice were harvested at sacrifice then fixed in 4% paraformaldehyde solution, femurs were then scanned using a desktop high-resolution Scanco μCT35 scanner (Scanco Medical AG, Bruttisellen, Switzerland). The scanning of specimens was standardized to a measurement voxel size of 20 μm at 70kVp and 200 μA. Datasets were reconstructed, and images were analyzed using the system-provided by the manufacturer (Scanco Medical). Values were obtained for BMD, and total bone volume.

Histological and histomorphometry analysis:

Femurs and mandibles were fixed in 4% paraformaldehyde for 48 hours then decalcified with 10% EDTA (pH 7.4) for three weeks undergoing a twice-weekly change of EDTA to maintain the exposure of femurs to 7.4 pH levels. Following decalcification, paraffin embedding was completed, then ten μm thick sections were produced using a microtome and prepared for staining. The first stain selected for both mandibles and femurs, was the Hematoxylin and Eosin (H&E)

stain in order to quantify the trabecular bone percentage in the distal metaphyseal region of the femurs, as well as the retromolar area distal to M3 in the mandibles. Results are reported in the form of trabecular bone percentage in relation to the total standardized area of interest (%/area). Further testing for femurs alone includes Tartrate-resistant acid phosphate (TRAP) staining assay to label TRAP+ cells. TRAP staining kit was purchased from (Sigma-Aldrich), and samples were prepared by deparaffination then stained according to manufacturer instructions. TRAP staining images were also acquired from the distal metaphyseal region of the femur. The results are shown as the number of osteoclasts per square millimeter of bone surface area (N.Oc/BS). For dynamic bone histomorphometry, double calcein labeling was utilized, the mice were intraperitoneally injected with calcein (Sigma, 15 mg/Kg body weight) which was prepared in 2% sodium bicarbonate solution at two intervals 13 and 3 days before sacrifice. After sacrifice, femurs were fixed n 4% paraformaldehyde for 48 hours then decalcified with 10% EDTA (pH 7.4) for a period of two weeks and samples were prepared for cryo-sectioning and slides were created and mounted with fluoroshield mounting medium with DAPI (Ab 104139) and pictures were obtained for the bone dynamic histomorphometry analyses for MAR. Mineral apposition rate is the distance measured between the two labels divided by the time between the two IP injections.

In vivo Oil red O staining / Fatty marrow analysis:

For the analysis of adipocytes surrounding the trabecular areas of the metaphyseal region of femurs, they were fixed in 4% paraformaldehyde and decalcified with 10% EDTA (pH 7.4) for a period of two weeks. After that, femurs were prepared for cryo-sectioning by processing the samples in 15% sucrose in 3 hours, then 30% sucrose overnight. The following day, samples were embedded in 1:1 OCT and 30% Sucrose for 3 hours, then moved and embedded into OCT solution and stored in -20 C. Freezing microtome machine was then used to create 12 Micrometer sections. The staining of sections was then completed using the Oil Red-O solution. Positive areas were quantified under the microscope and illustrated as a percentage of the total area.

Enzyme Linked Immunosorbent Assay (ELISA):

In order to determine the blood serum levels of the different markers, IFN-γ, IL-17, ALP, and RANKL, peripheral blood was collected at sacrifice, and the serum was extracted from blood samples by letting the blood samples sit for one hour then undergoing the centrifugation process to complete the serum extraction. Samples were then stored in -20 °C until testing was performed. For ELISA testing, mouse ELISA MAX™ Deluxe kits for IFN-γ, IL-17, and RANKL were purchased from (BioLegend), ALP was purchased from G-biosciences (IT5507), and ELISA testing was performed according to the manufacturer's instructions for each assay. Results are shown as levels of markers present in serum in picogram/milliliter.

Isolation and culture of mouse BMMSCs.

Harvesting of single suspension of bone marrow-derived all nuclear cells (ANCs) from femurs was completed after sacrifice, and 15×106 cells were seeded in 10 cm culture dishes (Genesee) then incubated at 37℃ with 5% CO2. After 48 hours, dishes were washed with PBS to remove any non-adherent cells. Adherent cells were cultured for additional 14 days in alpha minimum essential medium (α-MEM, Invitrogen) supplemented with 20% fetal bovine serum (FBS), two mM L-glutamine (Invitrogen), 55 μM 2-mercaptoethanol (Invitrogen), 100 U/ml penicillin with 100 μg/ml streptomycin (Invitrogen). Cells were incubated until experiments were performed using Passage one and two mouse BMMSCs.

Transplantation of BMMSCs into immunocompromised mice.

After the sacrifice of mice, around 4.0×106 mouse BMMSCs from OVX, GMSC-treated, and control mice were incorporated with 40 mg of hydroxyapatite/tricalcium phosphate (HA/TCP) powder (Zimmer Inc., Warsaw, IN, USA) and subsequently implanted subcutaneously into the dorsal surfaces of eight-week-old female immunocompromised Prkdcscid mice. The implants were implanted for a period of twelve-weeks, then mice were sacrificed, and transplants were harvested. After that, they were fixed in 4% paraformaldehyde then decalcified with 10% EDTA (pH 8.0) for two weeks in preparation for paraffin embedding. Ten uM paraffin sections of the transplants were then created using a microtome, then deparaffinized, rehydrated, and stained with the hematoxylin and

eosin (H&E) stain. Results are shown as a proportion of the bone formed to the total area (Bone/total area).

Proliferation capacity analysis / BrdU labeling assay.

BMMSCs were seeded into 8-well chamber slides (Thermo Scientific) with a concentration of 2×104 per well. After two days of cell culture, A concentration of (1:100) BrdU labeling reagent (Invitrogen) was added to the medium, after which the cells were incubated at 37℃ with 5% CO2 for 24 hours. Cells were then fixed with 70% Ethanol, denatured with 2N HCl, and stained with anti-BrdU antibody (Invitrogen) overnight at 4℃. The second antibody was incubated at room temperature for 1 hour, then mounted and counterstained with fluoroshield mounting medium with DAPI (Ab 104139). The positive cells from four fields per sample were quantified under microscopy and presented relative to the total number of cells. (positive/total cells).

***In-vitro* osteogenic differentiation capacity analysis.**

The culture of BMMSCs was done under osteogenic inductive conditions with a twice/week change of the medium. The growth medium composed of 2 mM β-glycerophosphate (Sigma-Aldrich), 100 μM L-ascorbic acid 2-phosphate (Wako), and 10 nM dexamethasone (Sigma-Aldrich). After four weeks of osteogenic

induction, matrix mineralization was detected by 1% Alizarin Red (Sigma-Aldrich) staining or cells of other wells were lysed for protein isolation and examination of osteogenic gene expression. For Mineralized nodule formation, stained positive areas were quantified using NIH ImageJ software and shown as a percentage of the total area (%/Total area).

***In-vitro* adipogenic differentiation capacity analysis.**

BMMSCs were cultured under adipogenic inductive conditions. The adipogenic medium contained 500 nM isobutyl methylxanthine (Sigma-Aldrich), 60 µM indomethacin (Sigma-Aldrich), 500 nM hydrocortisone (Sigma-Aldrich), 10 µg/ml insulin (Sigma-Aldrich), and 100 nM L-ascorbic acid phosphate. Eight days after adipogenic induction, wells were stained with Oil-red-O stain (Sigma-Aldrich) for quantification of adipocytes, and positive cells were counted under a microscope and shown as a percentage of the total cells (%/total number of cells). Additional good sets were also lysed for protein collection in order to examine adipogenic gene expressions.

Western immunoblotting.

After cells are washed using PBS and lysed in the RIPA lysis buffer system with protease and phosphatase inhibitors (Santa Cruz). Protein levels were then quantified using PierceTM BCA Protein Assay Kit (Thermo scientific). 20 µg of

proteins were separated by SDS-PAGE (Invitrogen) and transferred to 0.2 μm nitrocellulose membranes (Millipore). Then the membranes were blocked using a 5% non-fat dry milk and 0.1% Tween-20 for a period of one hour, followed by overnight incubation with primary antibodies diluted in blocking solution. Antibody to PPARγ (sc-7196) was purchased from Santa Cruz Biotechnology, Inc. Antibody to LPL (PA5-47033) was purchased from Thermo Fisher Scientific. Antibodies to RUNX2 (12556) were obtained from Cell Signaling Technology. Antibodies to ALP (ab108337) was purchased from Abcam, and β-Actin (A5441) were purchased from Sigma-Aldrich. The membranes were then washed and incubated for one hour in HRP-conjugated secondary antibody (Santa Cruz) diluted in blocking solution. Immunoreactive proteins were detected using SuperSignal™ West Pico PLUS Chemiluminescent Substrate, SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo) and Autoradiography Film (Labscientific, inc).

Flow Cytometry.

After mice were sacrificed, T-cells were collected from spleens. T cells were extracted by processing spleens and treating them with ACK buffer for red blood cells removal (Lonza, Switzerland), after that isolated T-cells were incubated for an hour on ice with 1ug/100ul of PerCP anti-mouse CD4 Antibody (100538, BioLegend), then for the T-reg testing, an additional 2 ug of APC anti-mouse CD25 (102012, BioLegend) was added as well. FoxP3 staining buffer kit was used to Fix and permeabilize the samples overnight using Intracellular Staining

Permeabilization Wash Buffer (BioLegend). Cells were then stained with 2ug of either PE anti-mouse FoxP3 (320008, BioLegend) for T-regs, or with APC anti-mouse IFN-γ for Th1 (505810, BioLegend) for 30 minutes in the dark at room temperature Then washing with FACS buffer was carried out. After that, all samples were analyzed using FACSCalibur with CellQuest software (BD Bioscience).

Immunofluorescent microscopy.

To test the GMSCs expression of MSC markers CD90, CD 105, and SCA-1. The anti-mouse CD-90 antibody was obtained from Abcam (ab3105), anti-mouse CD 105 antibody was obtained from BD Biosciences (550546), and anti-mouse SCA-1 antibody was also obtained from BD Biosciences (553334). For immunostaining of PD-L1 expression. PD-L1 antibody (#MA5-29672) was obtained from ThermoFisher Scientific. The samples were then incubated with the obtained specific or isotype-matched first antibodies (1: 200) overnight at 4 °C, and then the next day stained with the second antibody according to the manufacturer's instructions. Slides were mounted using Fluoroshield Mounting Medium with DAPI (Abcam). Pictures are shown for illustration purposes.

siRNA transfection.

For mechanistic testing, an additional treatment group of OVX mice treated with PDL-1 knockdown GMSCs was assigned and In vitro siRNA was performed, GMSCs (0.8×106) were seeded in 60 mm culture dishes and treated with Pdcd-1L1 siRNA (SC-39700, Santa Cruz) or vehicle siRNA control (Santa Cruz) with lipofectamine reagent (Invitrogen), according to the manufacturers' instructions. After successful transfection was achieved, and the protein expression level is verified with western immunoblotting, siPdl1 cells were collected and used for infusion into the siPdl1 mice group.

T-cell apoptosis assay

GMSCs or siPdl1 GMSCs (0.2X106) were seeded in a 24-well culture plate (Corning) containing Dulbecco's Modified Eagle's Medium (DMEM; Lonza, Basel, Switzerland) with 10% heat-inactivated FBS, 50 μM 2-mercaptoethanol, 10 mM HEPES, 1 mM sodium pyruvate (Sigma-Aldrich), 1% non-essential amino acid (Cambrex, East Rutherford, NY, USA), 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. After incubation of GMSCs for 24 h, Spleens from wildtype were harvested and processed ACK buffer for red blood cell removal (Lonza, Switzerland) and extraction of T-cells. After isolation of T-cells, (1X106) T-lymphocytes were pre-stimulated by seeding them in anti-mouse-CD3ε (100331, BioLegend) antibody with a concentration of 5 μg/mL and soluble anti-mouse-CD28 (122004, BioLegend) antibody with a concentration of 2 μg/mL. After 2-3

days of T-Cell activation and confirming aggregation and growing was achieved. T-Cells were then directly loaded onto WT GMSCs or siPDL1 GMSCs and co-cultured for three additional days. Additional wells for control contained plated T-cells only in medium with no cells. After that, apoptotic T-cells were detected by staining with a CD3 antibody, followed by the use of the Annexin-V Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) and then samples were run and analyzed by the use of a FACSCalibur flow cytometer equipped with CellQuest software.

Statistics:

All the data we have shown were expressed as the mean ± SEM. Statistics were performed using the software SPSS V.26. (IBM corporation. Armonk, New York) Comparisons between two groups were analyzed using the independent two-tailed Student's t-tests. As for the comparison between > two groups were analyzed in IBM SPSS V.26 using one-way ANOVA/Fisher LSD. P-values less than 0.05 were considered statistically significant.

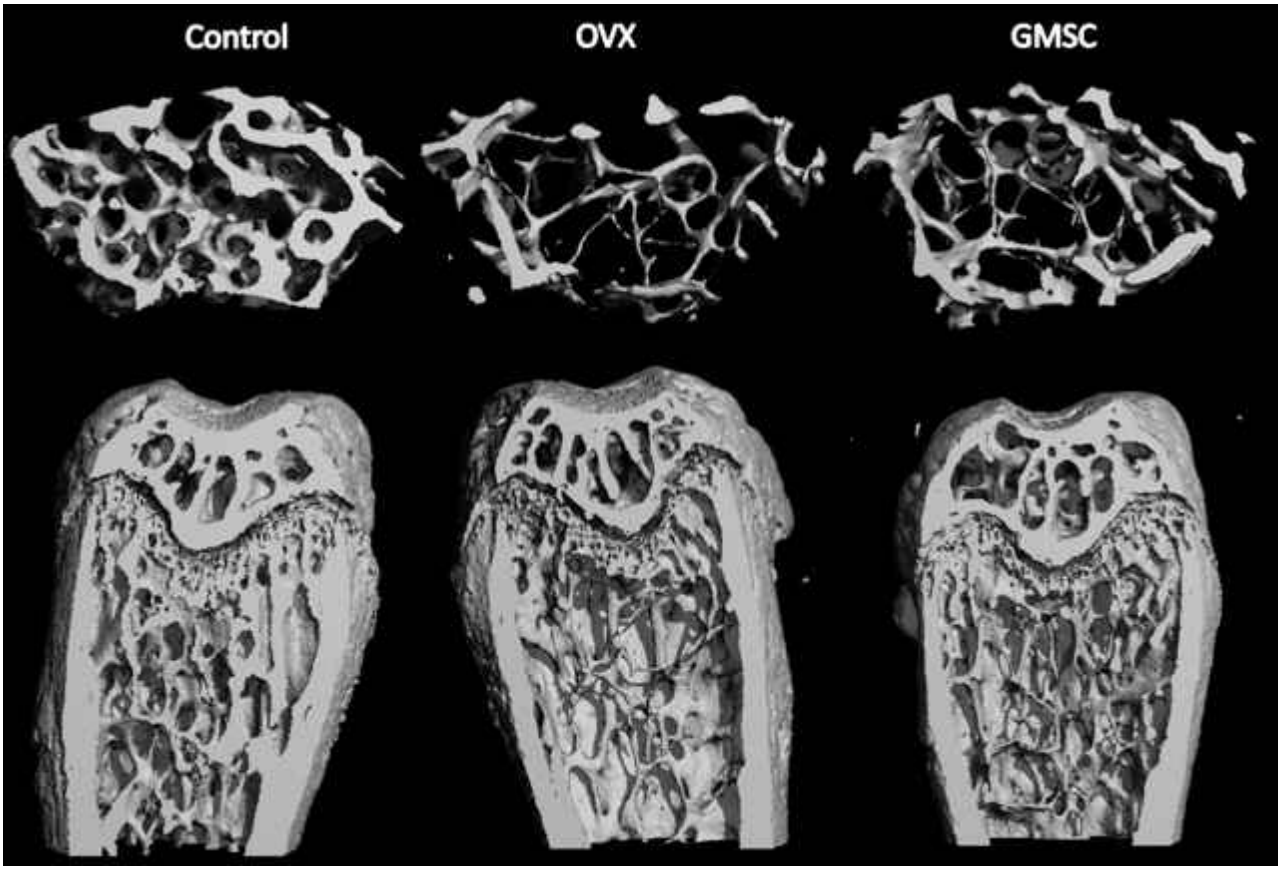
Results

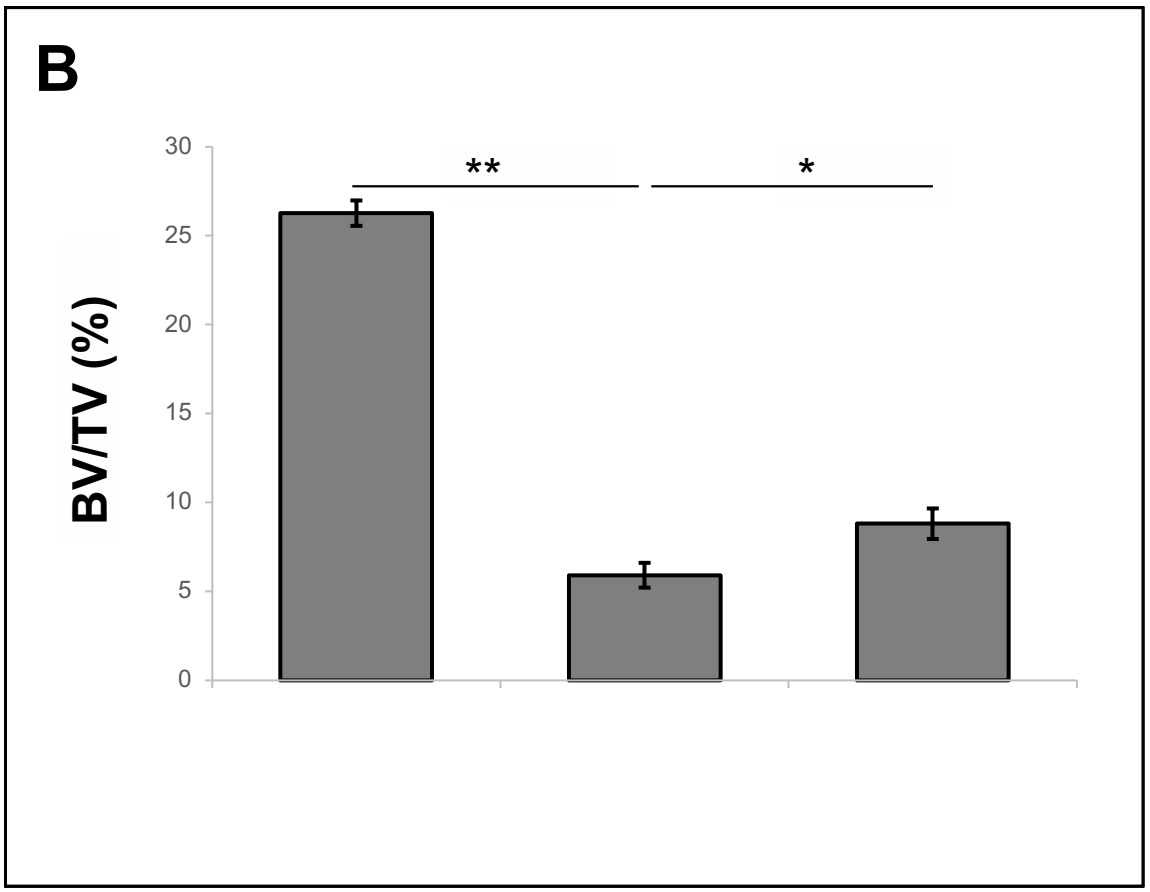
A single infusion of GMSCs successfully improved the bone phenotype of the ovariectomized mouse model

To explore the therapeutic effects of a GMSC transplantation on the OVX mouse, an OVX disease model for treatment was established. The timeline of the treatment consisted of obtaining 8-week-old mice from Jackson labs, the mice would be then assigned to 3 different groups. After allowing mice to acclimate into their groups for a week. An ovariectomy procedure would be completed to 2/3 groups, the OVX group and the GMSC-treatment group. Mice would then be allowed a period of two weeks for the osteoporosis associated with ovariectomy to develop. After two weeks the GMSC treatment group would receive a single GMSC transplantation. After 4 weeks of the transplantation, all mice would be sacrificed for experimentation purposes. The computed microtomography analysis of mice femurs (Fig.6) reveled a significant deterioration in the bone phenotype of the OVX mice in comparison to the negative control group as shown by a decrease in the measurements of bone mineral density (BMD), total bone volume (BV/TV), Connectivity density (Conn.D) as well as an increase in Structure Model Index (SMI) values. This reported effect validates the OVX osteoporosis model and provides additional assurance of the validity and the effect of ovaries removal on bone. On the other hand, our group of interest, the GMSC-treatment group showed

that a one-time GMSC transplantation intravenously produced a marked improvement in the bone phenotype of mice femurs as seen by the elevation in the bone BMD, total bone volume, Conn.D values, as well as the decrease in SMI values.

A




B
BV/TV (%)
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25
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10
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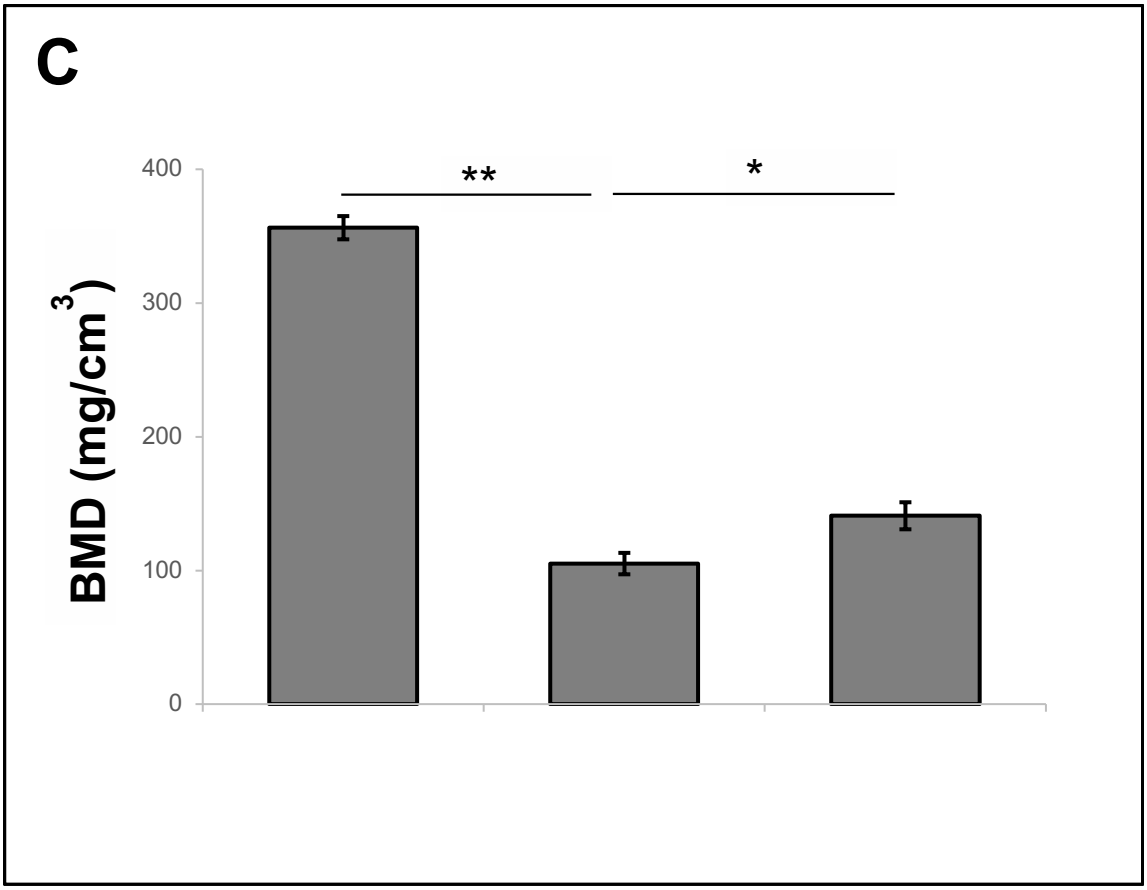
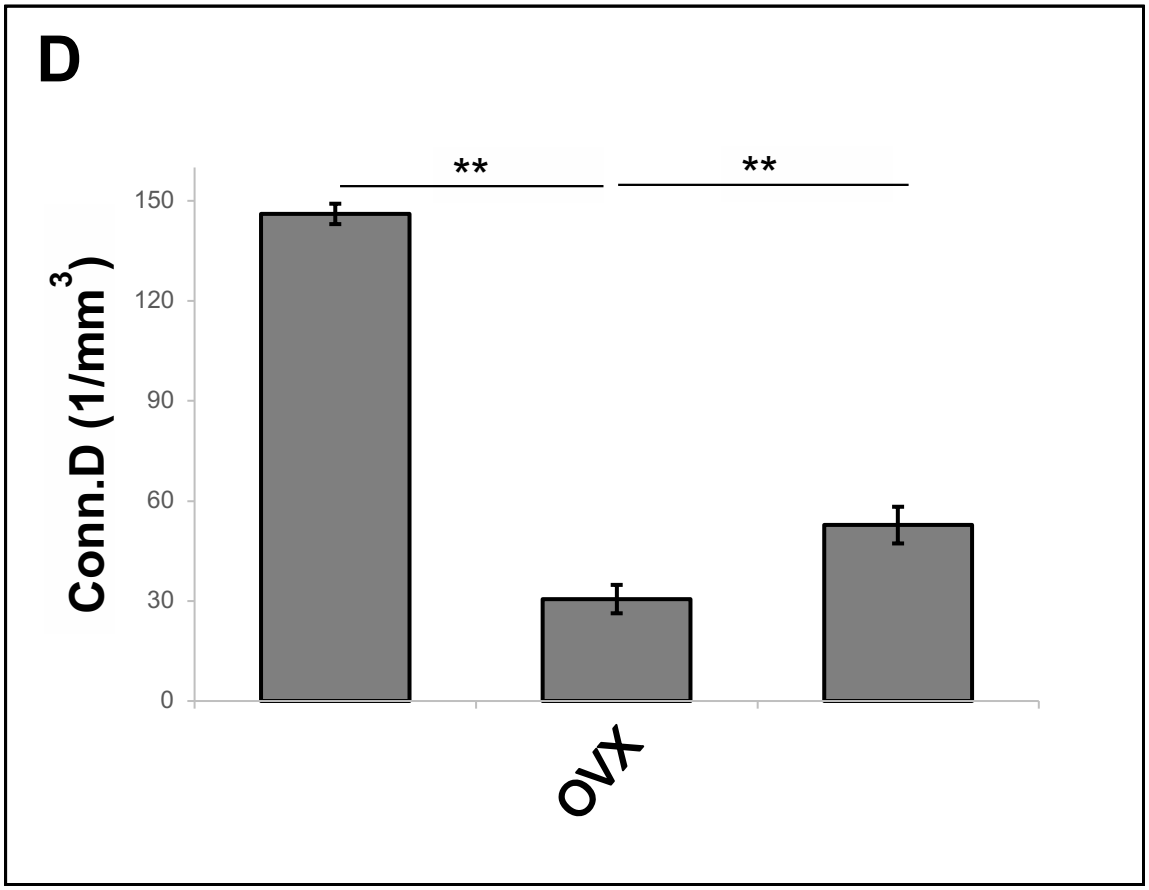

C
BMD (mg/cm3)
400
300
200
100
0
**
*


D
**
**
150
120
90
60
30
0
Conn.D (1/mm3)
OVX

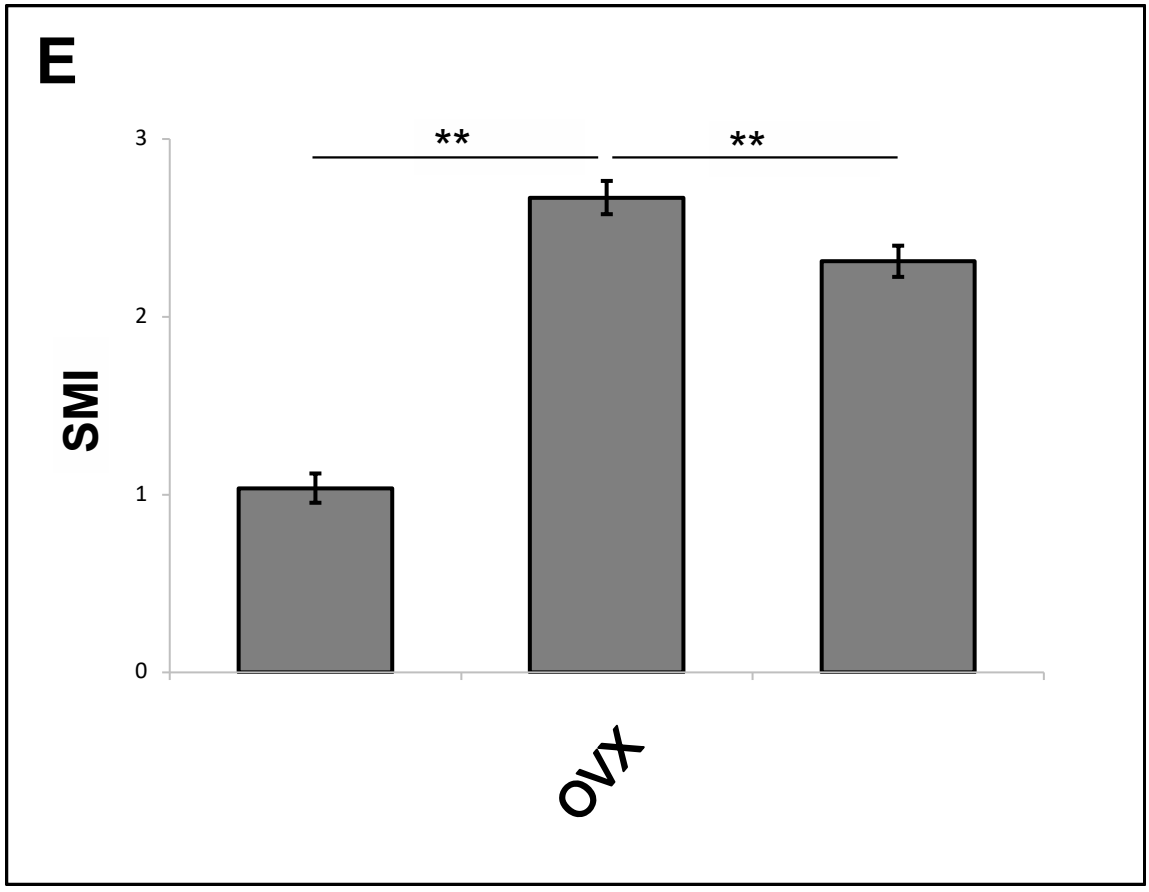

E
**
**
3
2
1
0
SMI
OVX

Figure 6. Computed microtomography analysis of femurs. (A) Images from the scanned femurs showing difference in trabeculation between different groups. **(B)** Bone mineral density measurements revealing improved bone phenotype in GMSC-treatment group in comparison with OVX group. **(C)** Total bone volume measurement also showing an improvement in the GMSC-treatment group when compared with OVX. **(D)** Conn.D measurement values of different groups. **(E)** SMI measurements of study groups.

To further investigate the effects of ovariectomy and GMSC treatment of the bone phenotype in the femurs. Histological analysis of the femurs was completed to analyze the trabeculation levels of distal metaphyseal regions. Our H&E analysis indicated a similar outcome and mirrored the finding of computed microtomography of significant improvement in the distal femur trabeculation which is seen the GMSC group when compared with the OVX group (Fig.7).

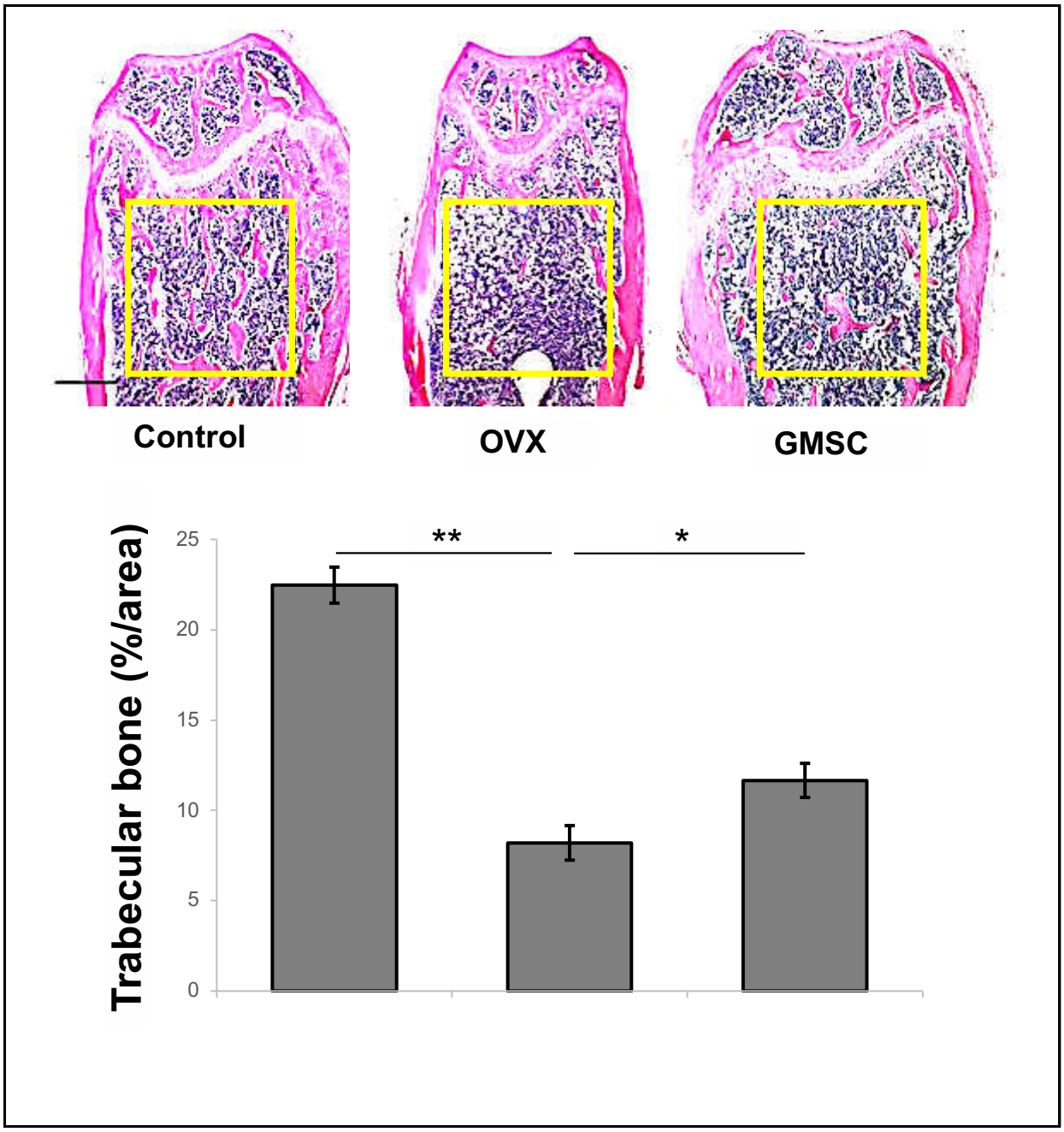
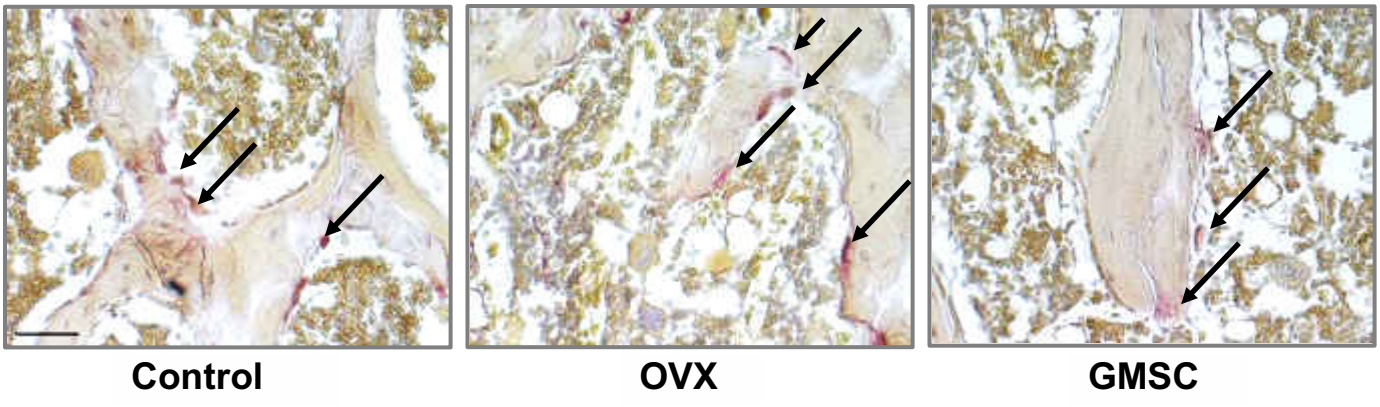


Figure 7. Histological analysis of femurs. H&E staining of distal femoral metaphyseal region showing less trabeculation levels of the ovariectomized group in comparison to both the control and GMSCs-treated group. Scale bar, 500 μm.

To test whether the osteoclastic activity would be evaluated in bone of those mice, we further examined the effect of GMSCs transplantation on the number of osteoclasts in femurs collected from study groups, we found that the number of osteoclasts is markedly reduced in the control group when compared to the ovariectomized mice, this reduction of osteoclast number is also seen after the GMSCs transplantation, this was tested through the observed decrease in number of Tartrate-Resistant Acid Phosphatase positive cells (TRAP) (Fig.8).



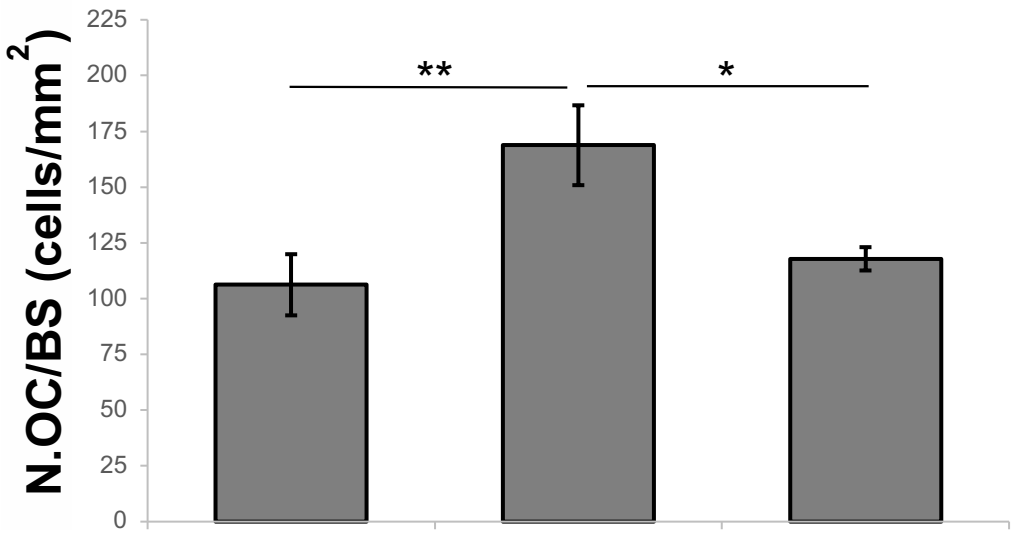


Figure 8. Trap$^+$ cells analysis of femurs. Trap$^+$ cells staining shows a significant increase in Trap$^+$ cells in the ovariectomized mice group in comparison to both the control and GMSCs-treated groups. Scale bar, 100 μm

To further test whether there was fatty marrow presence in femurs, Oil red O staining was utilized for this test. Our results reveal the presence of higher Fatty marrow levels in the femurs of the OVX group in comparison with the control group. The GMSCs-treated group femurs show a significantly reduced fatty marrow area in comparison to OVX group (Fig.9). We then moved to measure the bone formation rate in femurs by performing a calcein double labeling assay aimed at determining the daily rate of bone formation, we found that the control group had the highest bone formation rate as indicated by the double labeling of calcein, the OVX group on the other hand showed the lowest mineral apposition and GMSCs-treated group showed a significantly higher level of MAR than OVX group (Fig.10).

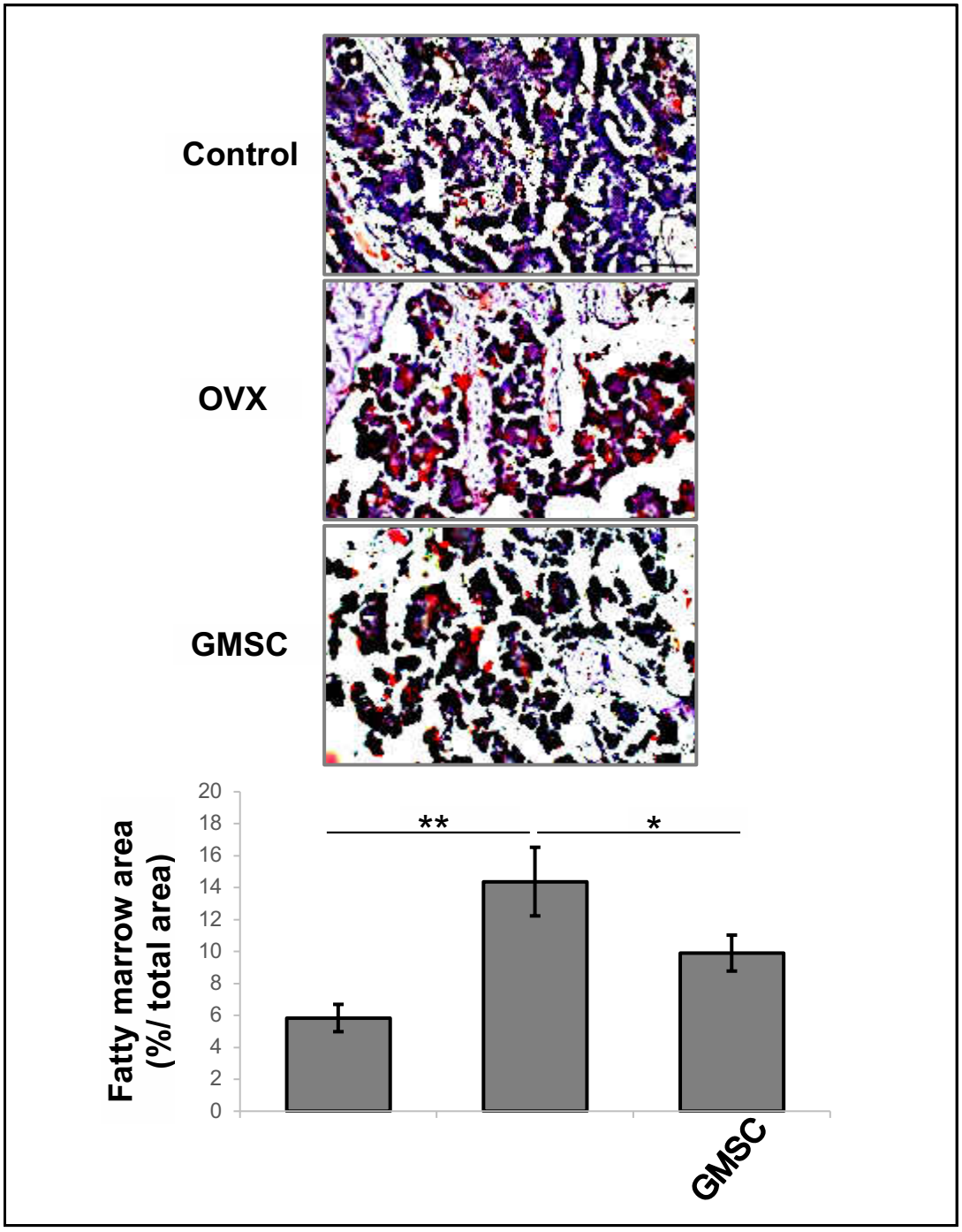


Figure 9. Oil-Red-O staining/in-vivo analysis of fatty marrow. Fatty marrow levels as tested by Oil-Red-O staining show an increase in the levels in the OVX group when compared with the control group. The fatty marrow level is also reduced in the GMSCs-treated group when compared to the OVX group. Scale bar, 100 μm.

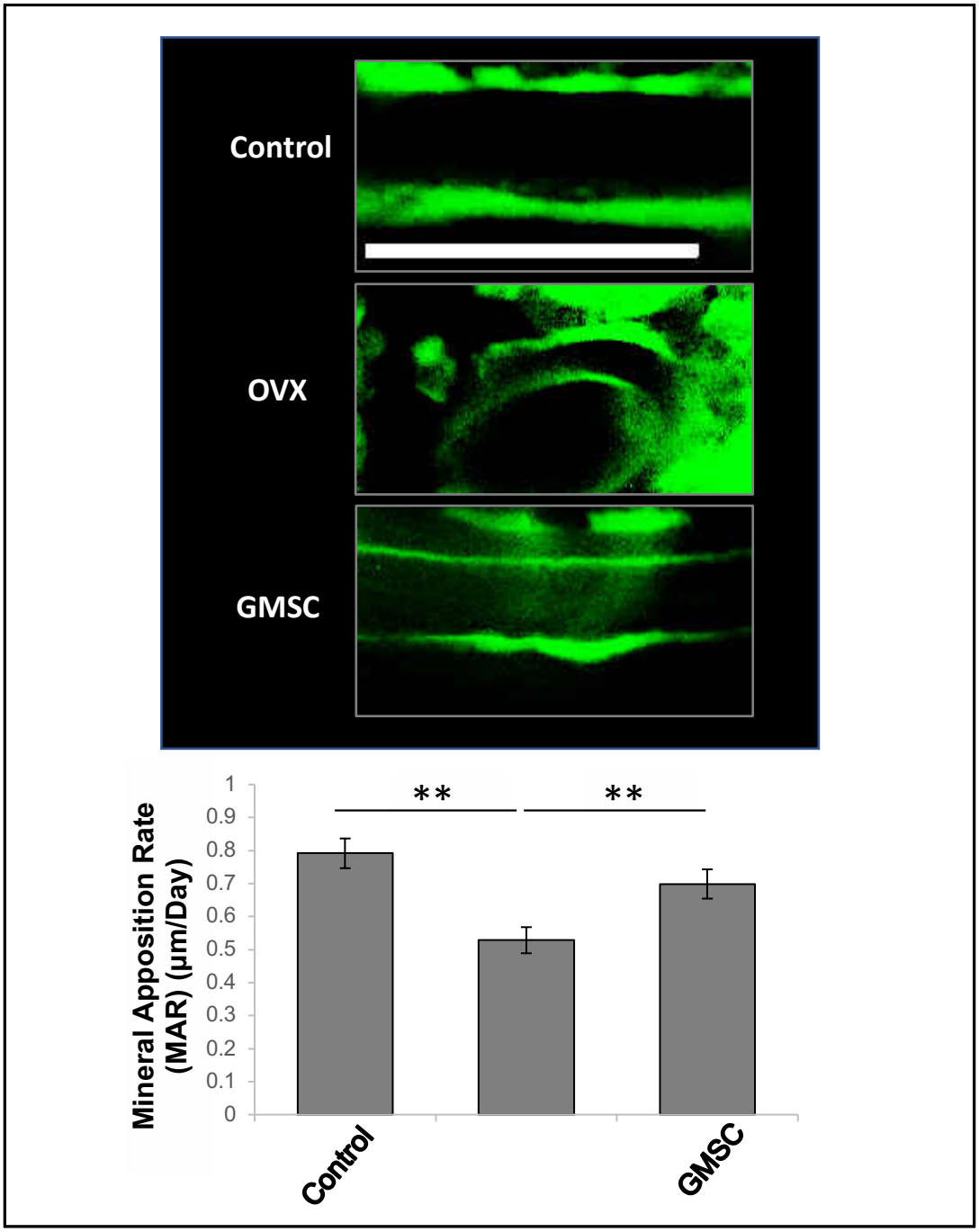
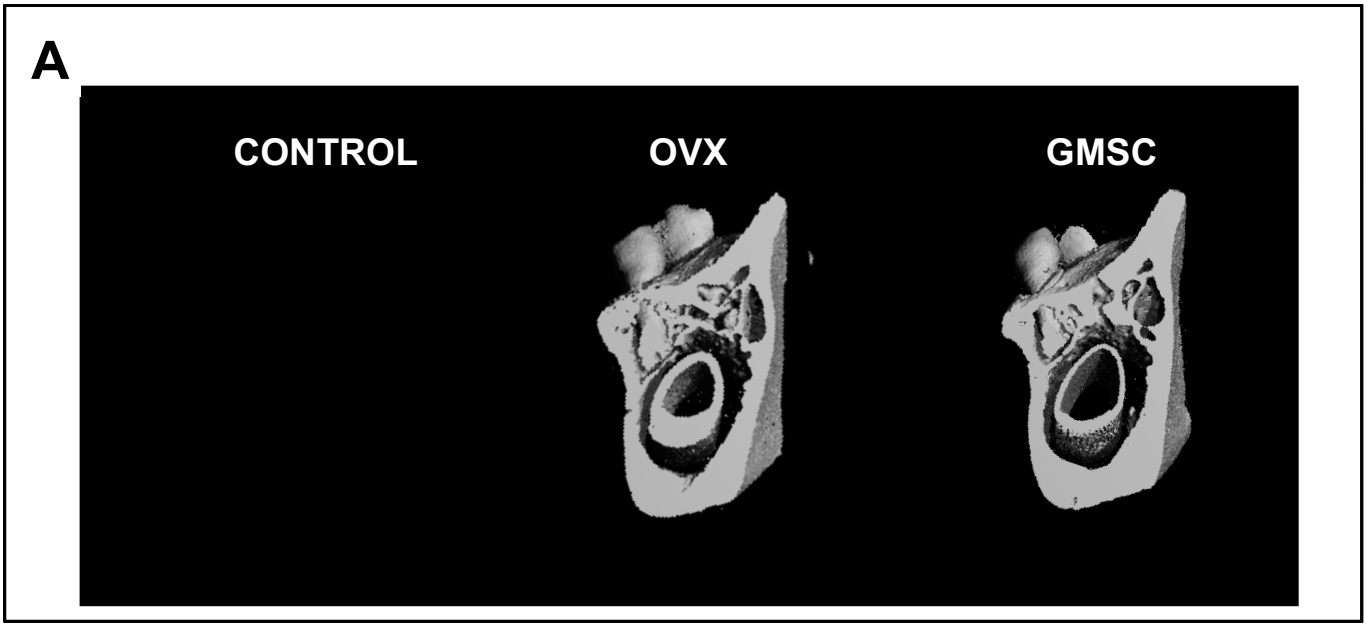


Figure 10. Dynamic bone histomorphometry. The mineral apposition rate was tested by calcein double labeling showing an increased MAR in control and GMSC-treatment groups when compared with the OVX group. Scale bar, 50 µm.

After testing the effect of transplantation on the femurs of mice, we next explored the effect of OVX and GMSCs transplant in the mandibles of mice. The computed microtomography scans and analysis of the retromolar area distal to M3 in mice mandibles reveled a significant decrease in the bone phenotype of the OVX mice in comparison to the control group as shown by a decrease in the measurements of BMD and BV/TV mirroring the effect seen in femurs. The GMSC-treatment group as well showed a marked improvement in the bone phenotype of the mice mandibular jawbone when compared to the OVX group (Fig.11 A-C). This is further confirmed by H&E stain and the analysis of the trabeculation levels in retromolar area of mandibles showing the same trend as the one seen in the computed microtomography (Fig.12).



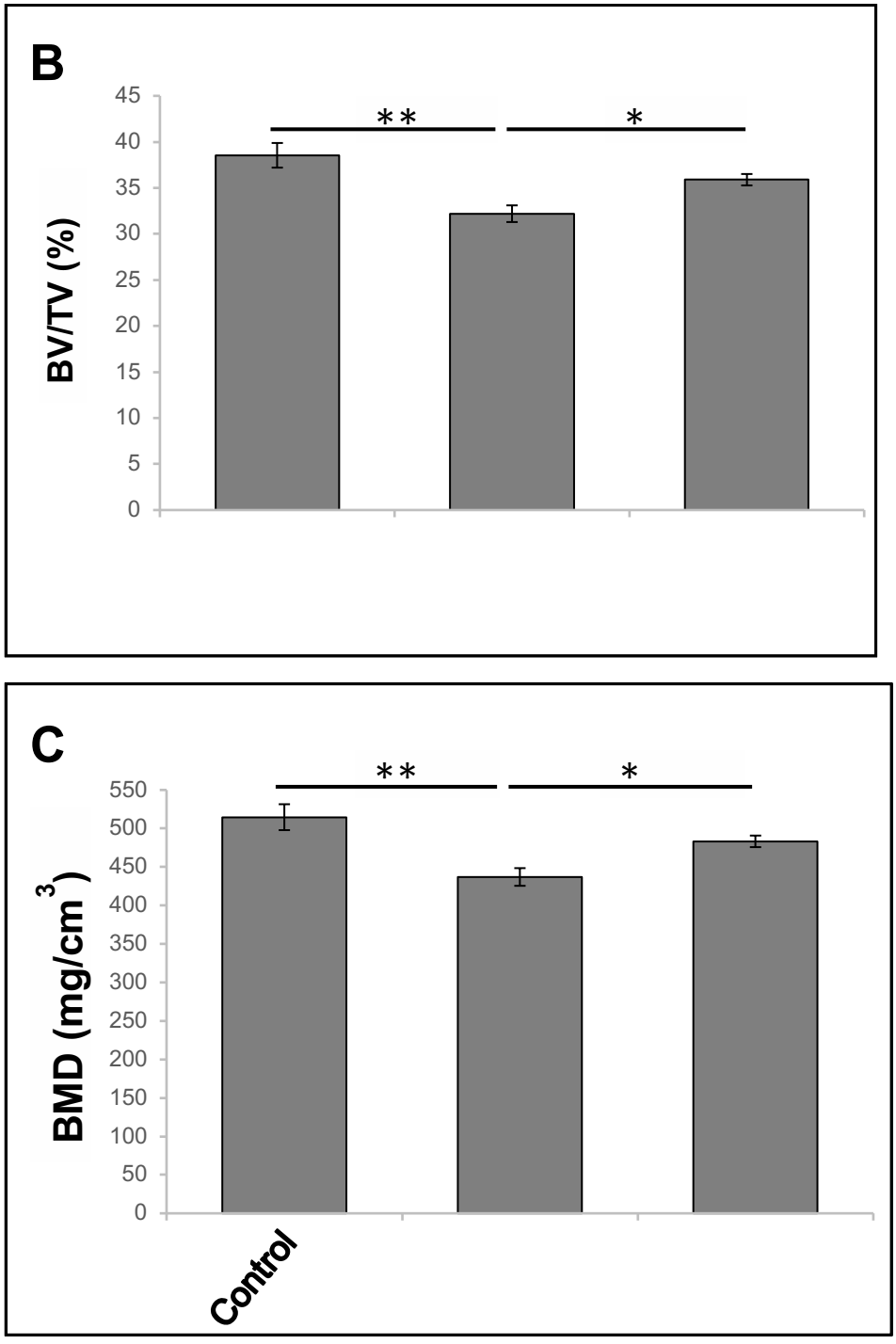


Figure 11. Computed microtomography analysis of mandibles. (A) Images from the scanned mandibles showing difference in trabeculation between different groups. **(B)** Bone mineral density measurements revealing improved bone

phenotype reflected in GMSC-treatment group mandibles in comparison with OVX group. **(C)** Total bone volume measurement also shows an improvement in the GMSC-treatment group when compared with OVX.

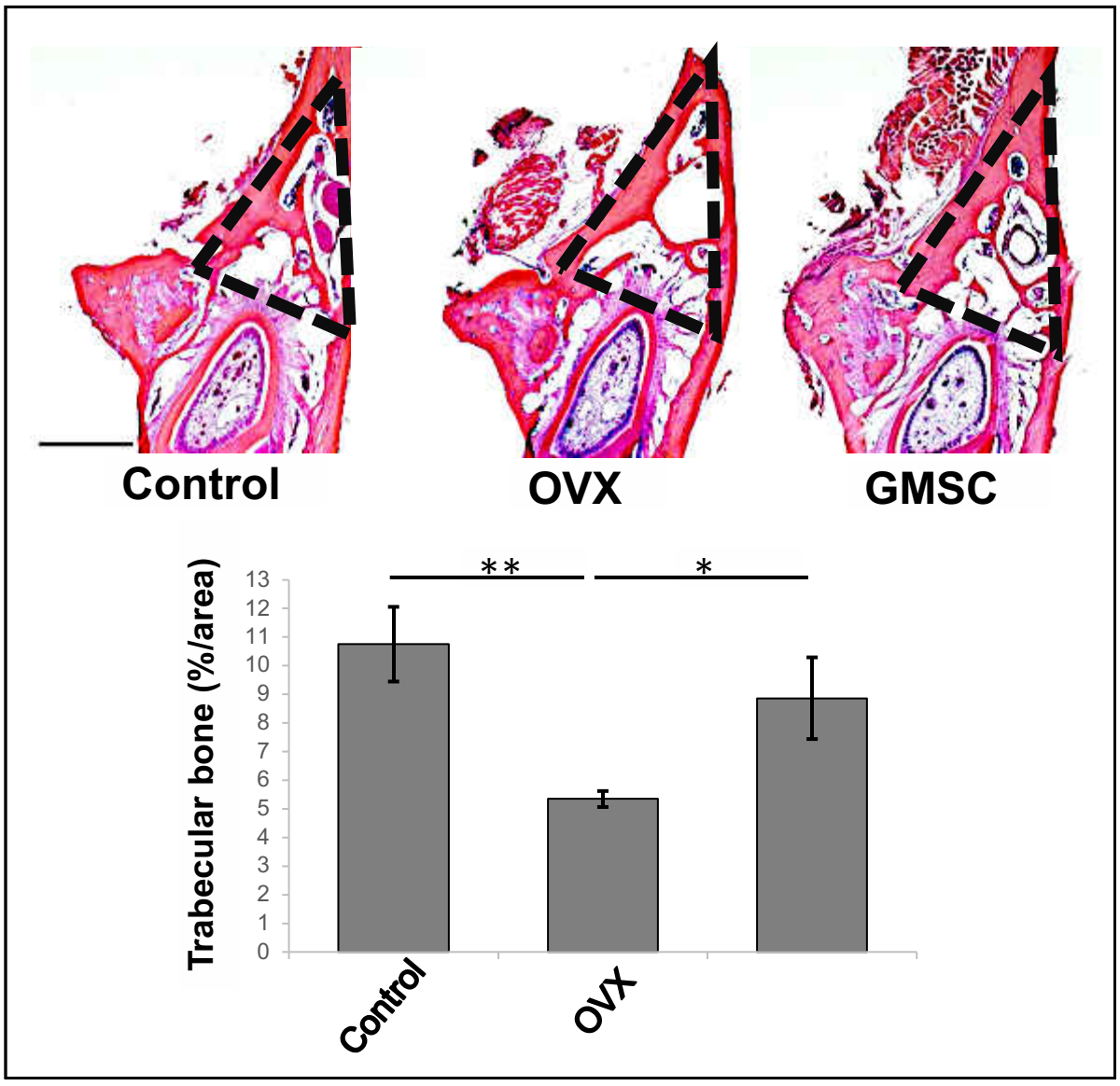
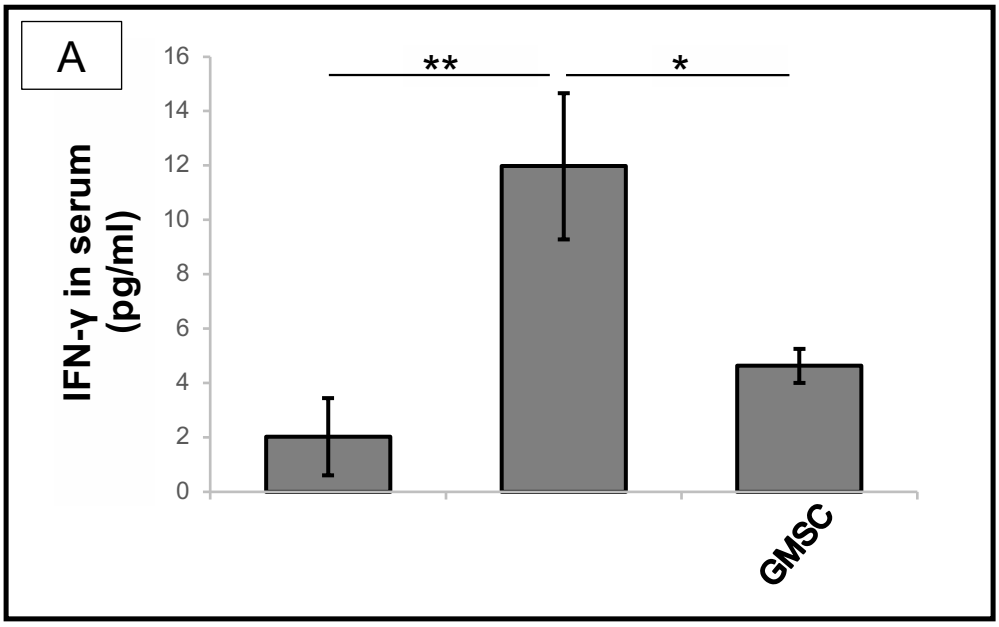
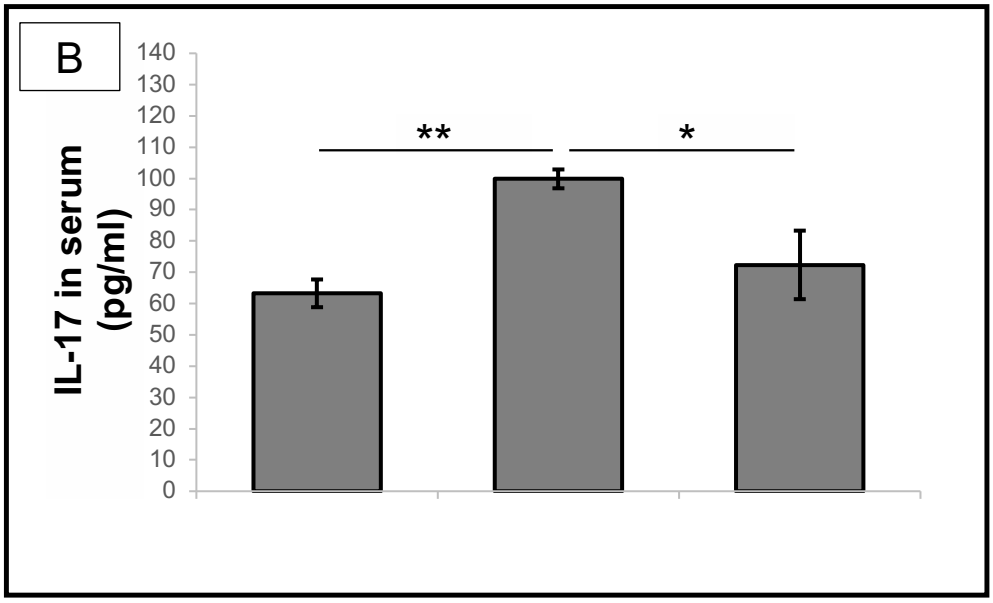


Figure 12. Histological analysis of mandibles. H&E staining of retromolar area of M3 showing less trabeculation levels of the ovariectomized group in comparison to both the control and GMSCs-treated group. Scale bar, 500 μm.

We next explored the effect of the transplantation on blood serum levels of different inflammatory and osteoclast markers. We saw that levels of Serum IFN- γ, and IL-17 is markedly decreased in the GMSC treated group compared to the OVX group which is in turn significantly higher in expression when compared to the control group mice (Fig 13.A, B). Osteoclast marker RANKL was also significantly decreased in the GMSC treated group (Fig 13.C). These Elisa results suggest that GMSC infusion successfully reduced the inflammation in the ovariectomized mice and downregulated the osteoclastic activity. Additionally, Serum ALP levels were measured in the blood of mice after sacrifice and show a similar trend of a marked increase in serum ALP in those mice of the OVX group when compared to the control group, the level of ALP was seen less in the GMSCs-treated group than OVX but this decrease is not of significance (Fig 13.D).




B
IL-17 in serum (pg/ml)
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130
120
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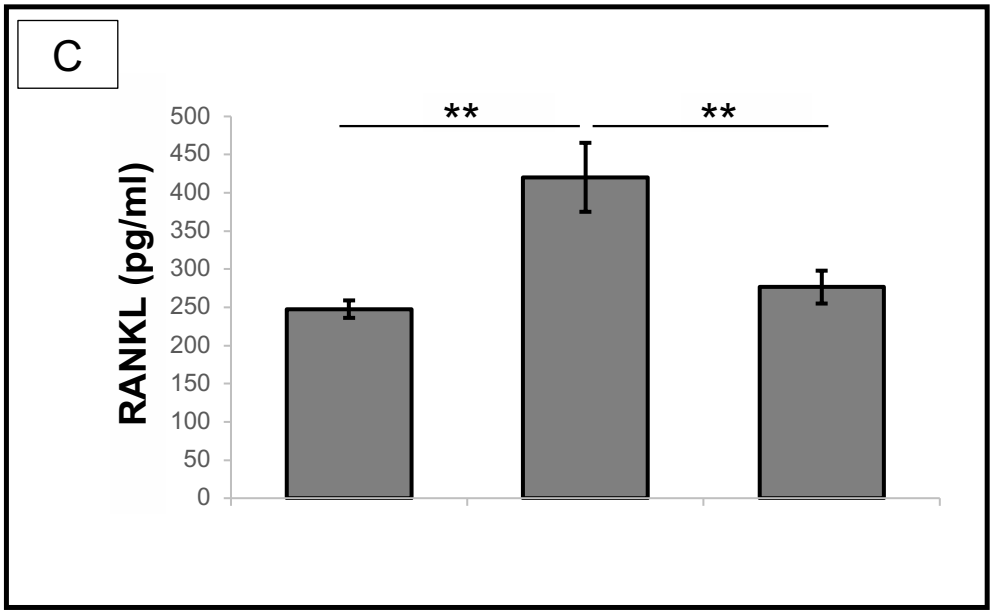
100
90
80
70
60
50
40
30
20
10
0
**
*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RANKL (pg/ml)
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400
350
300
250
200
150
100
50
0
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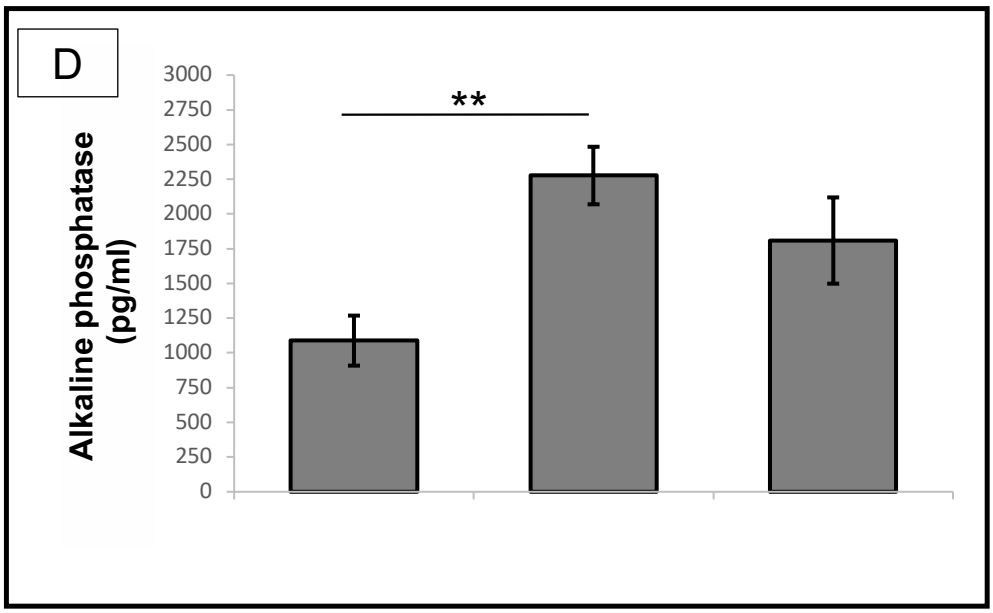

D
Alkaline phosphatase (pg/ml)
3000
2750
2500
2250
2000
1750
1500
1250
1000
750
500
250
0
**

Figure 13. Blood serum markers analysis. (A). IFN-γ levels were overly expressed in the serum of OVX mice as seen by the Elisa testing, the control group possessed the lowest levels of IFN-γ expression, and GMSCs treatment significantly reduced the expression versus the OVX group**. (B). IL-17** level was expressed at about 100pg/ml in the ovariectomized mice blood serum, this was significantly less in the control group and the GMSCs treatment significantly reduced the expression level compared to OVX group. **(C).** RANKL blood serum levels in the OVX mice was almost double the level found in the control mice blood, and the GMSCs treatment reduced the blood serum level of RANKL. **(D).** ALP levels in blood serum tested showing a significant increase of ALP expression in serum of OVX mice when compared with the control mice, ALP is mildly decreased in GMSCs treated mice when compared to OVX mice.

GMSCs transplantation into OVX mice rescued the function of the endogenous population of BMMSCs.

After addressing our first aim of the study where It was hypothesized that the ovariectomy would lead to significant bone loss and that the single transplantation of GMSCs would improve that osteoporotic bone phenotype. Next we hypothesized that the GMSCs transplantation into the OVX mice would improve the function of the endogenous population of BMMSCs. This part of the study aimed at examining the effects of OVX on the BMMSCs of mice and how GMSCs transplantation influences that effect. To run functionality measuring in-vitro experiments, the collection of mice BMMSCs from the three different groups was

completed after sacrifice at 15 weeks. Several experiments were performed in order to form a generalized idea about the functional capacity of BMMSCs and what would a transplantation achieve. This included analyzing the ability of harvested cells to form Colony forming unit-Fibroblasts (CFU-F) by performing a CFU-F assay, measure the ability of the cells to proliferate utilizing a cell proliferation assay, testing their adipogenic differentiation capacity with the measurement of their expression levels of adipogenic proteins Lipoprotein Lipase (LPL) Peroxisome proliferator-activated receptor gamma (PPAR-γ), testing of their osteogenic differentiation capacity with the measurement of their expression levels of osteogenic proteins Runt-related transcription factor 2 (RUNX2), Alkaline Phosphatase (ALP), and finally by further testing the ability of BMMSCs harvested from mice to form new bone by transplanting them into immunocpromised mice after being mixed with particulate ceramic scaffold.

First test after harvesting the BMMSCs from mice, a simple experiment was completed to determine the ability of the cells harvested to form colonies. A CFU-F assay was completed to examine the formation of colonies. While there is no significant difference observed in the abilities of different groups, the BMMSCs harvested from the OVX group showed the highest number of colonies formed, and while the negative control group showed the lowest ability to form colonies, the GMSCs group lies in between both groups in terms of ability to form colonies as shown in (Fig.14).

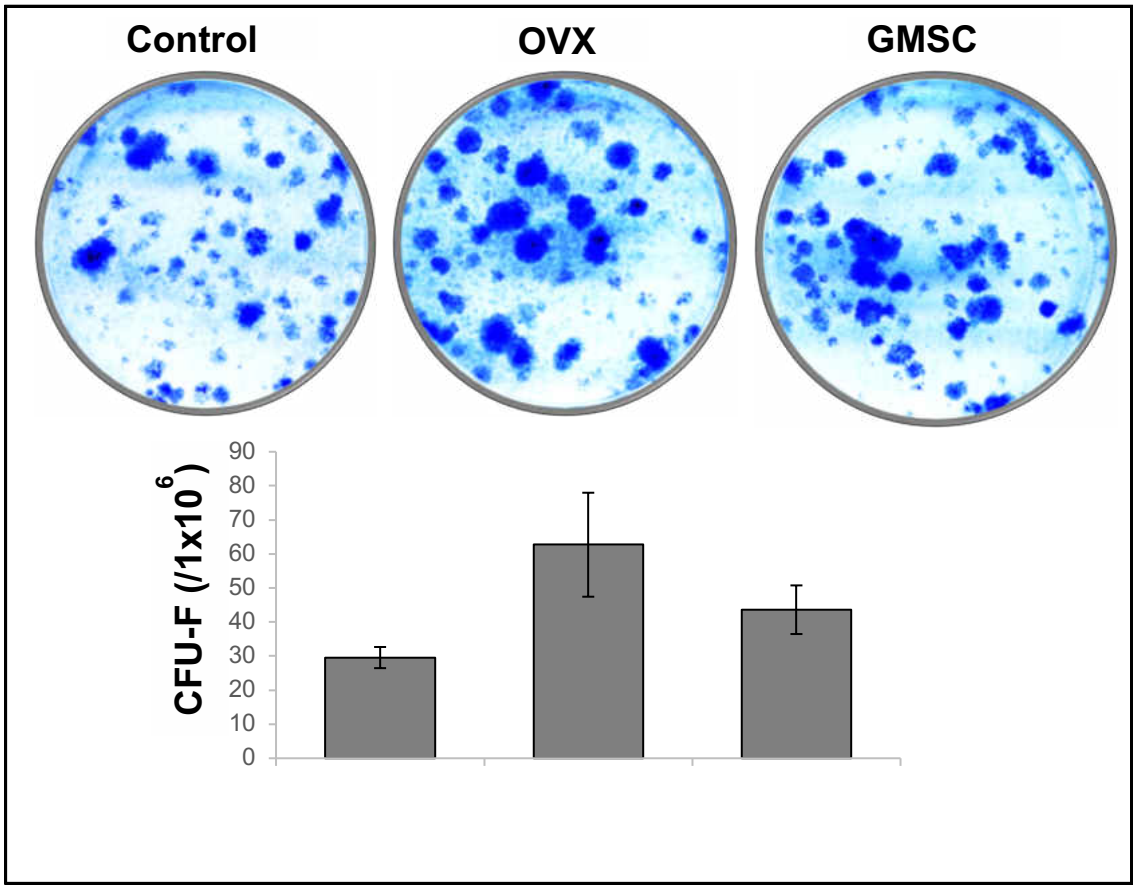


Figure 14. CFU-F Assay. Analysis of colony formation abilities of cells from different groups shows OVX possess the highest capacity of CFU-F.

After verifying the ability and measuring the capacity of BMMSCs to form CFU-F's as do MSCs. we examined the proliferation capacity of mouse BMMSCs trying to measure their division and doubling abilities. We saw that cells from the OVX group had a significantly higher proliferation rate as shown by an increase in BRDU-positive-cells, this elevation in proliferation capacity is not seen in the

GMSC-treated group as it was of reduced proliferation compared to OVX and at an almost comparable rate to those of the control group (Fig 15).

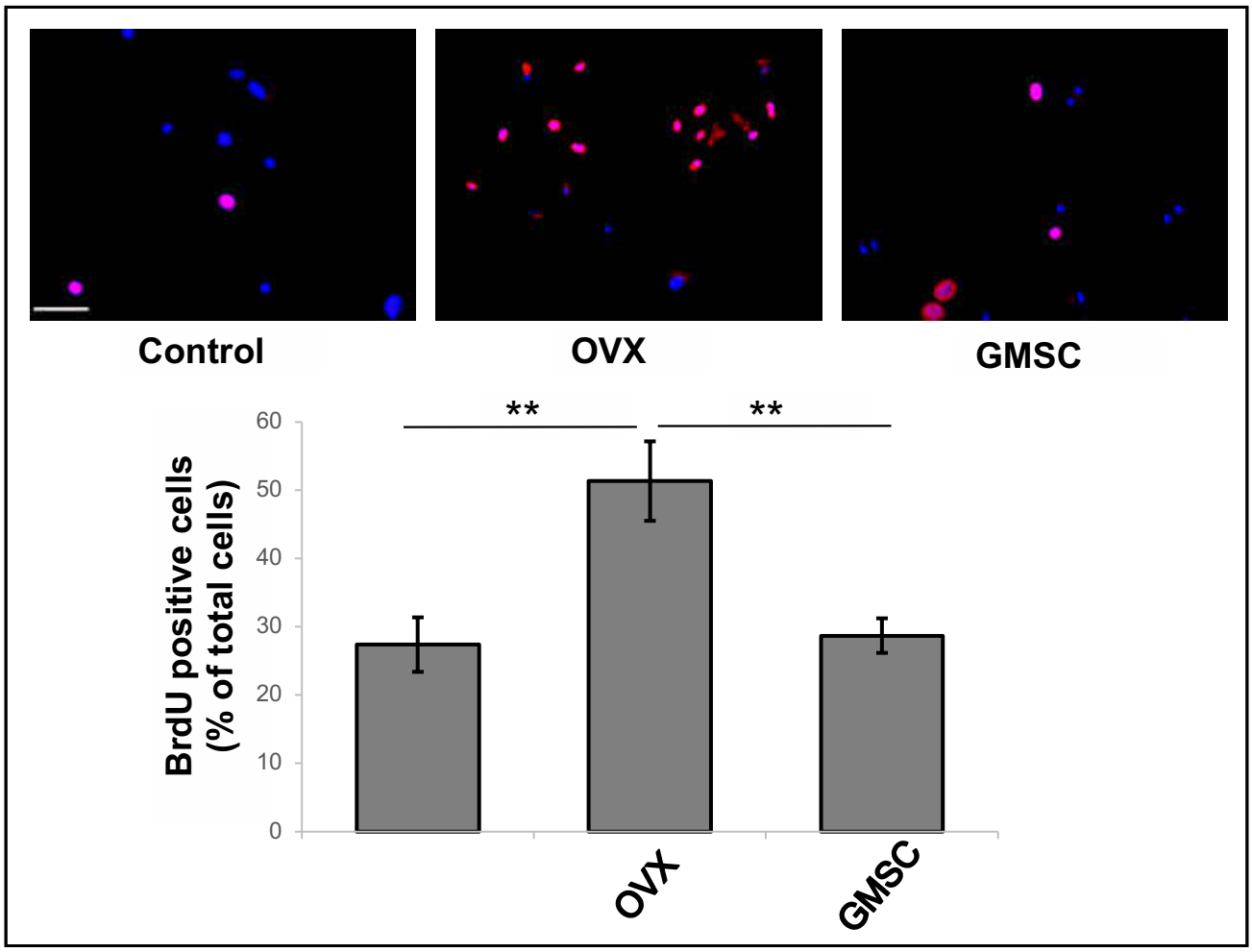


Figure 15. BrdU proliferation assay. Analysis of proliferation capacity of cells from different groups shows BMMSCs from GMSC-treated mice has a proliferation rate comparable to the control while the BMMSCs from OVX mice showed the highest proliferation rate. Scale bar 50 μm.

In the testing of osteogenic differentiation potential of BMMSCs. The OVX group showed a significant reduction in osteogenic differentiation capacity, while the GMSC-treated group showed a significantly higher potential as shown by the mineralized nodules formation evaluated by Alizarin red staining (Fig.16), the decrease in OVX osteogenic potential is accompanied by a decrease of Runx2 and ALP expression, On the other hand an increase in the expression is seen in both GMSC-treated and control groups as assessed by western blot analysis (Fig.17).

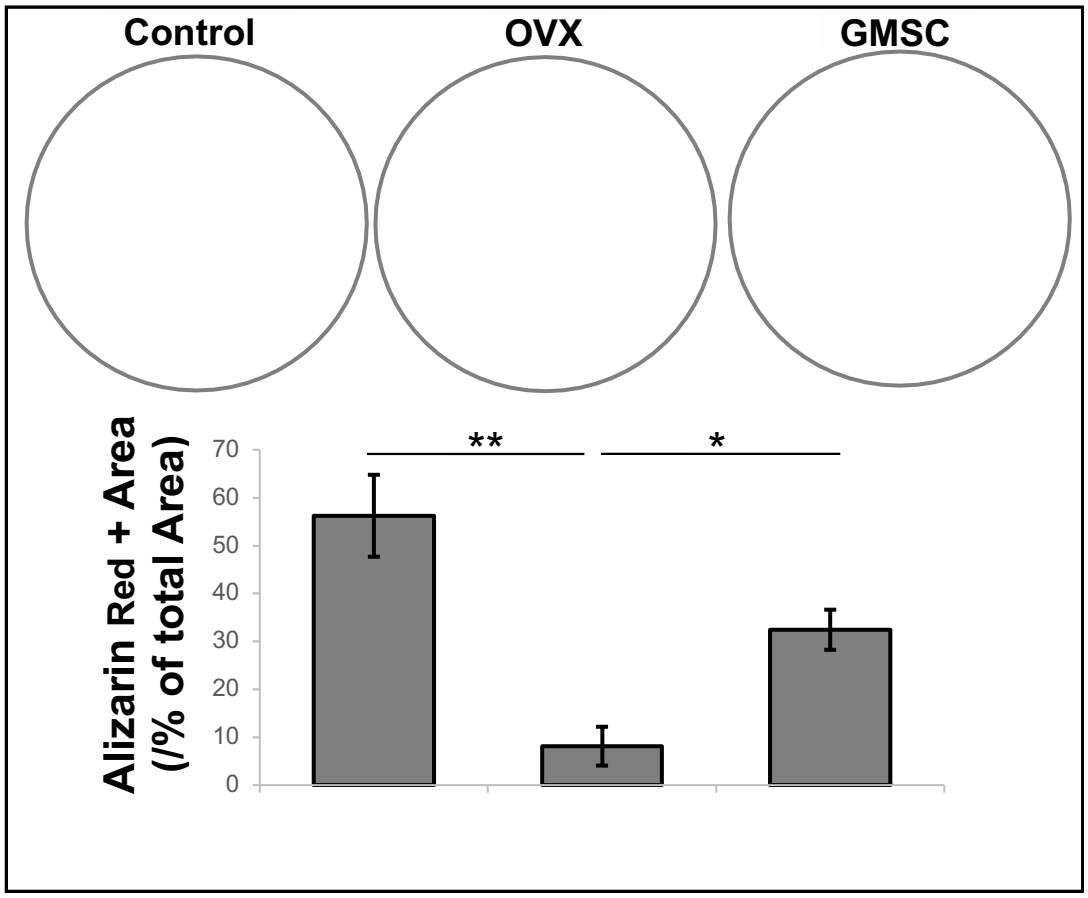


Figure 16. Osteogenic differentiation capacity assay. Osteogenic differentiation capacity of cells from different groups shows BMMSCs from GMSC-

treated mice has a markedly higher osteogenic differentiation capacity than the BMMSCs from OVX mice.

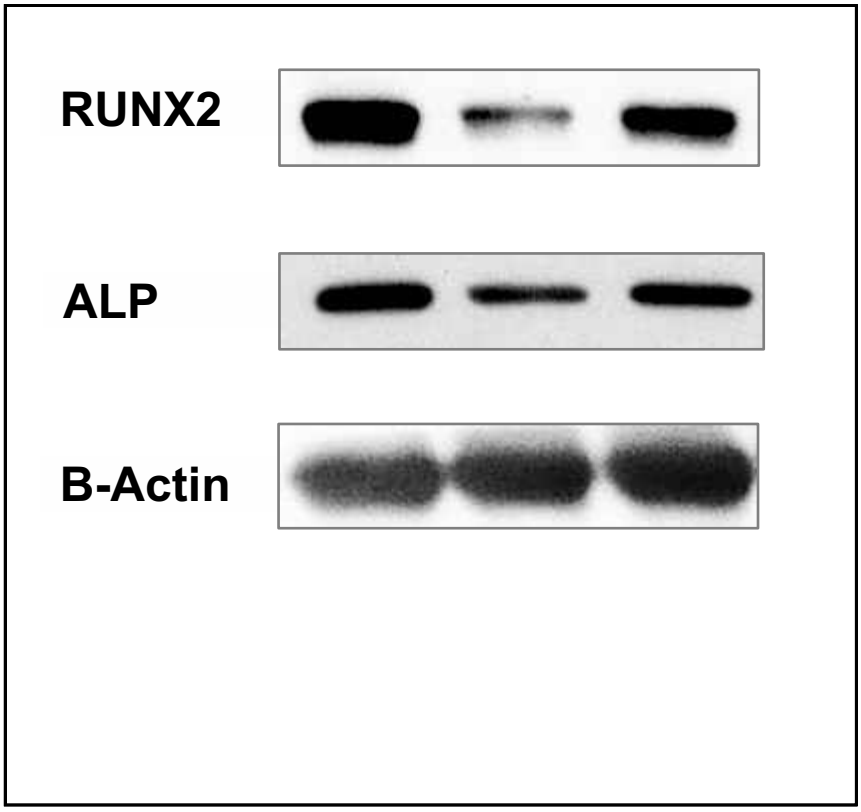
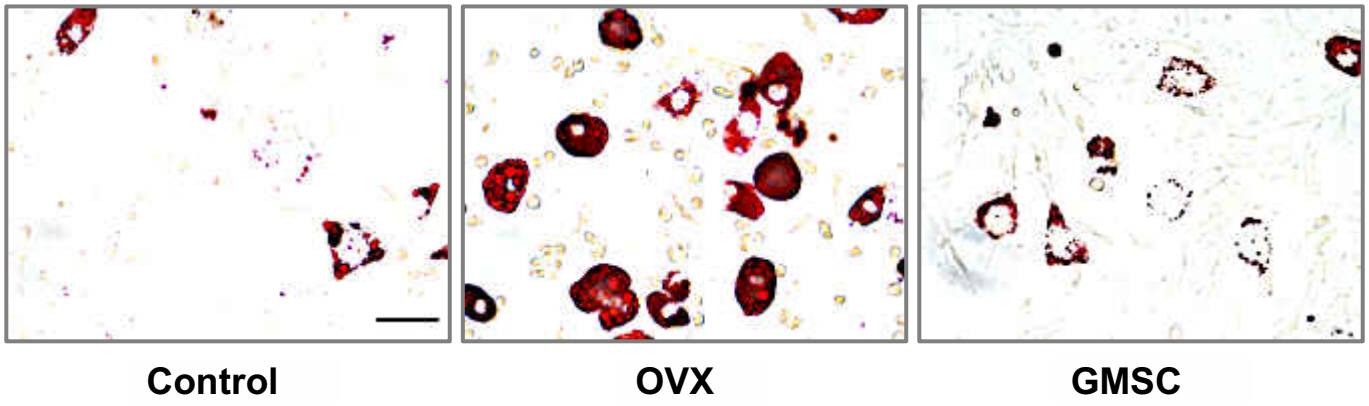


Figure 17. Osteogenic proteins expression levels. Protein level analysis reveal that levels of RUNX2 and ALP proteins are expressed at higher levels in the BMMSCs from GMSCs-treated mice when being exposed to osteogenic inductive conditions, whereas those from OVX group express the same proteins at significantly less level.

Furthermore, when finished testing the osteogenic differentiation capacity, the adipogenic differentiation potential of BMMSCs was also evaluated after inducing the cells and staining them with Oil-red-O staining, the BMMSCs of OVX group mice showed a significantly higher adipogenic differentiation potential when compared with BMMSCs from both the control and GMSC-treated groups (Fig 18). This was also further verified by the observed increase in the levels of the adipogenic proteins PPAR-G and LPL in the OVX group. These proteins were expressed by BMMSCs from control and GMSC-treated mice as well but at a significantly reduced level (Fig 19).



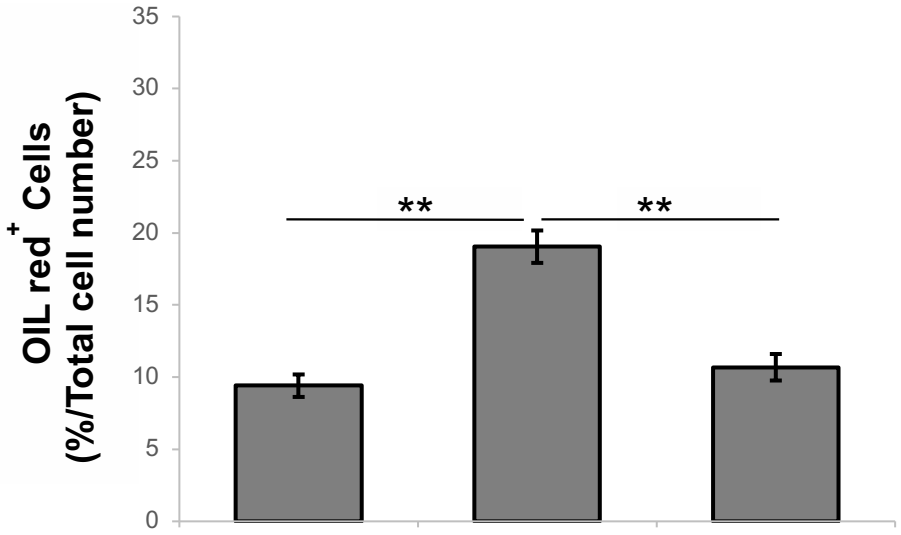


Figure 18. Adipogenic differentiation capacity assay. Adipogenic differentiation capacity of cells from different groups shows BMMSCs from OVX mice having a markedly higher potential for adipogenic differentiation than those from BMMSCs of control or GMSC-treated mice. Scale bar 50 μm.

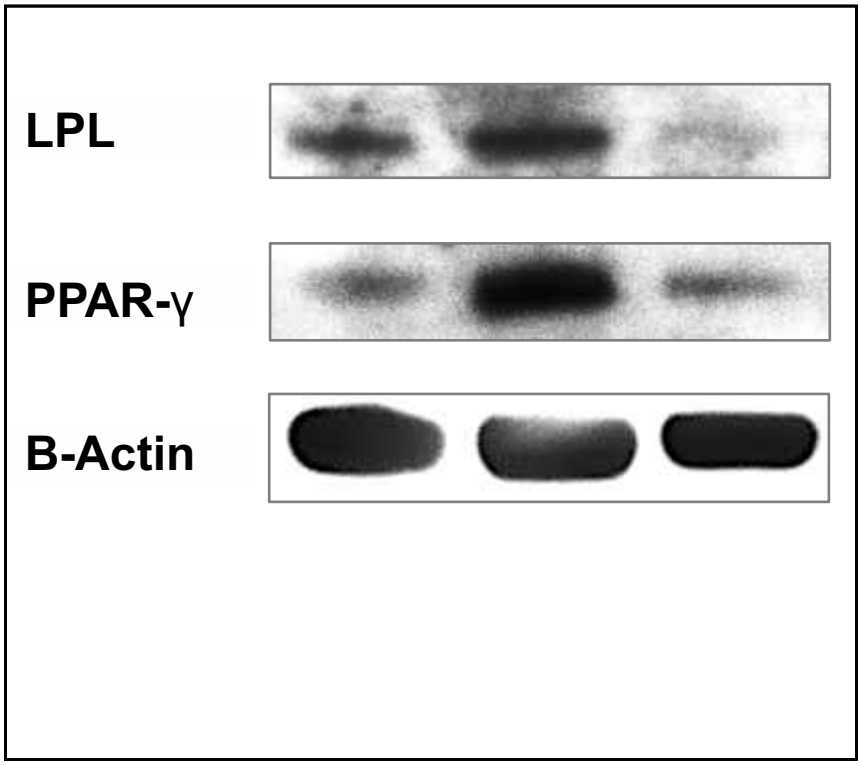


Figure 19. Adipogenic proteins expression levels. Protein level analysis reveal that levels of LPL and PPAR- γ proteins are expressed at significantly higher levels in the BMMSCs from OVX mice when being exposed to adipogenic inductive conditions, whereas those from both GMSC-treated and control groups express the same proteins at significantly less level.

In the last part of determining the influence of OVX and GMSC treatment on the function of BMMSCs, we wanted to test the abilities of BMMSCs from our groups to form new bone in vivo. The test involved using immunocompromised mice and the implantation of BMMSCs from test groups to determine their bone formation rates after incorporating them into HA/TCP grafts. When analyzed, grafts from GMSCs-treated and control group exhibited significantly higher level of new bone formation in comparison to OVX groups (Fig.20).

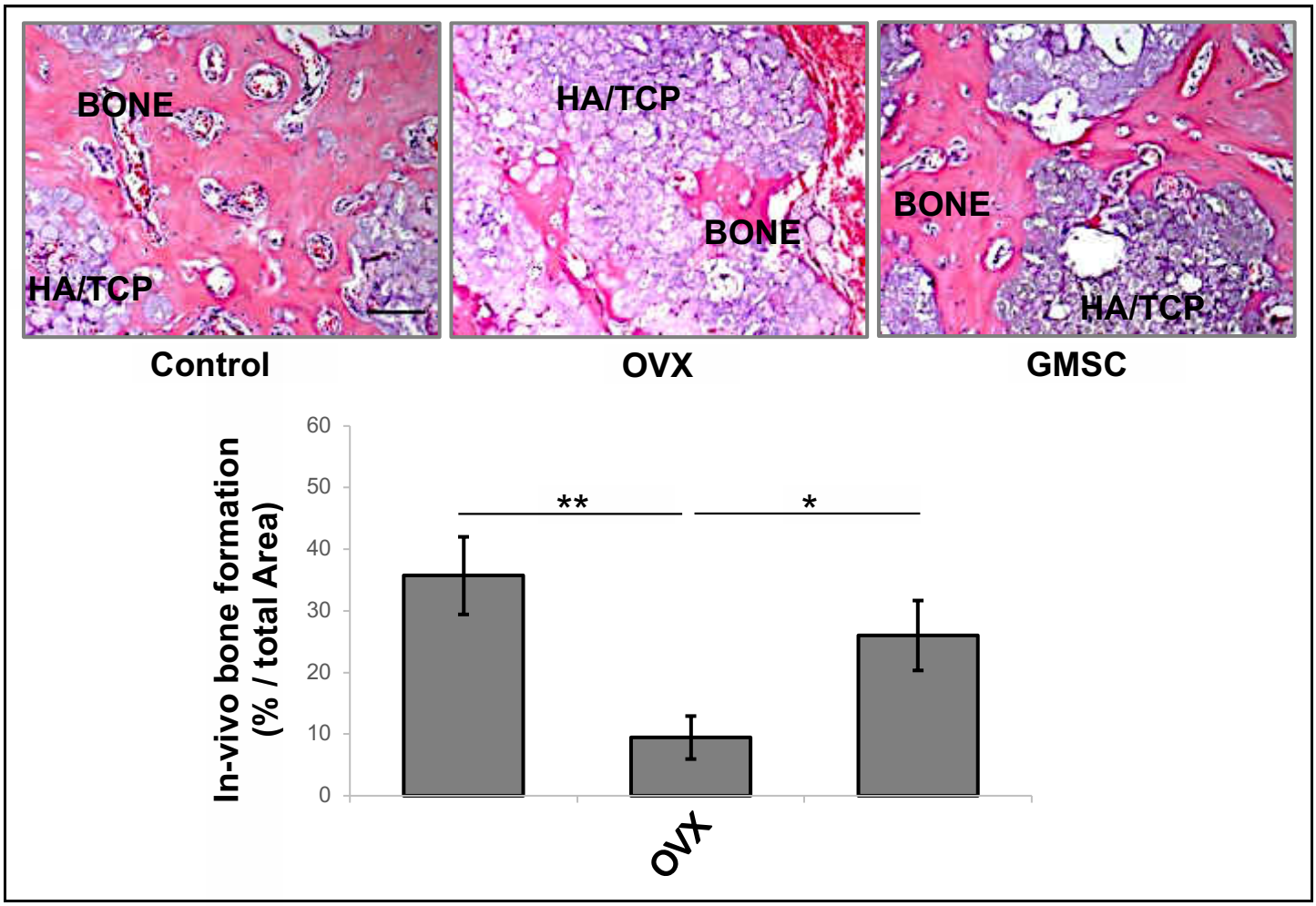


Figure 20. In-vivo bone formation capacity assay. Testing of the bone formation capacity revealed higher bone formation observed in those BMMSCs from control

and GMSC-treated mice, grafts containing BMMSCs from OVX mice show a severe deficiency in the ability to form new bone. Scale bar, 20 μm.

These results and findings of our functional testing involving BMMSCs of study groups suggest that the endogenous BMMSs population harvested from the OVX mice group were of significantly compromised function in all the experiments completed in this part. This function is of normal expected levels in the mice of the control group. The BMMSCs from the GMSCs-treated mice revealed there was and significant improvement in function and the BMMSCs were rescued as a result of the GMSC transplantation.

Mechanistic Investigation

GMSCs effect in the OVX mouse model is independent of Fas/FasL cell death pathway

To test the mechanism behind GMSCs effect in the OVX mouse model. First, we tested a potentially involved pathway. New groups to test FASL/FAS pathway are shown in (Fig.21).

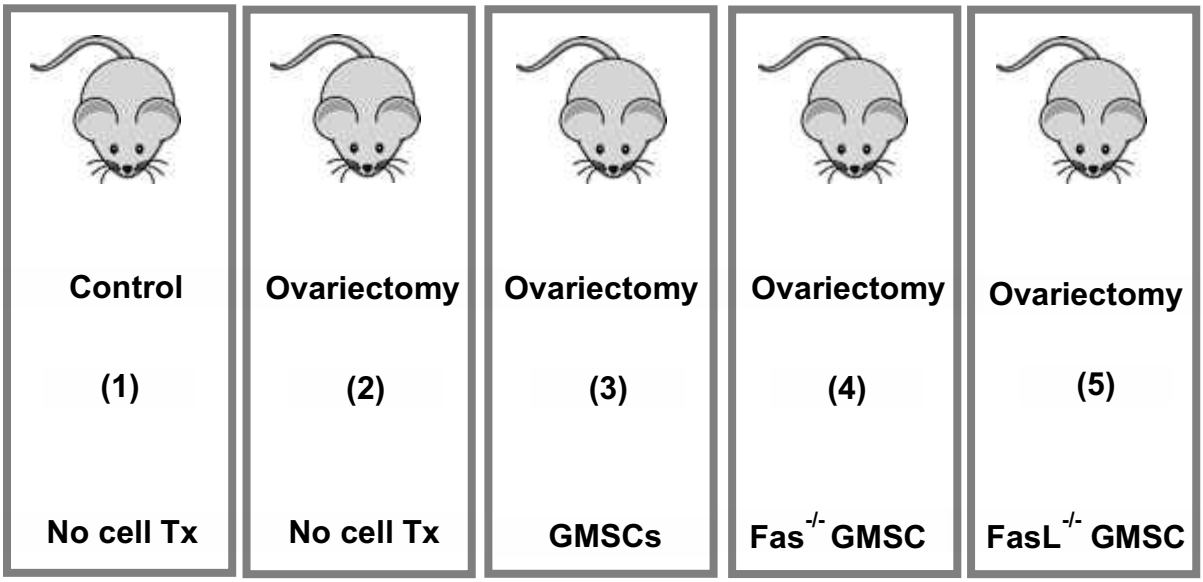
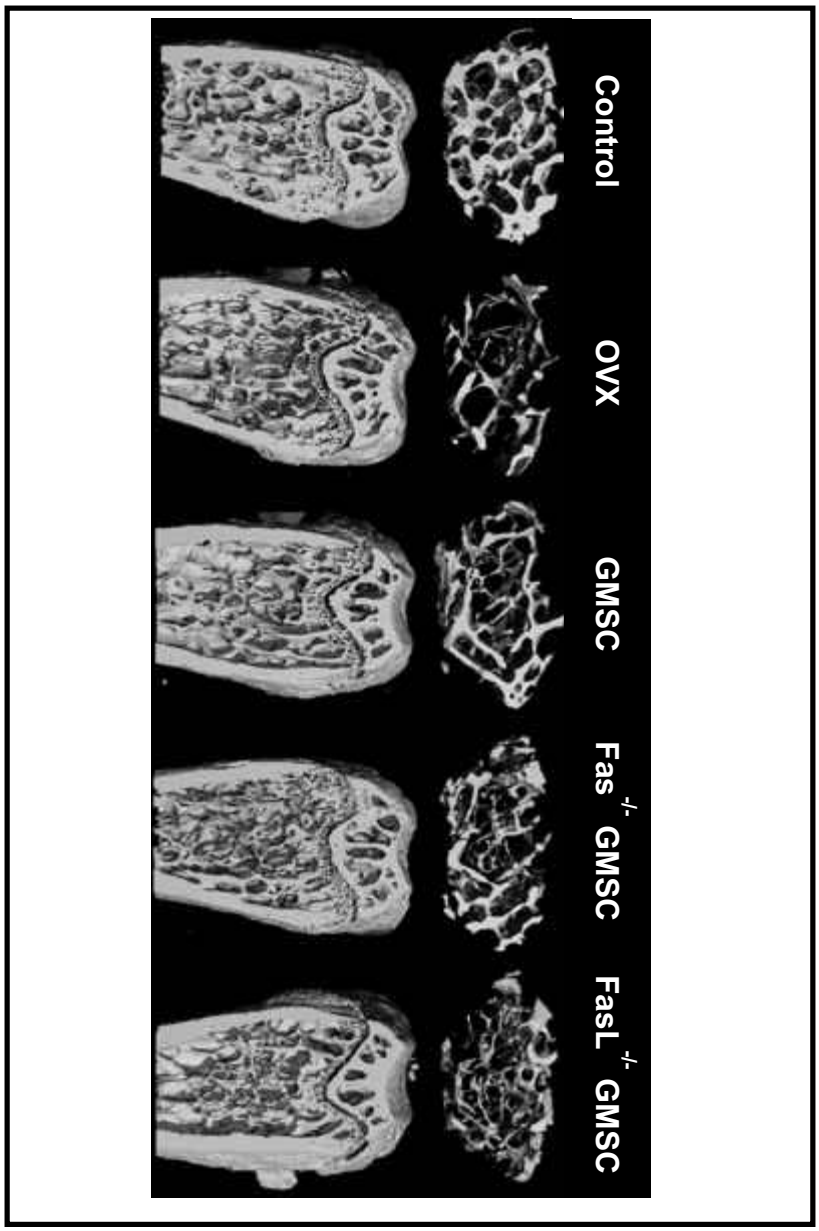


Figure 21. FAS/FASL pathway testing group assignment. (1) Mice not undergoing ovariectomy procedure, nor transplantation. (2) Mice undergoing ovariectomy procedure, but no cellular transplantation. (3) Mice undergoing ovariectomy procedure, then a single transplantation of GMSC. (4) Mice undergoing ovariectomy procedure, then 1X transplantation of FAS deficient GMSC obtained from LPR mice (5) Mice undergoing ovariectomy procedure, then 1X transplantation of FASL deficient GMSC from obtained GLD mice.

Following the same outline as previously describe in Fig.4 and after the experiment concluded and mice were sacrificed, new femurs were scanned and analyzed. the compute microtomography images and analysis also revealed a significant deterioration in the bone phenotype of the OVX mice in comparison to the negative control group as shown by a decrease in the measurements of bone mineral density (BMD), total bone volume (BV/TV). While a GMSCs treatment improved the bone phenotype in the OVX mice, so did $Fas^{-/-}$ and $FasL^{-/-}$ GMSCs (Fig.22).



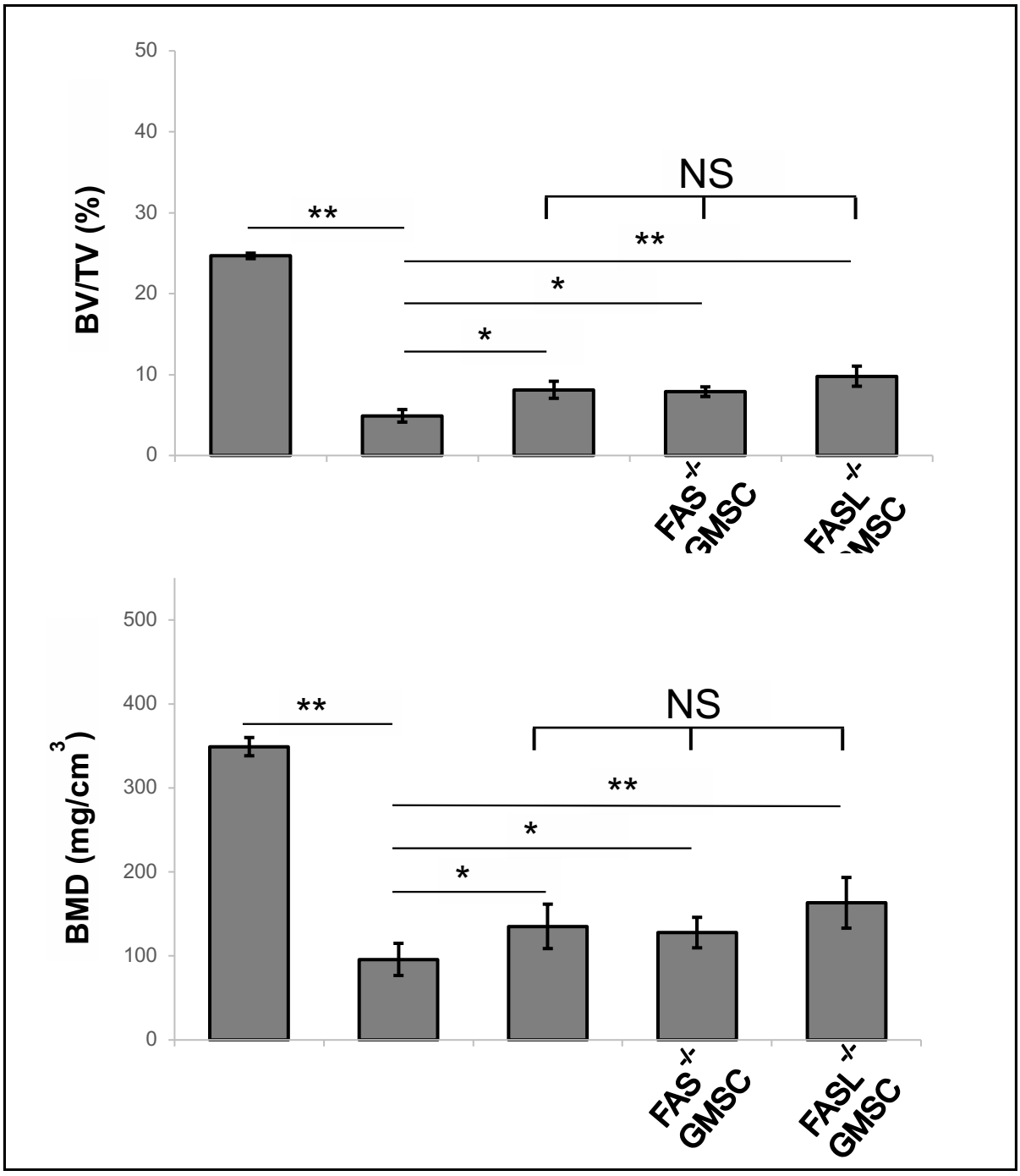
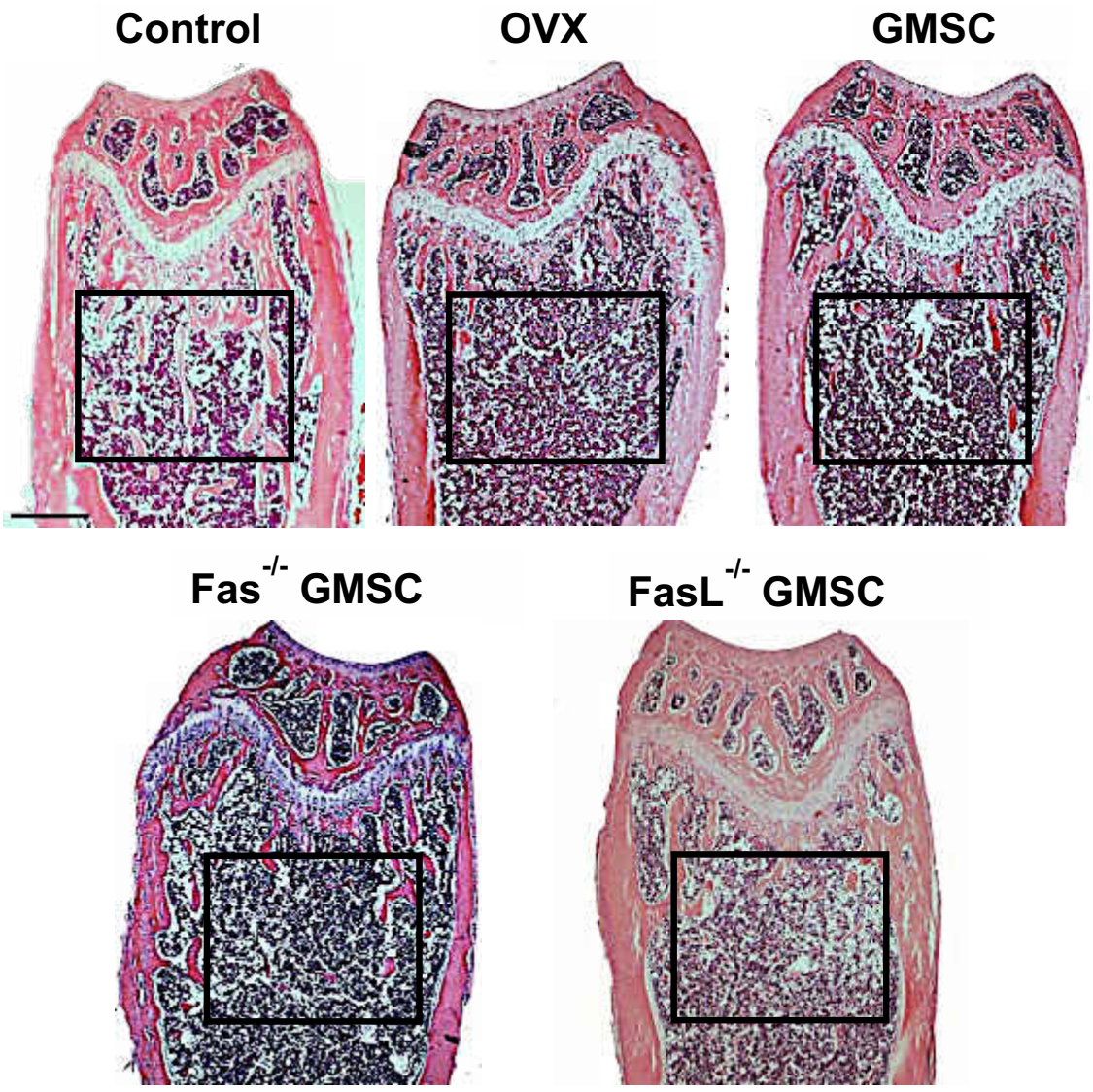


Figure 22. Computed microtomography analysis of Fas$^{-/-}$, FasL$^{-/-}$ GMSCs treatment. Femurs of Fas$^{-/-}$, FasL$^{-/-}$ GMSCs show similar effect in BMD and BV/TV values as that observed as an improvement of the phenotype of the femurs of GMSC-treatment group.

Additional analysis of femurs further revealed that the trabeculation levels of those from mice treated with $Fas^{-/-}$, and $FasL^{-/-}$ GMSCs are similar to those treated with wild type GMSCs (Fig.23) as shown by H&E analysis of the sections created.



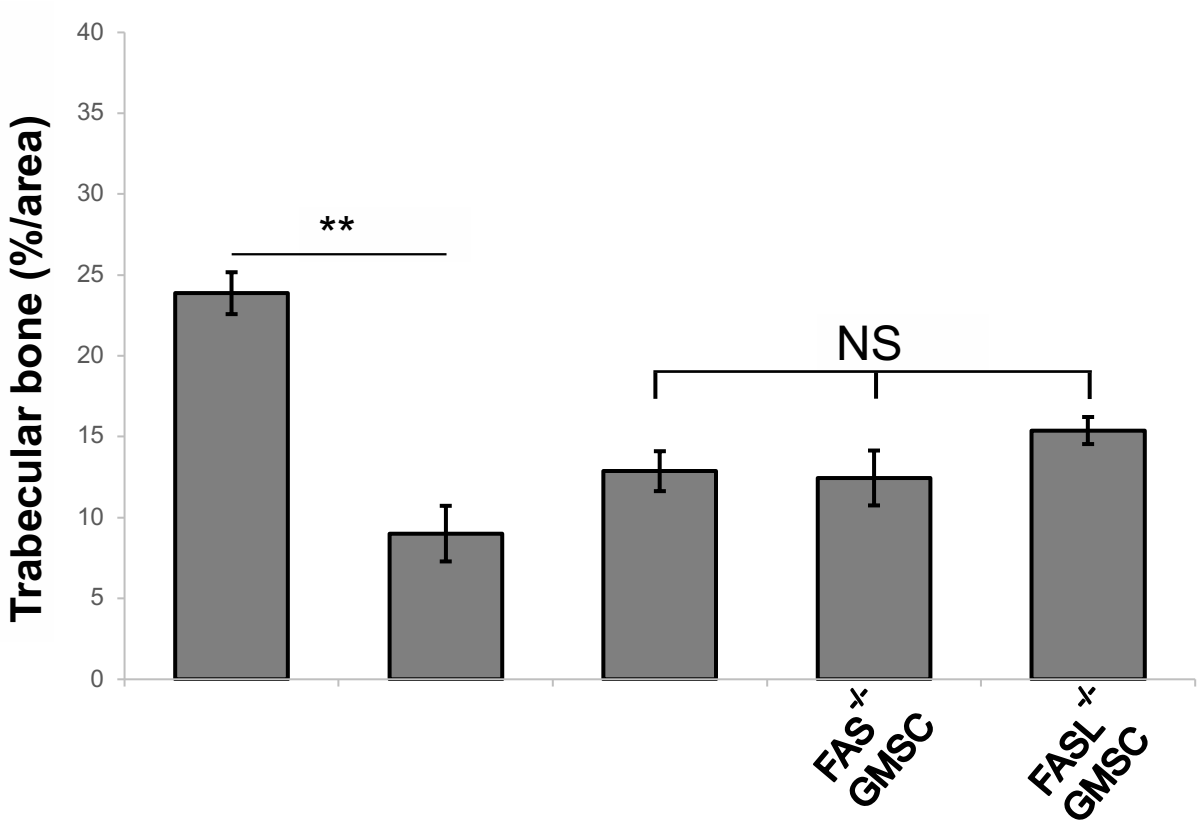


Figure 23. Histological analysis of femurs. $Fas^{-/-}$, $FasL^{-/-}$ GMSC treated mice showing an improvement in the trabeculation levels of femurs compared to the femurs of GMSC-treatment group. Scale bar, 500 μm

This was further confirmed with TRAP+ cell testing to quantify the number of osteoclasts in the distal metaphyseal region, we found that TRAP+ cell numbers are in agreement with the previous finding of trabeculation levels in H&E sections of Femurs as those of $Fas^{-/-}$, $FasL^{-/-}$ GMSCs-treatment show a similar level of TRAP+ cells as WT GMSCs-treated group (Fig.24).

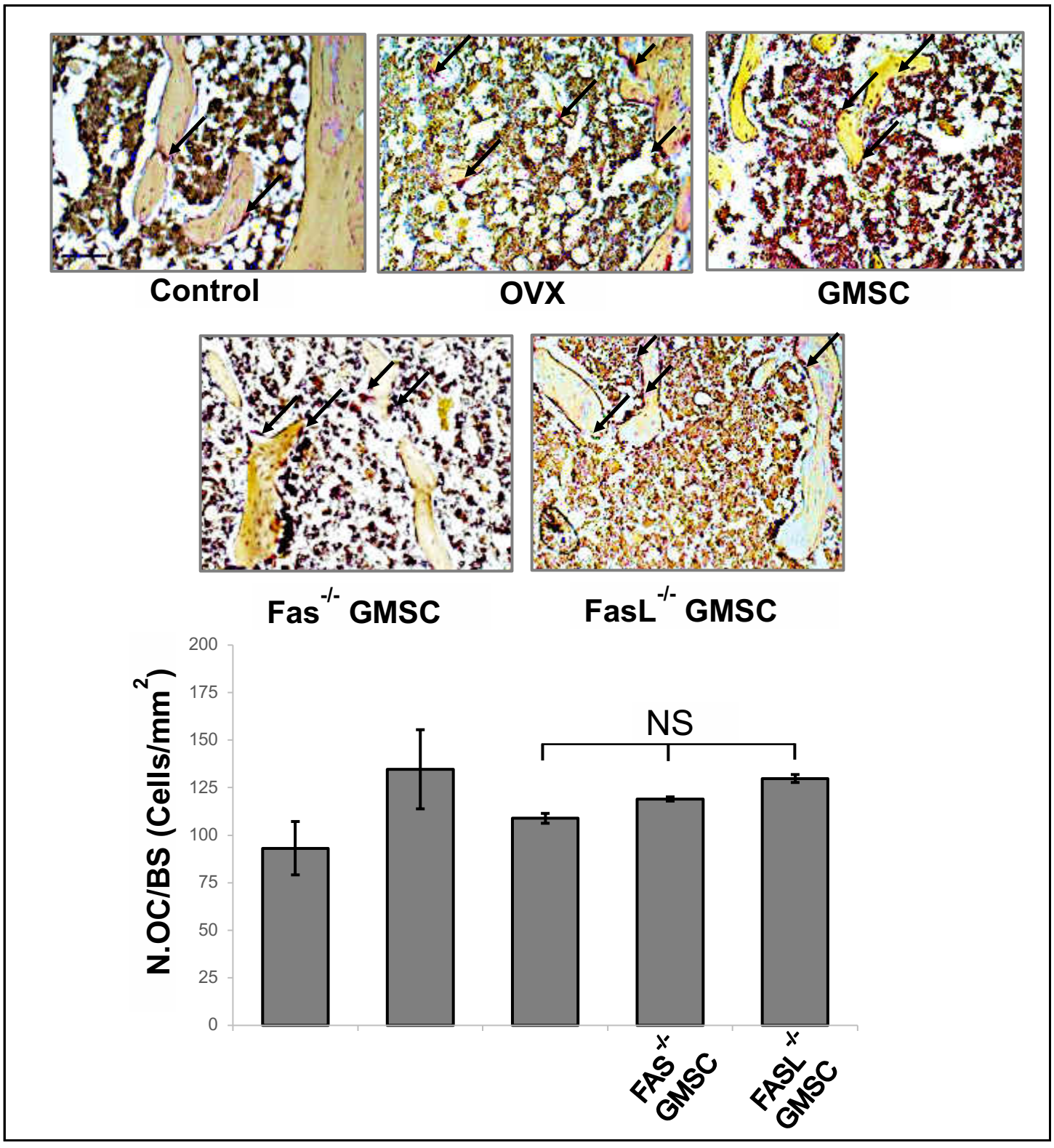


Figure 24. TRAP^{+} cell analysis of femurs. Femurs of Fas$^{-/-}$, FasL$^{-/-}$ GMSCs treated mice show similar levels of osteoclasts to those of the femurs of GMSC-treatment group. Scale bar, 100 μm.

The testing of Fas/FasL deficient GMSCs on the bone phenotype showed a similar effect as the one seen in treatment of mice by wild type GMSCs. This indicates that GMSCs-mediated effect in the OVX mouse is independent of Fas/FasL pathway. The next pathway investigated as a pathway to play a potential role is a similar pathway called PD-1/PD-L1 pathway.

GMSCs influences T-cells in the OVX mice via the PD1/PDL1 pathway

Programmed death-1 (PD-1) is a protein first described by Ishida; it is a member of the immunoglobulin gene superfamily and plays a role in cell death by apoptosis (67). PD1 is known to be expressed by T-cells, B cells, as well as other types of cells. PD1/PDL1 pathways application has been investigated in infections, cancer therapies and immunotherapies due to its role in immune regulation (68,69). Investigations of PD-1/PD-L1 pathway show that PD-1 ligation restored immune tolerance potentially by the suppression of T-cells, inducing their apoptosis as well as its promotion of T-reg function (70,71) MSCs are also known to express PD-L1 and induce T-cell suppression via the PDL1/PD1 pathway accentuating the importance of PD-L1 in MSC mediated immunoregulation (72). This prompted us to propose that PD-L1/PD-1 pathway is of a significant role in GMSCs immune-mediated effect in the OVX mice and that GMSCs are directly influencing activated T-cells leading to their apoptosis (Fig.25). To test and confirm this we formulated four study groups to examine our theory of PD-1/PD-L1's pathway involvement (Fig.26).

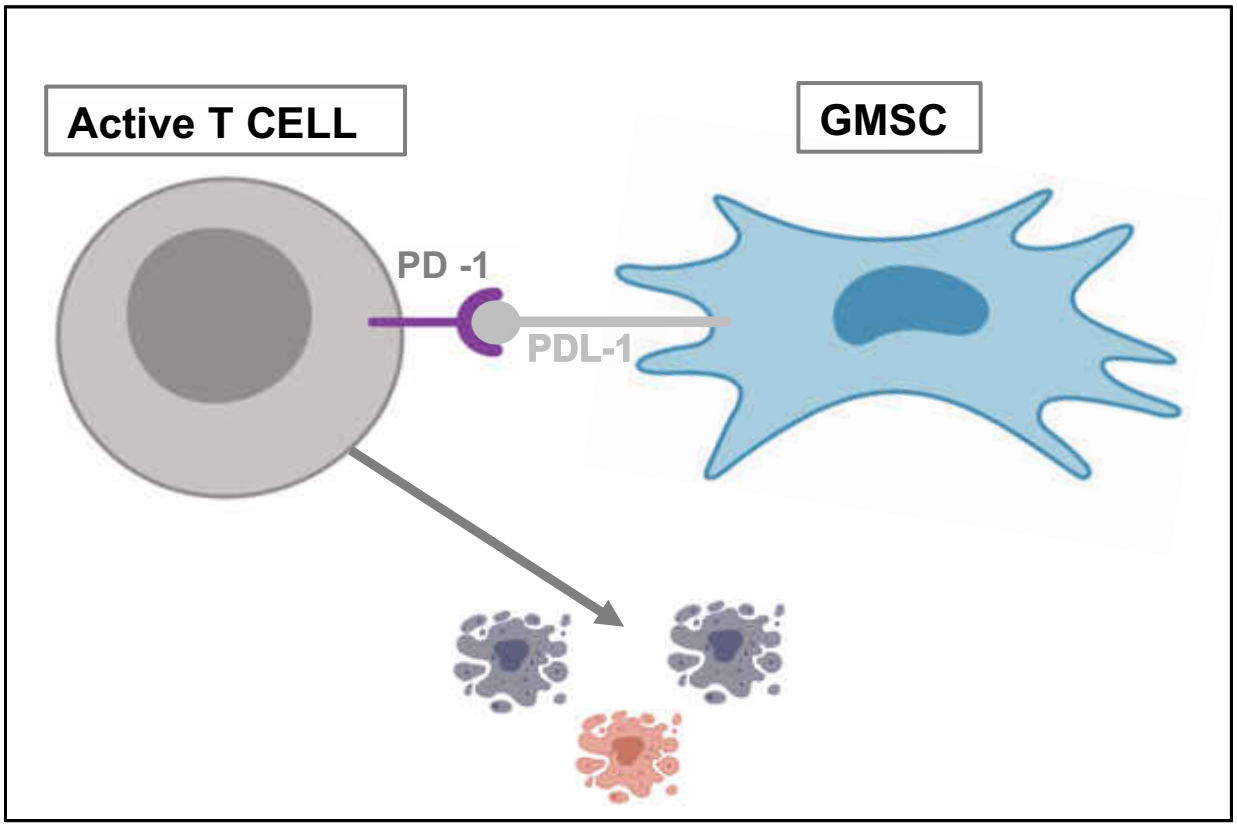


Figure 25. Proposed mechanism involving GMSCs suppression of T-cells. GMSCs interact with T-Cell via PD-1/PD-L1 pathway and induced T-cell apoptosis.

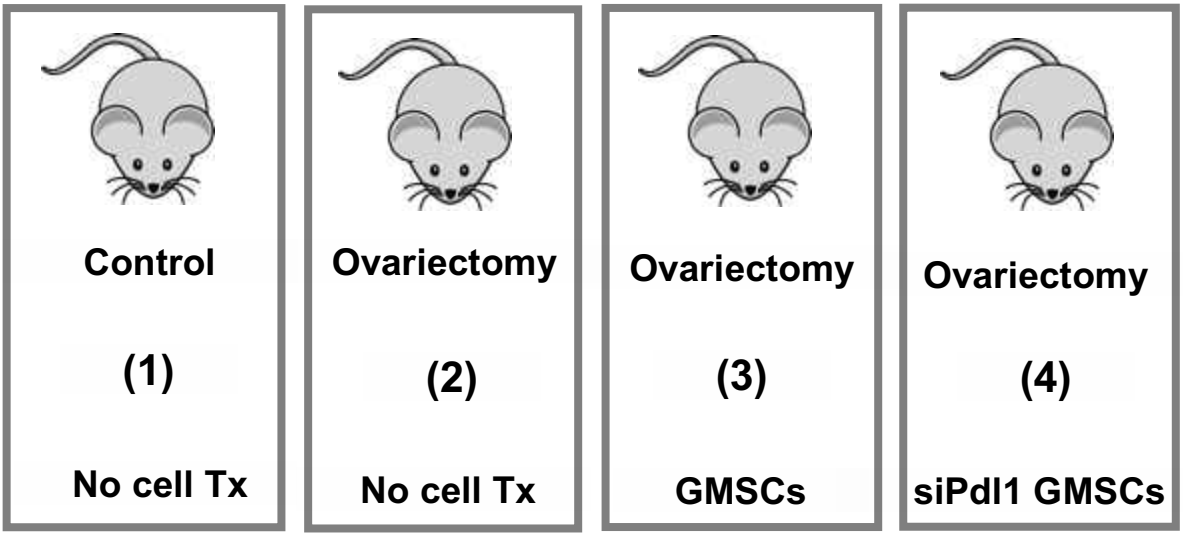


Figure 26. Study groups of PD-1/PD-L1 pathway mechanistic investigation. (1) Mice not receiving ovariectomy, nor transplantation. (2) Mice receiving ovariectomy but without cellular transplantation. (3) Mice receiving ovariectomy procedure and a single transplantation of WT GMSC. (4) Mice receiving ovariectomy and a single transplantation siPDL1 GMSCs.

To verify the knockdown of PD-L1 gene in GMSCs after using siRNA approach to reduce its expression, protein was analyzed to detect the level of protein expression (Fig 27).

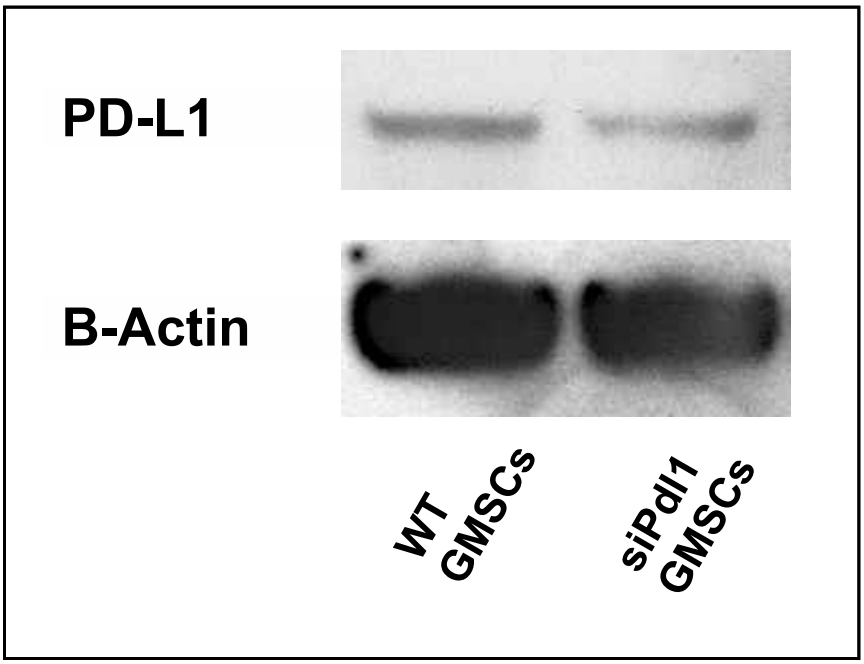


Figure.27 Expression levels of PD-L1 protein. Verification of protein levels after utilization of siRNA approach to the knockdown the expression of PD-L1.

To further verify the successful knockdown, we attempted to test the expression of the protein utilizing an immunohistochemistry approach to stain and we saw higher

levels of siPdl1 in the WT GMSCs (Fig 28). After that we used either GMSC or siPDL1 GMSC to transplant into the OVX mice with same previously followed timeline (Fig.4), that included sacrifice of mice after 15 weeks for further analysis.

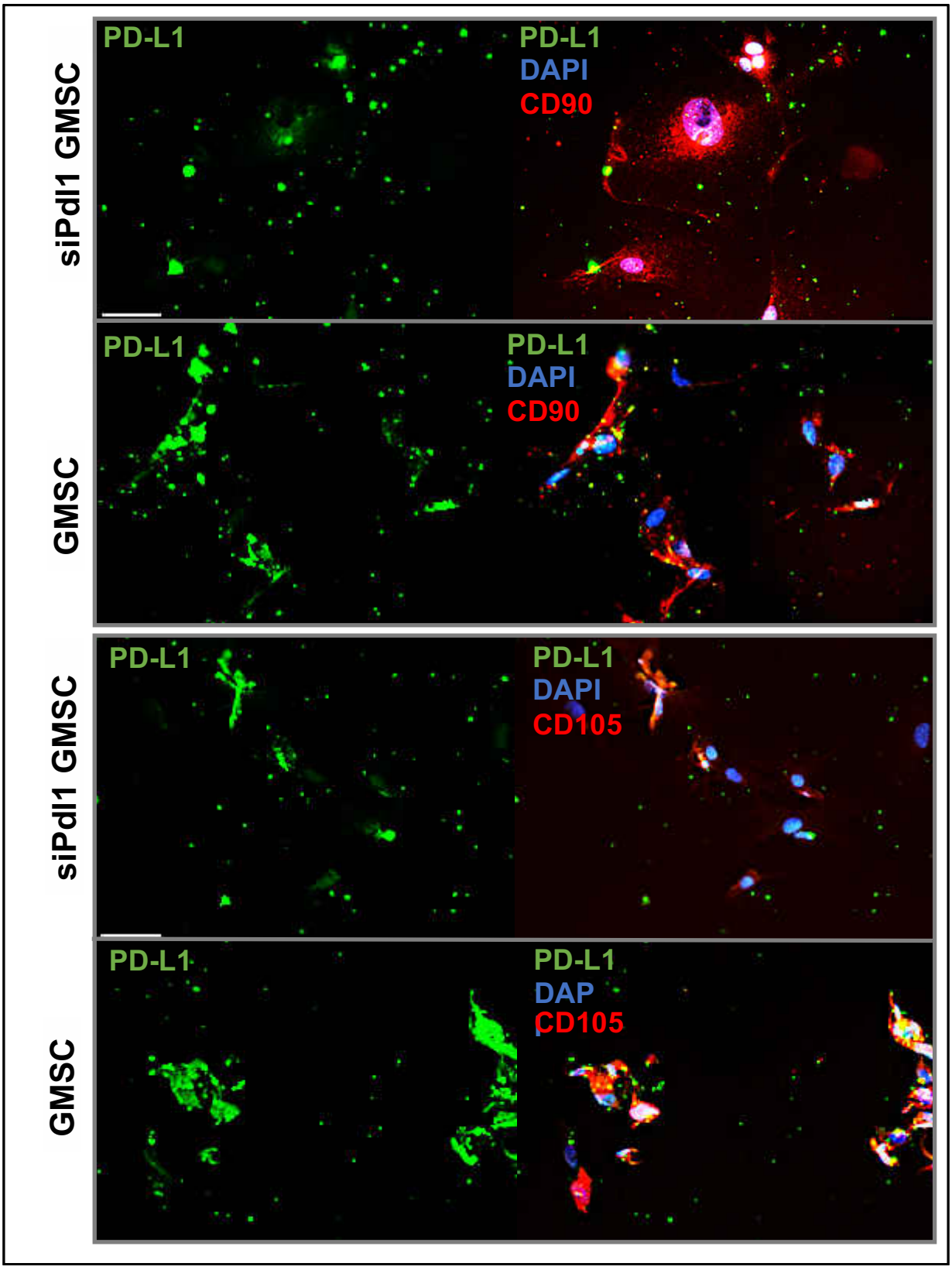
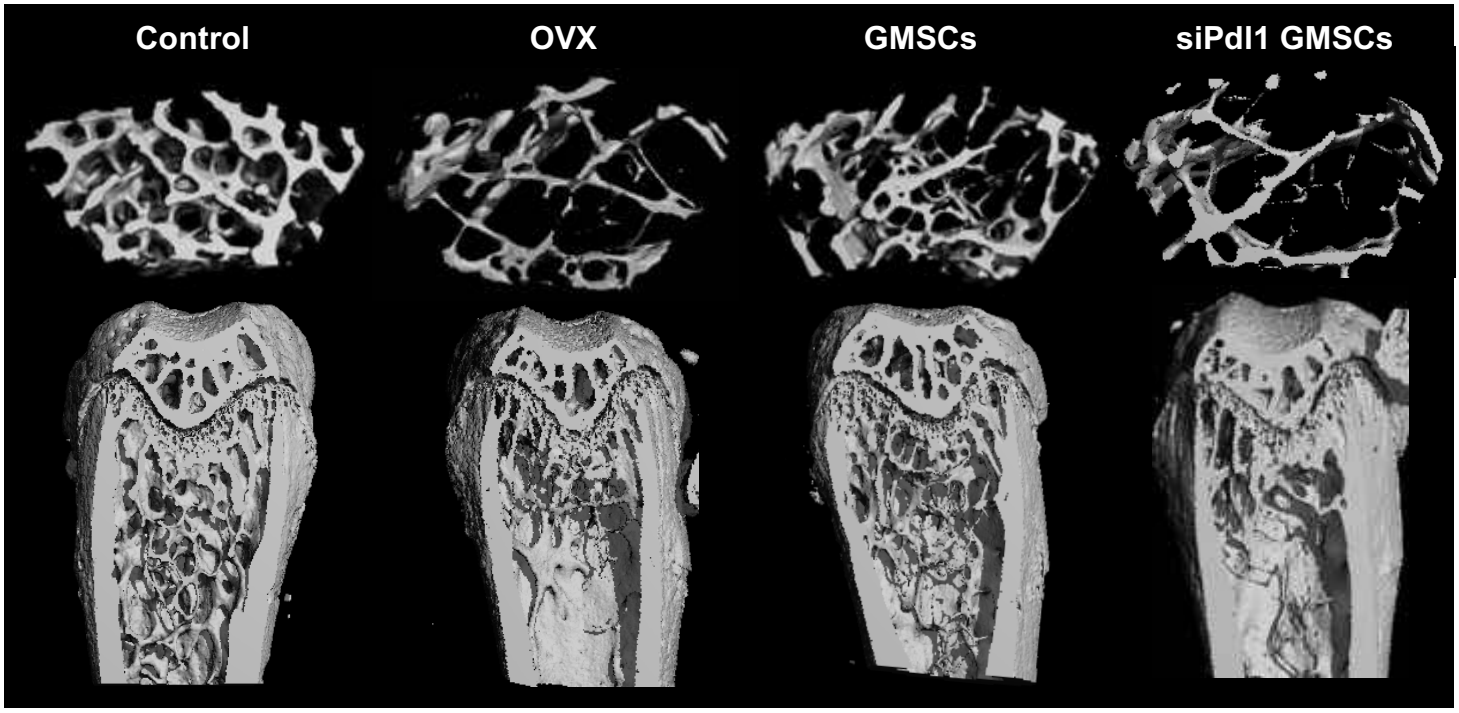


Figure.28 Immunohistochemistry analysis of PD-L1/MSC markers expression levels. PD-L1 protein appears to be expressed at lower rate as seen by the decreased density in PD-L1 signal in siPdl1 knockdown GMSCs in comparison to that in WT GMSCs. Scale bar, 50 μm.

After testing whether GMSCs do express PD-L1 and successfully knocking down its expression, it was then used in treatment. After sacrifice, the femurs from mice were scanned and analyzed. We saw that siPdl1-GMSCs failed to improve the trabecular bone phenotype in the OVX mice in comparison to the WT-GMSC treated group as shown by the μCT scan and analysis the measurements BMD, as well as BV/TV (Fig.29).



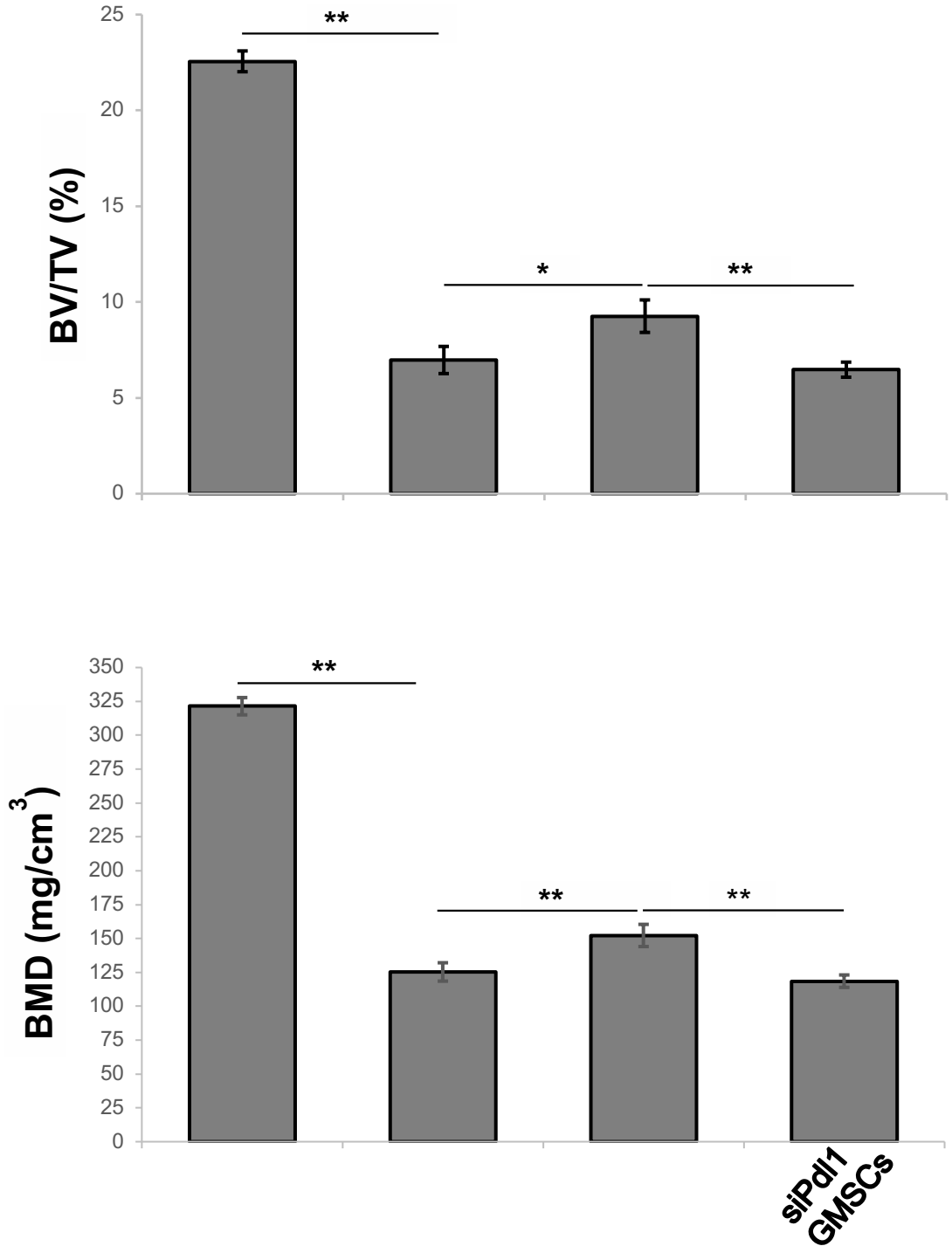


Figure.29 Computed microtomography analysis of siPdl1 GMSCs treatment. Analysis of femurs from siPdl1-GMSC treated mice have a marked deficiency in the trabeculation of the distal metaphysis region just as seen in OVX mice whereas the WT GMSCs-treated group show a marked improvement.

In addition to the scans of femurs, Histological analysis was also carried to verify the trabeculation levels of the distal metaphyseal region. We saw that the trabeculation in femurs from siPDL1-GMSCs group is significantly compromised just like those from the OVX mice when compared with those from the GMSCs treated group (Fig.30).

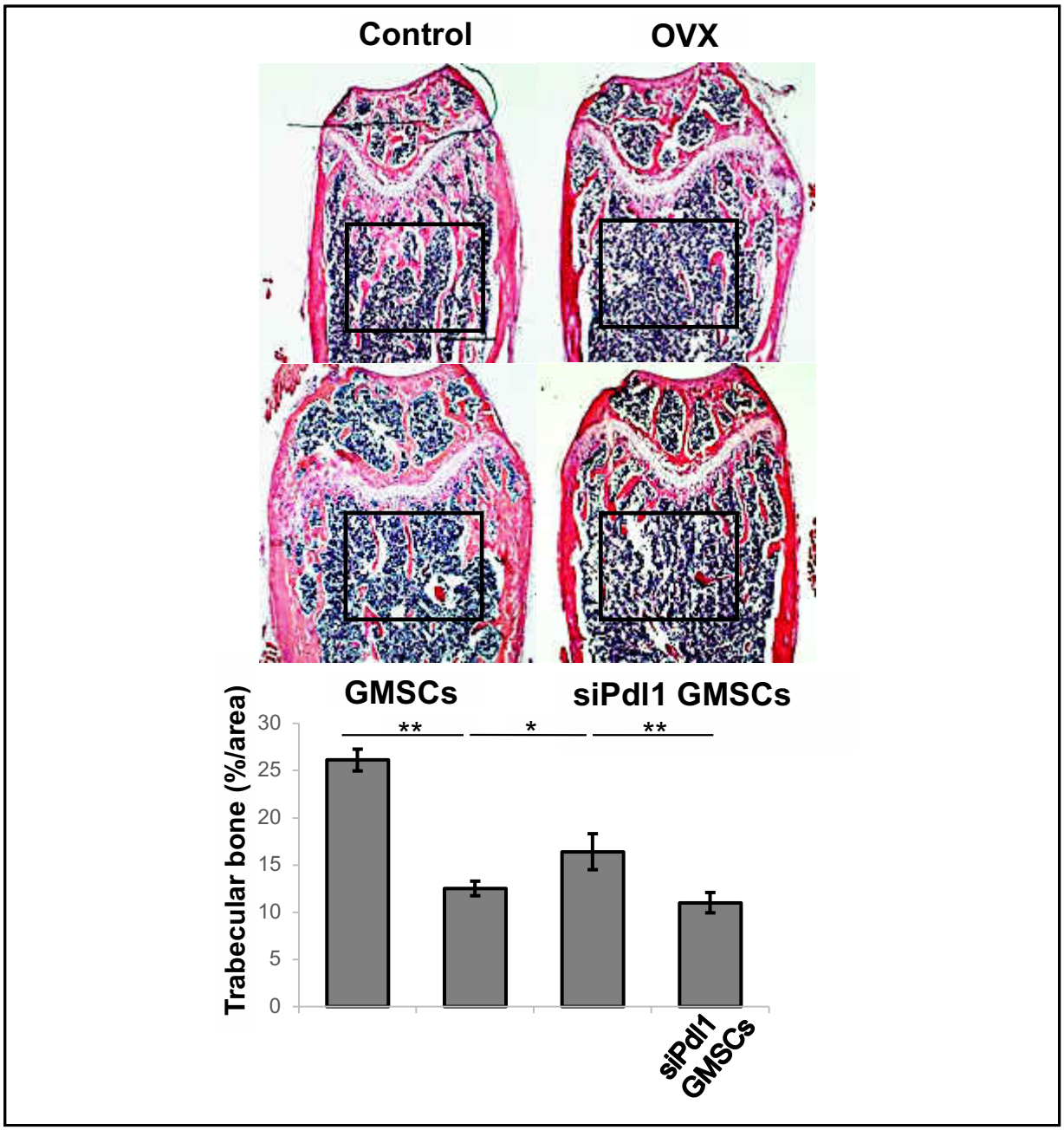
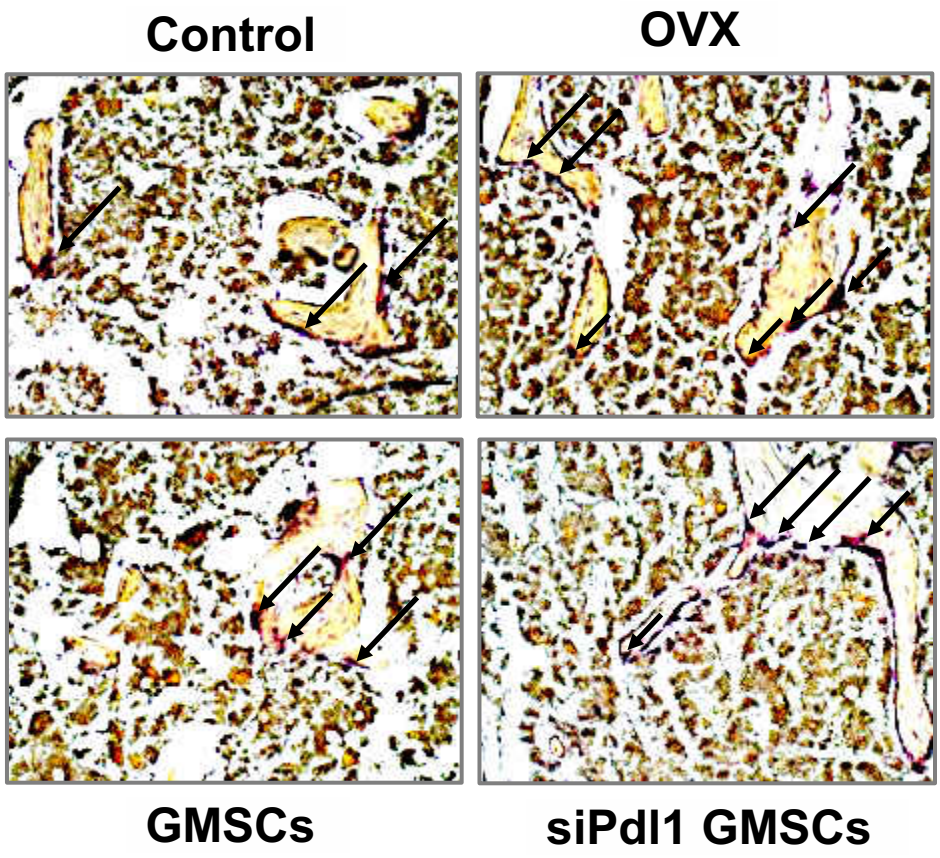


Figure.30 H&E analysis of femurs. Trabeculation levels shown to be severely compromised in the siPdl1-GMSC treated femurs in comparison to WT GMSCs treated ones, they are further comparable to the femurs from the OVX group. Scale bar, 500 μm.

Because the trabeculation levels are compromised in the siPdl1-GMSC-treated femurs, we would expect the osteoclasts to be higher in number in synergy with it. High osteoclast levels per square millimeter of areas analyzed in femurs lead us to confirm the defect of siPdl1-GMSCs by their failure to reduce TRAP+ cells in the femurs (Fig.31).



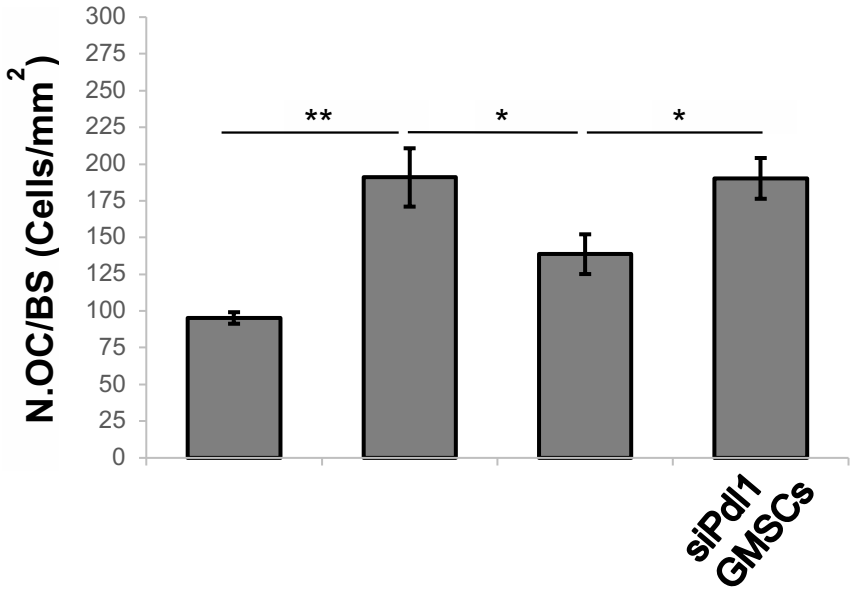


Figure.31 TRAP+ cell analysis. TRAP+ cells are of increased numbers in analyzed areas of femurs from siPdl1-GMSCs treated mice, as well as those from the OVX group. Scale bar, 100 μm.

Additionally, to test the levels of adipocytes that is associated with osteoporosis in femurs and the increased risk of fractures. An in-vivo test of the levels of fatty marrow in the distal metaphyseal region of femurs was completed. Femurs of siPDL1-GMSCs treated group showed a higher level of fatty marrow as show by increased levels by Oil Red O positive areas. This increased level of fatty marrow is also seen in the OVX treated group. GMSC-treated group femurs showed a significantly lower levels that are comparable to the control group (Fig.32).

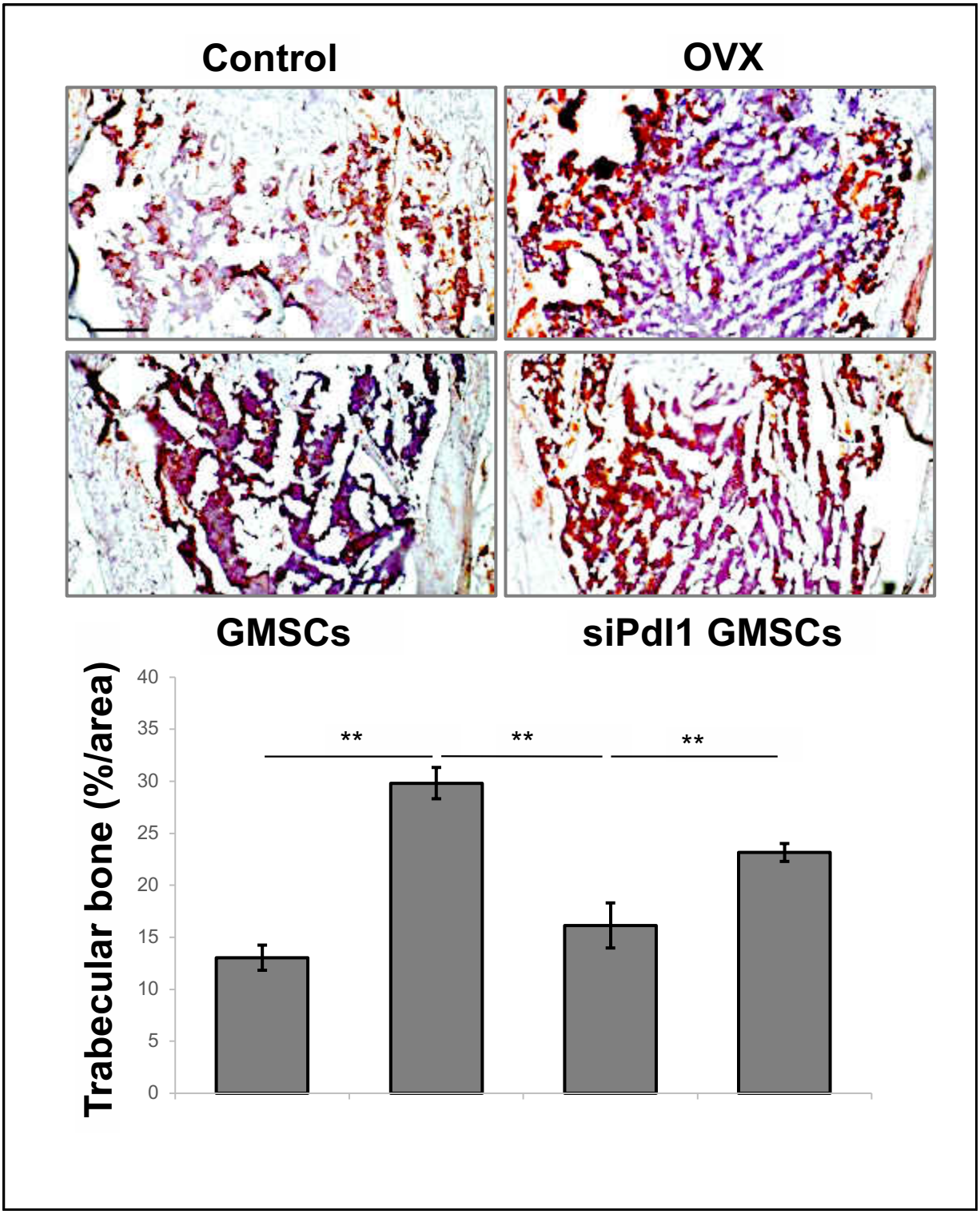


Figure 32. in-vivo femur fatty marrow analysis. An increase in fatty marrow seen siPdl1-GMSCs treated mice similar to those of OVX treated mice. Scale bar, 500 μm.

Testing of an Anti-PD-L1 drug therapy in combination with GMSCs infusion of OVX mice.

After confirming the failure of siPdl1 GMSCs to improve the bone phenotype in the OVX mice, we wanted to verify that PD-L1 is an immune checkpoint for GMSCs-mediated effect seen in the osteoporotic mice. so, we hypothesized that the administration of an anti-PDL1 drug (Atezolizumab) would block the therapeutic effect of the GMSCs transplantation and that there would be no improvement in the bone phenotype after GMSCs treatment. In order for us to test this hypothesis, we utilized two study groups as shown in (Figure.33) and formulated a timeline for our experiment (Figure.34) consisting of a double injection of the drug Atezolizumab in the test group and a placebo in the control group after GMSCs transplantation. After sacrifice, the mice femurs were collected and scanned with Scanco MCT35 scanner and the trabeculation levels are shown in (Fig.35). We also analyzed the BMD of femurs as well as Total bone volume (BV/TV) and we found a statistically significant difference in both values between the control and the test groups. This verifies our hypothesis that the drug treatment of Atezolizumab blocked the therapeutic effect of GMSCs and that PD-L1 is a pivotal immune checkpoint for GMSCs-mediated effect.

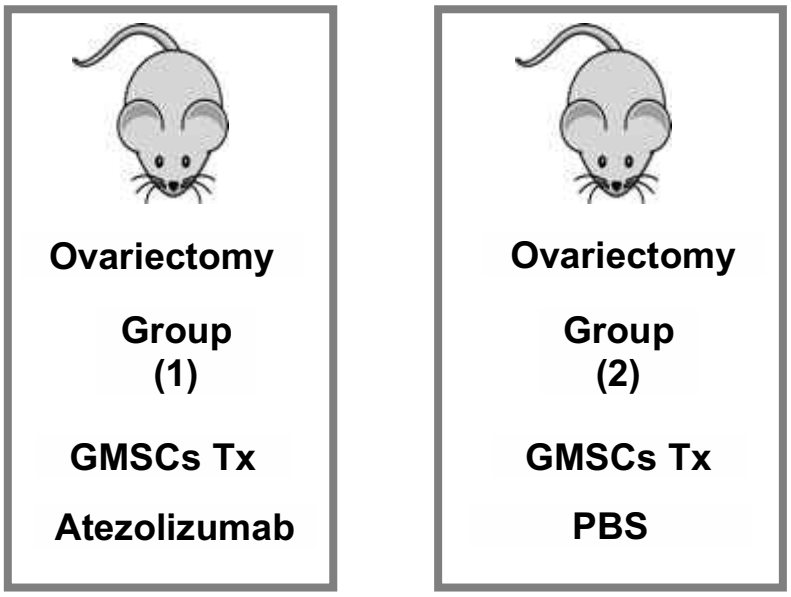


Figure 33. Study groups of PD-L1 drug therapy investigation. (1) Mice receiving ovariectomy, GMSCs transplantation, and Atezolizumab drug. (2) Mice receiving ovariectomy, GMSCs transplantation, and Placebo (PBS).

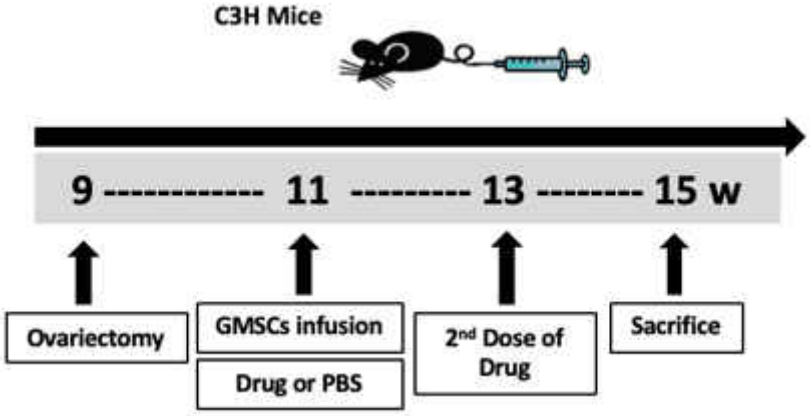


Figure 34. Experimental timeline of treatment. Ovariectomy procedure done at 9 weeks to designated groups, GMSCs were injected into designated group intravenously via the tail vein.

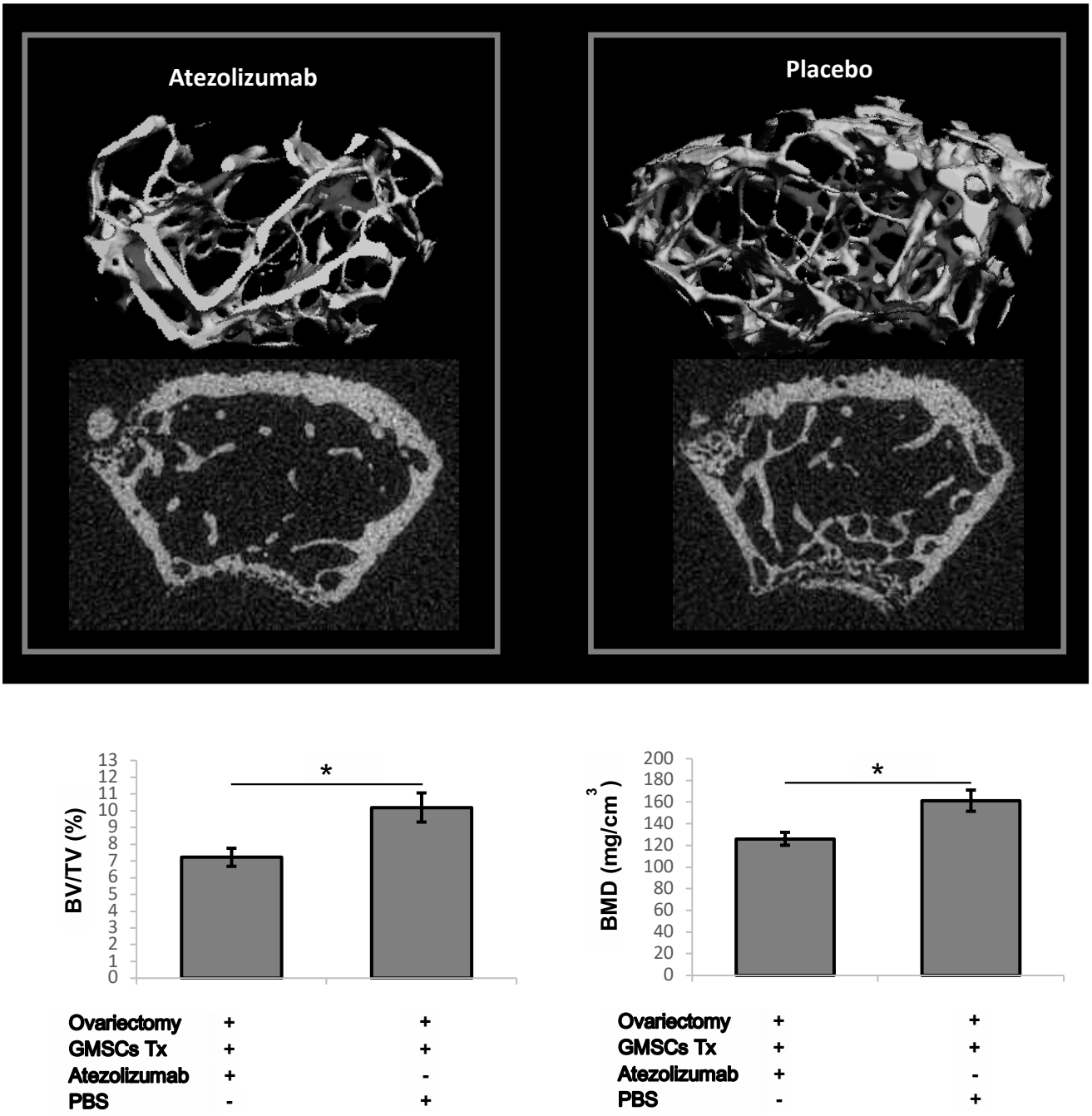
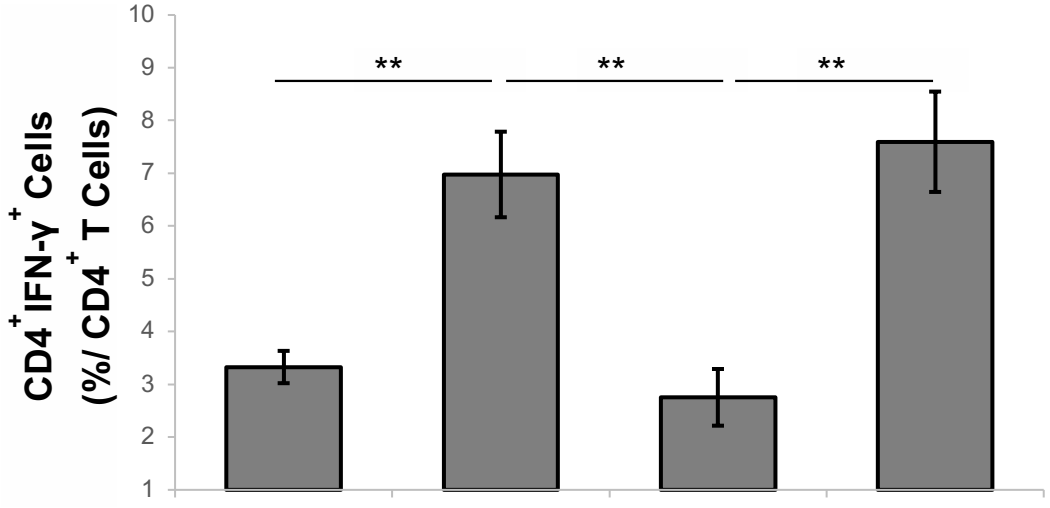
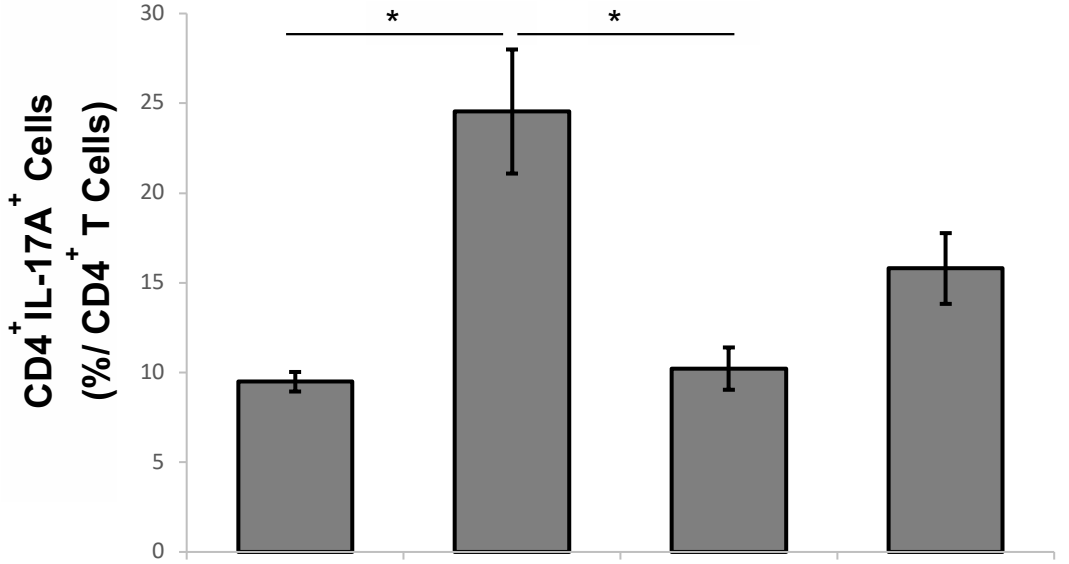


Figure.35 Femur Micro-CT images and analysis of Atezolizumab and placebo groups. Analysis of femurs shows an increased level of trabeculation in the placebo group vs Atezolizumab group as shown in BV/TV and BMD values.

Testing of GMSCs, siPdl1-GMSCs transplantations on subsets of T-cells in the OVX mice model.

We further tested the immune tolerance levels in the OVX mice treated with GMSCs and effect of GMSCs on the population of T-cells after the completion of the treatment period, We saw that at 4 weeks post-transplantation timepoint the GMSCs treatment had significantly downregulated Th1 and T17 levels in comparison to the levels seen in those of OVX mice. On the other hand, in siPdl1-GMSCs treated mice there was a marked increase in Th1 levels in comparison to the WT GMSCs treated mice, while there is still an increase in Th17 as well it wasn't as prominent as Th17. In addition to Th1, and Th17 levels, GMSCs treatment also significantly upregulated T-reg expression in the OVX mice and when testing the expression levels of T-regulatory cells in the siPdl1-GMSCs treated mice it was matching to those of the OVX mice confirming the failure of siPdl1 GMSCs to influence the expression of T-cells and the success of GMSCs in influencing T-cells in the OVX mouse model (Fig.36).




CD4+IL-17A+ Cells
(%/ CD4+ T Cells)
30
25
20
15
10
5
0
*
*

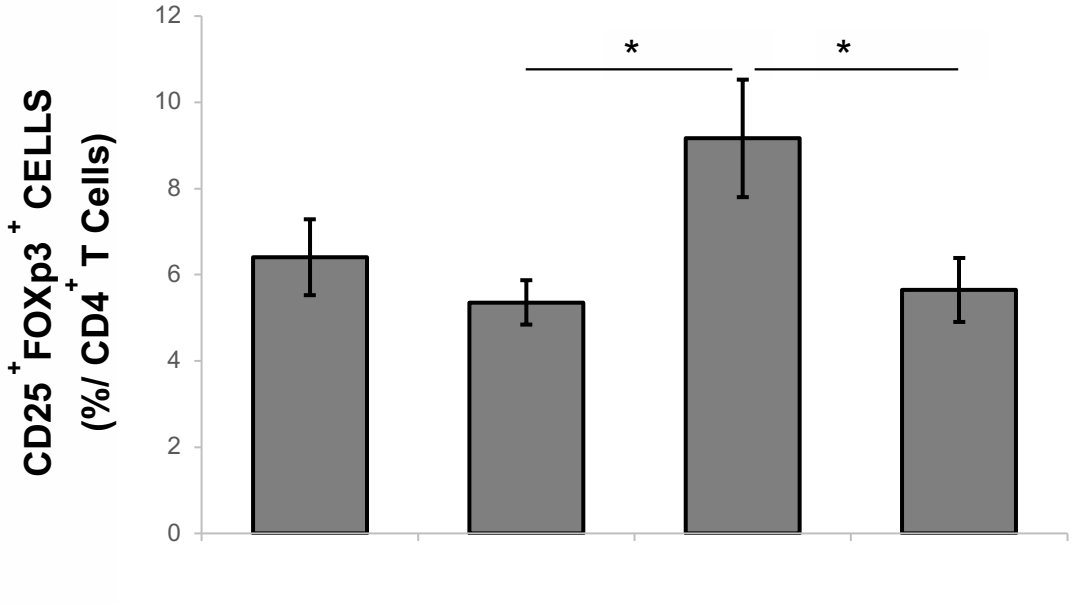
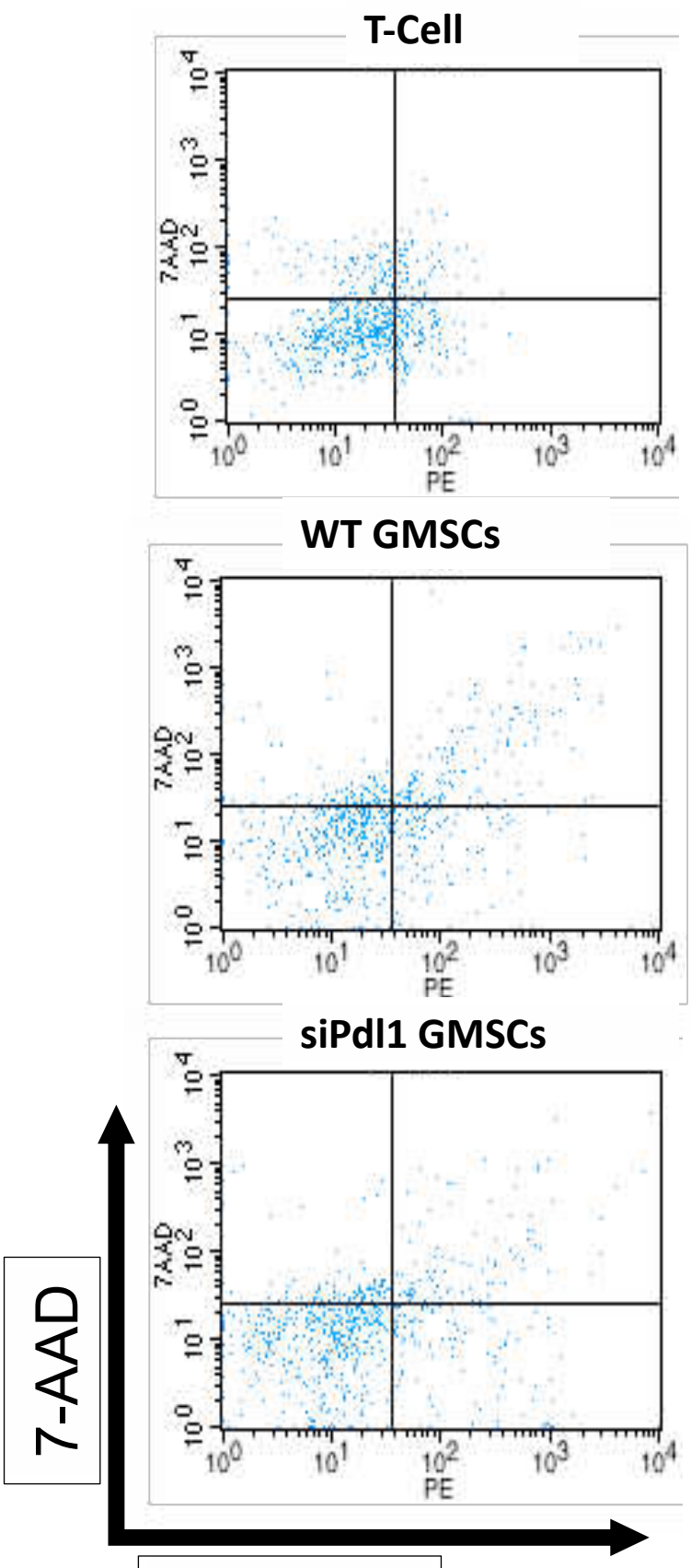

CD25+FOXp3+ CELLS
(%/ CD4+ T Cells)
12
10
8
6
4
2
0
*
*

Figure 36. Flow cytometry analysis of Th-1, Th-17 and T-reg levels. Th-1, Th-17 expression and T-reg levels in mice from different groups at sacrifice four-weeks post-transplantation.

Testing of GMSCs, siPdl1-GMSCs ability of inducing T-cells apoptosis in-vitro.

In order to examine the effect of GMSCs treatment on the population of T-Cells. We utilized an in vitro approach to test that effect. We harvested T-cells and activated them then cocultured them in vitro with wild type mice GMSCs, or siPDL1 GMSCs. T-Cells were then collected, and an Annexin V apoptosis assay/ flow cytometry analysis was completed. We noted that in after the 3 days of coculture, the apoptosis rate of T-cells only group was almost at 5%. The T-Cells collected from wells cocultured with GMSCs showed an apoptosis rate that is significantly higher at almost 30%, while the T-Cells harvested from the wells where they were cocultured with siPdl1 was significantly lower at around 19%. These findings confirm the ability of GMSCs to induce T-cell apoptosis as well as the finding reported previously that siPdl1 GMSCs are defective and unable to effectively induce T-cell apoptosis (Fig.37).


T-Cell
WT GMSCs
siPdl1 GMSCs
7AAD
PE
7-AAD
Annexin V

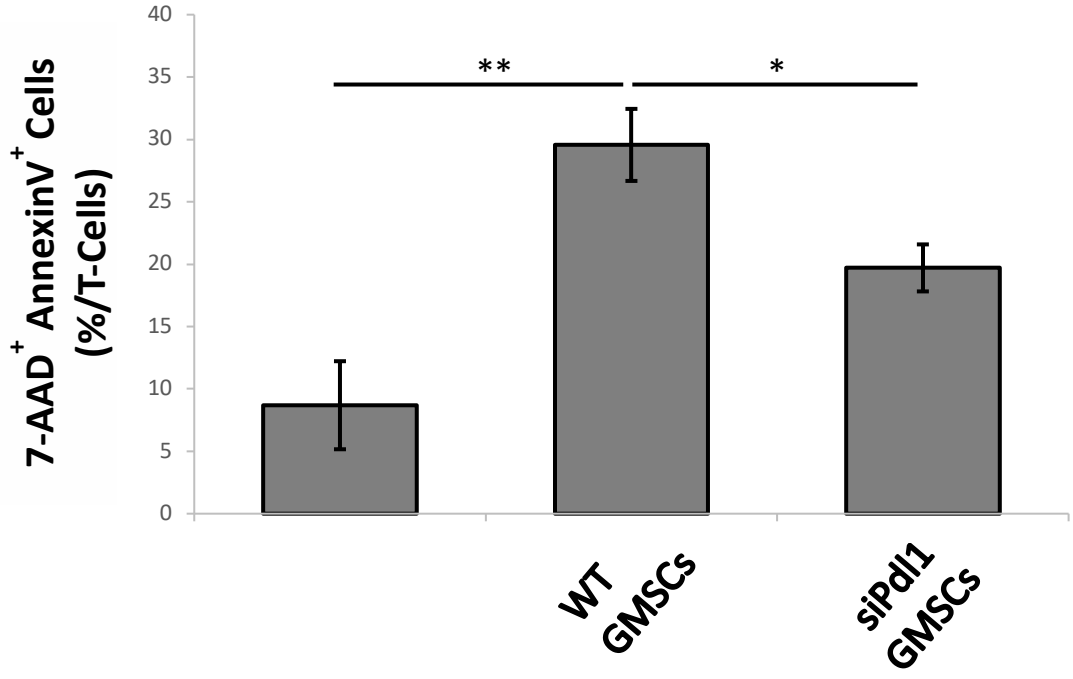


Figure 37. T-cell apoptosis assay. Coculture of siPdl1 and WT GMSCs with T-cells shows a higher ability of WT GMSCs to inducing T-cell apoptosis when cocultured with T-cells than the siPdl1 GMSCs.

Discussion

Osteoporosis is a skeletal system disorder that could occur in all ages at any given population resembling a great worldwide concern. The disease is also of a higher level of concern in particular in those populations of older men, and postmenopausal women as the osteoporosis-related fractures associated with the disease are of tremendous magnitude on healthcare systems. The treatment takes a toll on institutional financial resources. These fractures are also known to be of a higher risk of occurrence in women than in men (1,73,74). For the treatment of the disease, several interventional approaches are available to manage and treat those patients diagnosed with osteoporosis or at risk of developing it. Amongst the available and widely accepted treatment options are either pharmacological or non-pharmacological based treatments. The Non-pharmacological interventions are revolved mainly around dietary and lifestyle modifications such as the increase of calcium or vitamin D intake to achieve peak bone mass in an individuals' growth through life as well as the involvement of exercise routines, these interventions resembling more of a preventive approach and are aimed at reducing the risk of fractures (1).

On the other hand, pharmacologic interventions for diagnosed individuals include the utilization of antiresorptive agents in treatment such as the bisphosphonates class of medications used to reduces bone resorption by mainly increasing the apoptosis rate of osteoclasts (21). Different bisphosphonates class drugs that are used for the treatment of osteoporosis are effective in reducing bone

resorption rates (22-24), but their use has been associated with both short- and long-term adverse effects, with the latter including BRONJ, atypical femur fractures, and concerns of exaggerated suppression of bone turnover rates (25,26,75). Another class of antiresorptive agents available for use are estrogens and selective estrogen receptor modulators (SERMs) (27,28). their use as hormonal replacement therapy in treatment has been associated with reports of breast cancer and cardiovascular events (29,30). The third class of pharmacological, interventional drugs used in the treatment of osteoporosis are anabolic agents, they are different from the mechanism of action used by bisphosphonates as they act on the enhancement of bone formation rather than decreasing the bone resorption rates and an example of anabolic agents is the Parathyroid hormone (PTH). It is a well-known and potent inducer of bone matrix synthesis; it increases osteoblast activation and decreases their apoptosis rates (31). The use of the anabolic agent PTH in treatment of postmenopausal osteoporosis is deemed beneficial but the expense associated with the drug is a limiting factor for its use, in addition to that investigators found there was an incidence of osteosarcoma associated with its use in a two years animal study while testing different variety of doses leading to the recommendation of a limited time use (33,76).

Although the mentioned pharmacologic and non-pharmacologic interventions are currently employed as treatment methods for osteoporosis, the accompanying setbacks with their use could be considered a limitation to the effective utilization of these treatments. This increases the need for the

development of treatment approaches in the field as alternative methods to manage and treat the skeletal system disorder osteoporosis. Systemic infusions and cellular therapy investigations in disease models of chronic nature are of a wider potential for application nowadays; this is shown by the remarkable numbers of clinical trials investigating several diseases in different trial phases ranging from phase I to phase VI clinical trials. The investigations include mainly evaluating the use of MSC cell therapy in different fields like cardiovascular disease, autoimmune disease (i.e., SLE, GVHD, and SS), cutaneous skin defects, bone defects, and liver diseases (42). These present methods of investigation and the advancements of cell therapy applications in the field of research has made the search for a cell therapy-based approach popular. If a cellular infusion is effective therapeutically in the improving the bone phenotype of osteoporosis elevating its mass potentially without the adverse effects associated with those of pharmacological agents, it will lead to opportunities exploring and applying different approaches for treating osteoporosis in addition to a potential in the treatment of various types of immune-related diseases. MSCs in bone research are also deemed popular because of their abelites to directly replace damaged multiple tissues like that of bone, effectively influence cellular mechanisms of recovery, and exert a robust immunoregulatory effect (35,77-79).

In our investigation, the estrogen deficiency-related osteoporosis is an inflammatory disease in nature due to the increase in overall inflammation in the body caused by removal of ovaries or menopause, which is supplemented by an increase in the pro-inflammatory cytokines caused by estrogen deficiency and the

involvement of T-cells that is seen in ovariectomized mice (80-82), this background of the disease along with the immunomodulatory properties and multipotent differentiation potentials of MSCs impelled us to explore the therapeutic and immunomodulatory effects of a dental tissue-derived population of mesenchymal stem cells in a small animal model (OVX) which is a well-established model in bone disease research field (83). While the therapeutic outcome for our cell therapy approach can be investigated as a preventative measure against any OVX associated bone loss, and as a transplantation of SHED has already successfully prevented early phases of bone loss in OVX mice (59), we wanted our experimental timeline for the disease model to allow an adequate period for the defectiveness in the bone to develop. As for the reason we chose to use of GMSCs; they are available after birth, and their application has been explored in multiple disease models previously, and they have proven efficient in the immunomodulation of the host immunologic environment, their effect on the bone phenotype and application in the osteoporotic disease model has not been evaluated. Furthermore, additional reasons we chose to utilize mesenchymal stem cells from the gingiva in our study is that they hold several advantages over other sources of MSCs. They not only possess immunomodulatory properties and multipotent differentiation potential, the procedure of obtaining GMSCs from a source and their harvesting hold less morbidity in comparison to other types of MSCs, and it is high in proliferation.

In our investigation we found that a single systemic infusion of GMSCs was successful in causing a significant improvement in the osteoporotic bone

phenotype caused by estrogen deficiency in the OVX mice, this improvement was seen in the long bones of animals, and it was mainly demonstrated through studying and analyzing the distal metaphyseal region of the femurs as it has been reported to be amongst the commonly used methods to asses bone trabeculation in mice (84). This improvement was also shown in the mandibles of mice as evaluated by measuring and analyzing the retromolar area distal to M3, revealing an improvement in GMSCs treated mice mandibles in comparison to the significantly less trabeculation seen in the OVX mice. In femurs, in addition to our results showing an elevation of bone trabeculation levels of the distal metaphysis of femurs, an increase in the total bone volume, a decrease of the numbers of osteoclasts, a decrease of fatty marrow tissue occurred in femurs, and an increase in the MAR as measured by double calcein labeling, confirming that the overall bone phenotype was improved after the systemic infusion of mice. Mandibles also had a similar effect in the bone trabeculation of the mandibles, as shown by H&E stain and analysis. In addition to the femurs and mandibles analysis showing an improved phenotype, the blood serum analysis of mice showed elevated levels of IFN-γ, IL-17, and RANKL in the OVX mice as it was reported that after estrogen deficiency occurs, T-lymphocytes play a role in the bone loss (18,19). This increase was diminished by the infusion of GMSCs which did effectively reduce the level of these serum markers.

Furthermore, there is a deterioration of the endogenous populations of bone marrow stem cells in OVX mice, which contributes to the reduction in bone mineral density (85). Thus, after we confirmed the in-vivo effects of OVX in mice femurs

and mandibles, and we observed the improvement in the phenotype as a result of the GMSCs infusion as well as the reduction seen in some blood serum markers of inflammation, we wanted to investigate further if the deterioration of endogenous BMMSCs in OVX mice would be rescued and their function would be improved in response to the GMSCs infusion by functionally testing those BMMSCs harvested from animals at the end of our timeline. Our first step in testing was to check the colony formation ability of BMMSCs and test their ability to form colonies as well as if there was any difference between the groups, while cells from OVX mice had a slightly non-significant level of higher colonies formed in vitro than those of BMMSCs from other groups, this could be potentially explained that those from OVX mice are higher in colonies formed due to an overactivation or defectiveness of their BMMSCs leading to higher colonies formation. This trend was similar when measuring the proliferation capacities of BMMSCs of groups, OVX mice BMMSCs had a significantly higher level of proliferation, while those from GMSCs-treated mice had a comparable level of proliferation to those seen in the control group. The proliferation rate could be an additional confirmation to our explanation that BMMSCs from OVX mice are in an overactivated stage to compensate for the defectiveness in comparison with those from control and GMSCs-treated mice. Furthermore, BMMSCs are multipotent differentiation potential includes their capacity to be induced to adipogenic, osteogenic, or chondrogenic tissues under certain conditions and are identified as superior in their osteogenic differentiation potential (79,86).

In our study we tested the osteogenic and adipogenic potential of BMMSc harvested from mice to measure the differentiation potential, we found that those from the control group have the highest osteogenic differentiation potential and the lowest adipogenic potential of all groups, on the other hand, BMMSCs that were harvested from OVX mice had the highest adipogenic differentiation potential and the lowest osteogenic differentiation potential of all groups. This indicates a higher adipogenic tissue formation tendency related to those BMMSCs of the OVX mice and a less capability for osteogenic differentiation. When we looked at BMMSCs from the GMSCs-treated mice, we found that the differentiation potential was comparable to those from the control mice with a higher level of osteogenic differentiation potential than adipogenic differentiation potential. This was further confirmed by analyzing protein expression levels of adipogenic and osteogenic markers of BMMSCs undergoing induction by measuring osteogenic markers ALP, and RUNX2 and adipogenic markers LPL and PPAR-G. Osteogenic markers were expressed in a markedly higher level in BMMSCs from control and GMSCs-treated groups than the OVX group. In agreement with the differentiation capacity, adipogenic markers were highly expressed in BMMSCs from the OVX group, and that expression was seen less in BMMSCs from the control and GMSCs-treated groups. These findings suggest that the functionality of BMMSCs was improved from the GMSCs transplantation and that the defectiveness of BMMSCs cause by the ovariectomy is rescued. In order to further verify the function of BMMSCs was rescued, we tested the in-vivo bone formation ability of BMMSCs harvested from mice through transplanting the cells into dorsal surfaces of immunocompromised

mice after mixing them with HA/TCP. We saw that grafts that were incorporated with BMMSCs from the control or GMSCs-treatment mice had a significantly higher ability to form new bone when compared to those incorporated with BMMSCs from OVX mice, which had a significantly lower level of bone formation. These data showed the rescue of endogenous BMMCSs from the infusion of GMSCs and not only by in-vitro testing of BMMSCs function, but it was also observed in-vivo after grafts incorporated with BMMSCs from GMSCs-treated mice showed a higher level of new bone formation.

After we established the efficacy of GMSCs transplantation and explored the influence they have on improving bone phenotype and the function of the endogenous population of BMMSCs. We wanted to test whether there was an exerted effect of the GMSCs transplantation on the recipients' T-cell population and if the mechanism related to improvement seen by GMSCs transplants is due to its ability to suppress activated T-cells just as its capable of achieving in vitro, as it's already known that the T-cell activity is higher in postmenopausal osteoporosis in both humans and mice, highlighting the role of T-cells in the osteoporotic bone loss caused by estrogen deficiency (63,87,88). Furthermore, when it comes to systemic infusions and stem cell therapies, the mechanisms behind the therapeutic effect and immunomodulation achieved are still in the process of being fully understood. In our study, we established the presence of a positive effect from the GMSCs systemic infusion and further investigated possible pathways involved in the underlying mechanism leading to immune tolerance.

It is reported in the literature that the immunomodulatory properties achieved of a systemic infusion using BMMSCs involved the Fas/FasL cell death pathway, and it was revealed that BMMSCs expressed both FAS and FASL and that the FAS/FASL pathway is involved in the mechanism behind the BMMSCs-mediated therapeutic effect and the induction of T-cell apoptosis which leads to immunologic tolerance (89). For the cells of the body to undergo apoptosis, there are multiple known cell death pathways involved. Fas and FasL, a receptor and its ligand which are members of receptors in the subset of Tumor Necrosis Factor Receptor (TNF-R) superfamily and a part of the FAS/FASL pathway, also known as the CD95-CD95L pathway, and plays a significant role in which the immune system's programmed cell death by apoptosis is initiated achieving homeostasis (90,91). Since FasL is a type II transmembrane protein, the proteins forming as a consequence of its interaction with Fas initiates the Death Inducing Signaling Complex (DISC) (92,93). and because the Fas/FasL pathway is an identified method in BMMSCs-mediated induction of T-cell apoptosis and immunomodulatory homeostasis, as previously mentioned (89).

We wanted to investigate the FasL/Fas pathway and whether GMSCs would be similar to BMMSCs, and it would be the pathway involved in the GMSCs mediated immunomodulatory effect. To test that, we used gingival tissues from both FAS and FASL deficient mice B6Smn.C3-Faslgld/J and B6.MRL-Faslpr/J to harvest GMSCs for transplantation. We tested the effect of FasL or Fas knockdown GMSCs each separately in-vivo by transplanting them into the OVX mouse following the same previously mentioned experimental timeline (FIG.4). To

our surprise, our results and what we observed as a result of the transplantation after sacrifice was that neither FasL nor Fas knockdown GMSCs had a negative effect as we expected on the bone phenotype and they both had the same therapeutic effect as did the wild type GMSCs in the OVX mice, this was tested in femurs by measuring the BMD, Total bone Volume, bone trabeculation levels, and osteoclasts numbers. What this revealed to us was that GMSCs-mediated effect in the OVX mice was not established through the FAS/FASL pathway as seen in other types of MSCs (i.e. BMMSCs) this could be due to the difference in harvesting source with GMSCs being a type of dental tissue-derived stem cells and may mean they possibly use an alternative cell death pathway in the process of immune regulation or it could be that GMSCs transplantation did not affect the T-cell population of the host at all and the effect was seen in OVX mice is independent of any immune response and solely to the ability of GMSCs to directly differentiate into bone leading to an improvement in the overall bone mass and function of BMMSCs. After testing the Fas/FasL pathway, we looked further in-depth for other potential pathways that may be behind the GMSCs' effect in the OVX mice.

Another pathway we came across involves the PD-1 or programmed cell death one receptor, which is part of the CD28 family, and the PD-1/PD-L1 programmed cell death pathway is a popular cell death pathway in the research field. It is known to be an important pathway for apoptosis in many cell types, including T-cells, playing an effective role in immunomodulation, and immune tolerance as PD-1 has been shown to be expressed by different immune cells,

including B-cell, activated T-cells, macrophages, dendritic and natural killer cells (67,68,94). The pathway is also popular in the field of cancer research, and the reason it is heavily investigated is that the blockade of the PD-L1/PD-1 pathway exhibits an effect on activated T-cells, and clinical trials have already shown that anti-PD-1 drugs affected the regression of solid tumors. In addition to that, tumor cells may use the PD-1 pathway for evading the immune system, but by blocking the PD-1 pathway, the chance of cancer cells evading the immune system is eliminated. Clinical trials are underway investigating the targeted therapeutic blockade of PD-1 and PD-L1, such as the already approved FDA drugs nivolumab and ipilimumab, which are used for combination therapy of certain types of melanomas (69,95-97). As for MSCs, It has been reported in the literature that BMMSCs have the ability to express PD-L1 and directly induced T-cell suppression via the PDL1/PD1 pathway highlighting the importance of PD-L1 in BMMSC mediated immunomodulation and immune tolerance through T-cells (72). While BMMSCs are known to only express PD-L1 and not PD-1, dental tissue-derived stem cells like DPSCs and SHED are shown to express both PD-1 and PD-L1. For PD-L1, it has been shown to be expressed in the cytoplasm of the cells, while PD-1 is expressed in the membrane of the cells. PD-1 also has an important role in maintaining neural crest-derived dental pulp MSCs as it has been identified to participate in proliferation maintenance (98).

Furthermore, as the gingiva contains dental tissue-derived stem cells, and it also is known to contain roughly 90% neural crest-derived GMSCs and 10% from the mesoderm, and it had the ability to induce activated T-cell apoptosis in-vitro

(63). Therefore, we tested the PD-1/PD-L1 pathway, and we hypothesized that GMSCs being a dental tissue-derived stem cell population expresses PD-L1 like DPSCs and SHED and that PD-L1 pathway is the pathway in which GMSCs effect is seen in the ovariectomized mice model. To test this hypothesis, we utilized the siRNA approach to knockdown PD-L1 in GMSCs and used them for our transplantation. Our findings revealed that a single siPdl1 GMSCs transplantation was not successful in improving the bone phenotype in the OVX mice, this was also tested by scanning the femurs and analyzing the BMD, Total bone Volume, as well as testing measuring femurs levels of bone trabeculation, osteoclasts numbers, and fatty marrow levels which were confirmatory to the computed microtomography analysis of femurs. These data suggested that the effect on the bone and BMMSCs that we saw as a result of a GMSCs infusion in the OVX mouse model involves the PDL1/PD1 pathway.

Furthermore, to verify the involvement of PD-L1 in the GMSCs-mediated effect we utilized the use of an anti-PDL1 drug (Atezolizumab) and tested the effect on two groups, a wild type GMSCs-treated group receiving a placebo of PBS and wild type GMSCs treated group receiving two injections of Atezolizumab twice in a period of four weeks after the GMSCs treatment. The placebo group showed a significant increase in BV/TV, BMD values as measured and analyzed by computed microtomography in comparison to the Atezolizumab drug treatment group, this verifies the involvement of PD-L1 as in immune checkpoint for GMSCs mediated effect in the OVX mouse model, we utilized the drug Atezolizumab in our experiment as it has been approved recently by the FDA for treatment in multiple

cancer types pertaining to the lungs, and breasts in humans, it targets reestablishing anti-tumor T-cell activity by the blockade of PD-L1 protein which the expression of decreases T-cell anti-tumor activity (99-101). In our study, by witnessing a diminished effect of GMSCs infusion on the OVX bone phenotype, we confirm the role of PD-L1 as an immune checkpoint in the GMSCs-mediated effect in the ovariectomized mouse model. In addition to that, we tested the influence of GMSCs and siPdl1 GMSCs on the population of T-cells of mice and saw that the ovariectomized mice had a markedly higher level of Th-1 and Th-17, and a lower level of T-regs as determined by FACS. The levels of Th-1 and Th-17 in the GMSCs treated mice were significantly lowered when compared to the OVX group, and the T-regs where higher. The siPdl1 GMSCs treated mice had a similar expression of Th1, T-reg to that in OVX mice, and a less of similarity in Th-17. These data of T-cells subsets confirm the ability of GMSCs transplantation to reduce the levels of Th-1, Th-17 expression, and elevate T-reg expression leading to immune regulation. Lastly, when we tested the abilities of GMSCs and siPdl1 GMSCs to induce T-cell apoptosis in vitro, we observed a marked elevation in the levels and capabilities of WT GMSCs to induce T-cell apoptosis when compared to those of siPdl1-GMSCs. This further confirms the importance of siPdl1 in the identified influence of GMSCs on T-cells.

Conclusions

- Ovariectomy induced osteoporosis is a valid small animal model leading to significant deterioration of bone phenotype.

- A Single transplantation of GMSCs into the OVX model markedly improved the bone phenotype.

- A Single transplantation of GMSCs into OVX mice significantly improved the function of endogenous BMMSC population and rescued them.

- FAS/FASL pathway is not the pathway of which GMSCs exert its effect in the OVX mice.

- GMSCs immune-mediated effect in the OVX mice is through PD-L1 immune checkpoint and the influence of transplantation on T-cell populations leads to immune tolerance.

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