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14. ABSTRACT Bone is the most common site of metastasis for human breast cancer (BCa), which results in significant morbidity and mortality in patients with advanced disease. A vicious cycle, arising due to the interaction of BCa cells and cells in the bone microenvironment results in the activation of osteoclasts and increased osteolytic bone destruction. The major treatment to reduce the burden of bone metastasis in BCa patients is bisphosphonate therapy. Despite significant efforts to improve the potency of bisphosphonates, the complications are only retarded but not prevented. Thus, development of newer therapies that can both ameliorate the threshold of bone destruction and increase survival of patients with metastatic breast disease will be highly beneficial. The central hypothesis of the proposed work is bone-targeted delivery of genetically-engineered MSC, over-expressing OPG, will prevent osteolytic bone damage and restore skeletal remodeling. Further, based on the requirement of angiogenesis for tumor growth in primary and metastatic sites, in combination with a systemically stable anti-angiogenic therapy, long-term survival will significantly increase. These hypotheses will be tested in this proposal using an immunocompetent, preclinical mouse model of BCa dissemination to all major bones as in human patients.					
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INTRODUCTION

Bone is the most common site of metastasis for human breast cancer (BCa), which results in significant morbidity and mortality in patients with advanced disease. A vicious cycle, arising due to the interaction of BCa cells and cells in the bone microenvironment results in the activation of osteoclasts and increased osteolytic bone destruction. The major treatment to reduce the burden of bone metastasis in BCa patients is bisphosphonate therapy. Despite significant efforts to improve the potency of bisphosphonates, the complications are only retarded but not prevented. Thus, development of newer therapies that can both ameliorate the threshold of bone destruction and increase survival of patients with metastatic breast disease will be highly beneficial. The central hypothesis of the proposed work is bone-targeted delivery of genetically-engineered MSC, over-expressing OPG, will prevent osteolytic bone damage and restore skeletal remodeling. Further, based on the requirement of angiogenesis for tumor growth in primary and metastatic sites, in combination with a systemically stable anti-angiogenic therapy, long-term survival will significantly increase. These hypotheses will be tested in this proposal using an immunocompetent, preclinical mouse model of BCa dissemination to all major bones as in human patients.

Specific Aims:

- 1) To determine therapeutic effects of genetically-modified MSC, overexpressing OPG, for osteolytic bone damage using a bone-targeted delivery, in an immunocompetent mouse model of BCa dissemination to the bone
- 2) To determine the combined effect of MSC-OPG therapy with systemically-stable anti-angiogenic therapy for long-term survival.

BODY

During the first two years of the project, we identified domains in osteoprotegerin that bind to TRAIL and successfully abolished them to eliminate cancer cell survival, yet providing protection against cancer-induced bone loss. These studies were performed both in vitro and in vivo. Further, we tested the potential of OP, delivered using mesenchymal stem cells, engineered to overexpress OPG, in combination with an anti-angiogenic therapy. These studies were tested in a mouse model that exhibits osteolytic lesions upon tumor challenge only in limbs. Towards extrapolating the positive outcome to human situation, where breast cancer patients show osteolytic malignancy in all major bones, we developed a mouse model of osteolytic lesions in all major bones and tested the potential of modified OPGin reducing bone pathology.

In vivo analysis of OPG mutants in SCID animal model with osteolytic malignancy in all major bones: As in the first studies using MSC for in vivo expression of OPG, we used AAV-OPG-mutants to transduce hMSC to express OPG-wt or OPG-mutants (Y49R or F107A) 48 hrs before injection into tumor challenged mice. Cohorts of SCID mice were intravenously injected with $\sim 10^6$ of the human osteolytic cancer line, CAG^{hep}, followed by non-invasive imaging. Fourteen days post tumor cell injection and upon establishment of tumor growth within the skeleton, as confirmed by non-invasive image analysis, $\sim 10^6$ hMSC that were transduced via AAV to express either OPG-wt or OPG-mutants Y49R or F107A were administered via intra-cardiac route. Mice were then monitored by non-invasive bioluminescence imaging weekly for assessment of tumor growth kinetics. Results of this study demonstrated the localization of CAG^{hep} cells to both tibia and femur as early as day-7 and by day-14 the CAG^{hep} cells could be detected in the vertebra column. Fourteen days post hMSC administration, non-invasive imaging showed an overall delay of tumor growth in mice treated with either OPG^{wt} or OPG^{mut} when compared to the naïve control group.

Osteoprotegerin mutants protect against tumor-induced osteoclastogenesis in both spine and tibia: Post hMSC-OPG therapy, bones were harvested for micro-CT analysis. Results demonstrated that mice treated with either OPG^{wt} or OPG^{mut} (Y49R or F107A) significantly protected against osteolytic bone destruction induced by CAG^{hep} cells when compared to untreated mice (CAG^{hep} cells only). Results of these studies confirmed a preliminary finding, which also demonstrated bone protection with the use of OPG^{wt} or OPG^{F107A}. It was shown for the first time that the use of OPG^{Y49R} provides protection against cancer-induced osteoclastogenesis and that overall protection is comparable to both OPG^{wt} or OPG^{F107A}. Three dimensional reconstruction of micro-CT analysis showed significant destruction to the trabecular bone under the growth plate in the tibia of mice challenged with CAG^{hep} cells. Similarly, significant bone destruction was observed in the spine, particularly the L4 bone of the lumbar region in the spinal column; as 3D reconstruction images showed complete severance of the L4 bone in some of the naïve control mice (CAG^{hep} cells only). However, images of bone 3D reconstruction for cohorts treated with OPG therapy showed intact tibia and lumbar spinal bone with bone density and trabecular connectivity comparable to age matched control groups.

OPG-mutants protect against cancer-induced osteolysis through decrease activation of osteoclasts: Harvested bones were decalcified and sectioned for IHC staining and the presence of tumor was seen in both tibia and spine when H&E stain was performed. While there was not any observable difference in bone loss within the cohorts treated with the different OPG-mutants and OPG-wt when compared to its age-matched controls, there was however, visible bone destruction both in tibia and spine in the cohorts of mice that was challenged with CAG^{hep} cells. A TRAP stain was performed to assess the osteoclast activity and as expected in the tumor challenged cohort there was an observable increase in osteoclast activity when compared to the age-matched controls. Interestingly, when comparing the hMSC-OPG treated cohorts to the age-matched controls, there was a slight increase in osteoclast activity due to the presence of tumor within the bone cavity as seen with the H&E stains, but when compared to the tumor challenged cohort, there was noticeable decrease in osteoclasts, which can account for the decrease in bone destruction observed in these treated groups. Results of these studies are provided below.

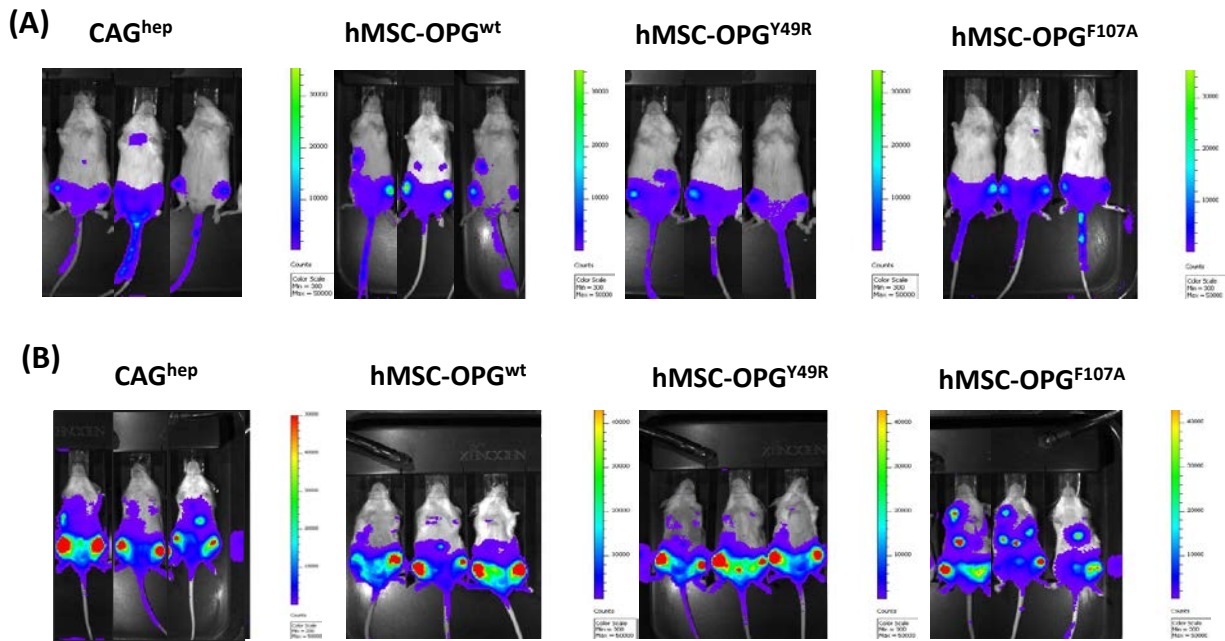


Figure 1. Monitoring of osteolytic tumor growth in both legs via noninvasive bioluminescence imaging during hMSC-OPG therapy. Cohorts of mice were challenged with $\sim 10^6$ CAG^{hep} cells via tail vein injections followed by noninvasive imaging for establishment of tumor cells within the tibia and femur. Cohorts were then administered hMSC-OPG^{wt}, hMSC-OPG^{Y49R}, or hMSC-OPG^{F107A} and continued being monitored for tumor growth in the tibia, femur and other skeletal regions on day-14, before OPG therapy (A) and day-28, 14 days after OPG therapy (B).

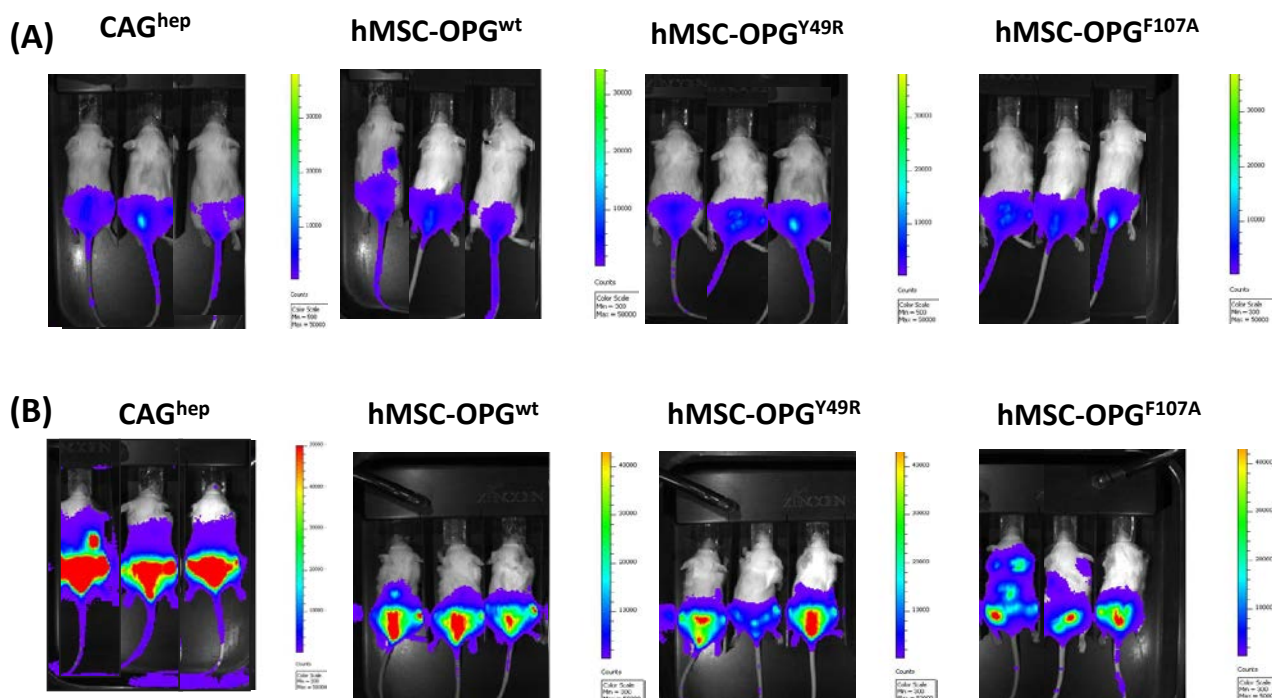


Figure 2. Monitoring of osteolytic tumor growth in spinal region via noninvasive bioluminescence imaging during hMSC-OPG therapy. Cohorts of mice were challenged with $\sim 10^6$ CAG^{hep} cells via tail vein followed by noninvasive imaging for establishment of tumor cells within the spine. Cohorts were then administered hMSC-OPG^{wt}, hMSC-OPG^{Y49R}, or hMSC-OPG^{F107A} and continued being monitored of tumor growth in the spine and other skeletal members on day-14, before OPG therapy (A) and day-28, 14 days after OPG therapy (B).

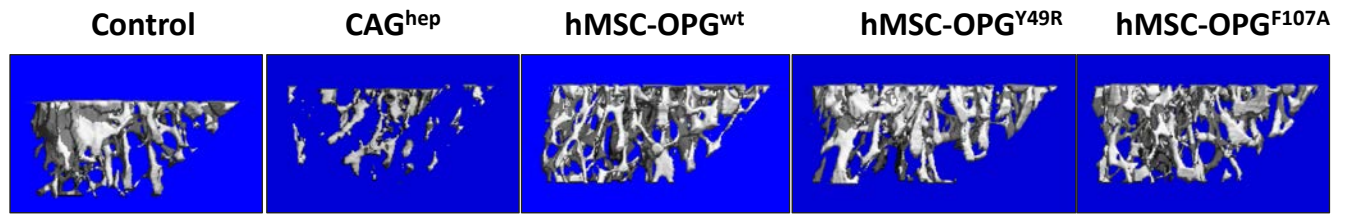


Figure 3. Evaluation of tumor-induced osteolysis by 3D reconstruction of the tibia post hMSC-OPG therapy. At the end of OPG therapy, bones were harvested from cohorts of mice for micro-CT analysis. Results of 3D reconstruction images demonstrated significant tibia destruction in cohorts of mice challenged with CAG^{hep} cells with overall bone protection observed in cohorts treated with hMSC-OPG^{wt}, hMSC-OPG^{Y49R}, or hMSC-OPG^{F107A}.

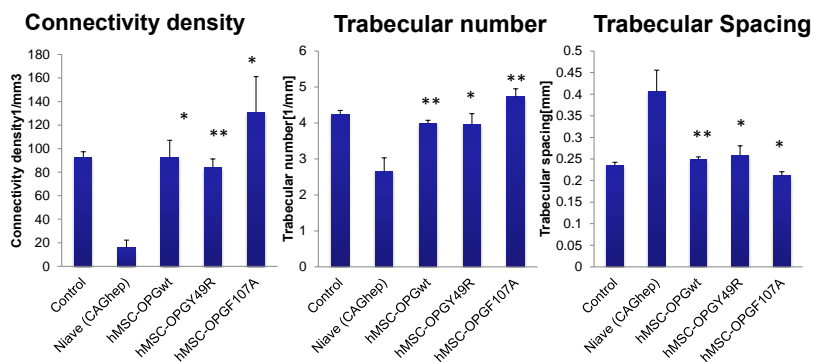


Figure 4. Micro-computed tomography analysis of tibia post hMSC-OPG therapy. Quantitative analysis revealed statistically significant differences in connectivity density, trabecular number, and trabecular spacing when comparing naïve treated group (CAG^{hep} cells only) to hMSC-OPG treated groups (B). (p* < 0.05, p** < 0.01)

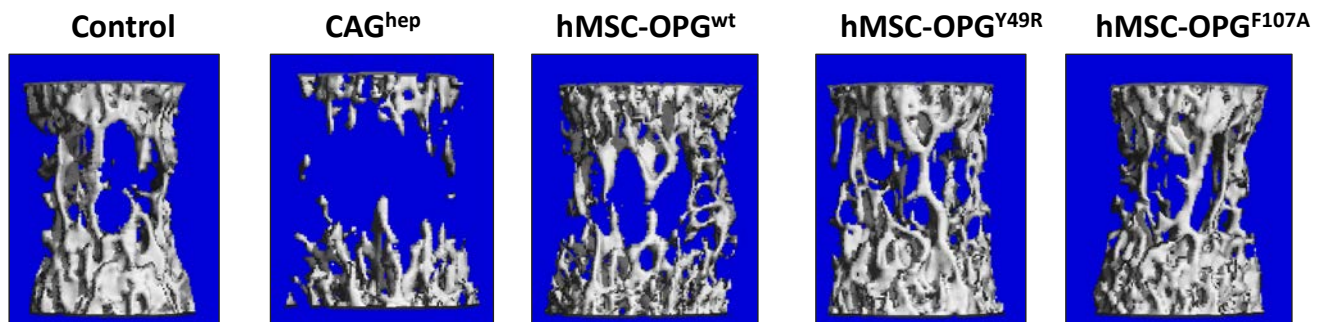


Figure 5. Evaluation for cancer-induced osteolysis by 3D reconstruction of the spine post hMSC-OPG therapy. At the end of OPG therapy, bone tissues were harvested from cohorts of mice for micro-CT analysis. Results of 3D reconstruction images demonstrated significant spinal destruction in cohorts of mice challenged with CAG^{hep} cells with overall bone protection observed in cohorts treated with hMSC-OPG^{wt}, hMSC-OPG^{Y49R}, or hMSC-OPG^{F107A}.

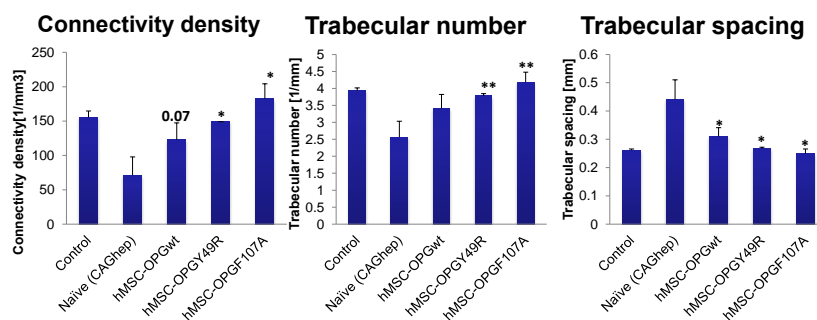


Figure 6. Micro-computed tomography analysis of L4 spinal bone post hMSC-OPG therapy. Quantitative analysis also revealed statistically significant differences in connectivity density, trabecular number, and trabecular spacing when comparing naïve treated group (CAG^{hep} cells only) to hMSC-OPG treated groups. (p* < 0.05, p** < 0.01)

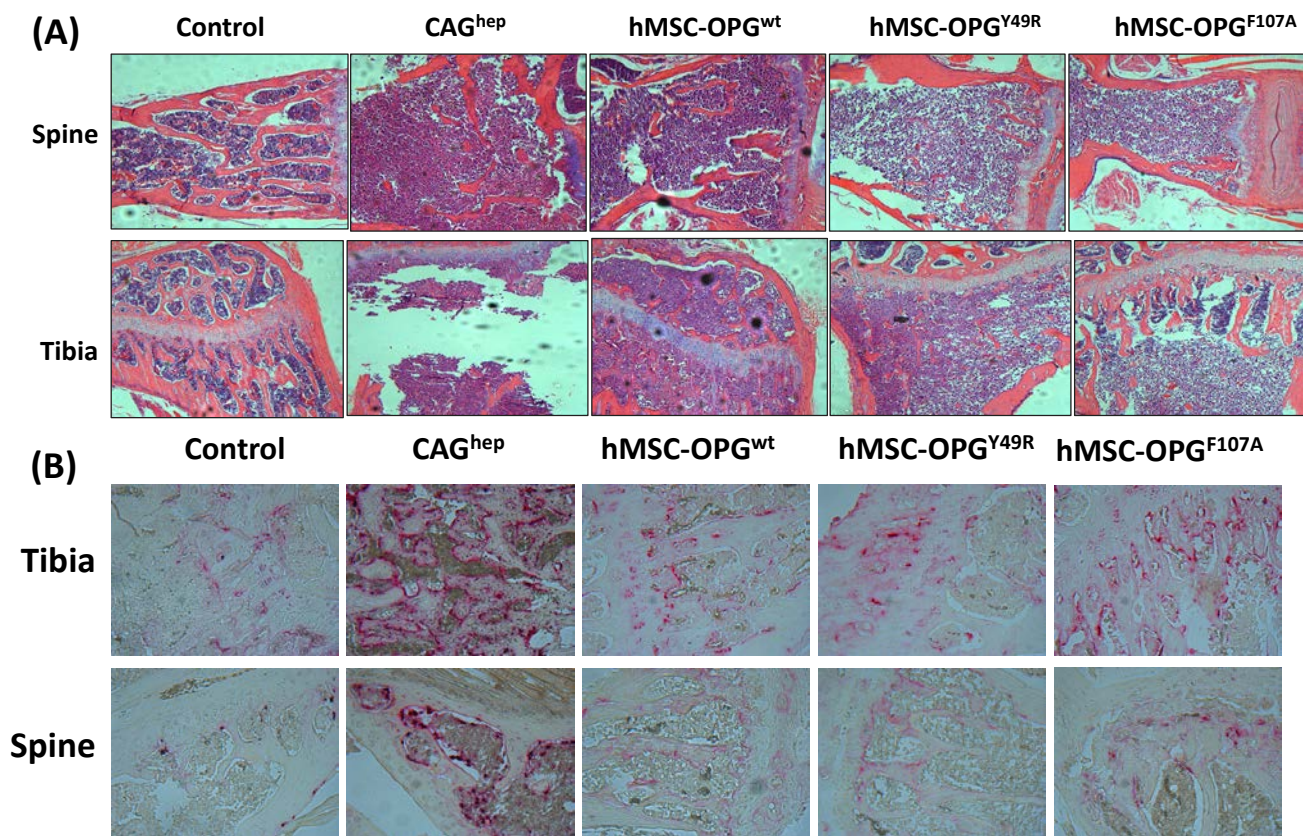


Figure 7. Immunohistochemistry staining of tibia and spine post hMSC-OPG therapy. Bone tissues were harvested post hMSC-OPG therapy, decalcified, and stained for assessment of the therapy. Hematoxylin and Eosin stain was performed for observation of tumor growth within the bone cavity of both tibia and spine (A). To assess overall osteoclast activity within the bone microenvironment, TRAP stain was performed on the both tibia and spine (B).

KEY RESEARCH ACCOMPLISHMENTS:

- Developed of a mouse model with osteolytic bone dissemination of tumor in all major regions of the skeleton
- Tested the potential of OPG mutant therapy using MSC
- Observed a significant decrease in osteolytic burden following therapy with OPG mutant in both limbs and spine.

REPORTABLE OUTCOMES

None

CONCLUSIONS

rAAV-OPG.Fc treatment was highly effective in inhibiting metastatic progression of osteolytic carcinoma cells, disseminated to various skeletal regions, similar to the human patients. Final validation of this will be performed in combination studies.

PERSONNEL RECEIVING PAY FROM THIS GRANT

Selvarangan Ponnazhagan, Ph.D.
Anandi Sawant, Ph.D.