

Figure 2. Immunoblot analysis of purified OPG mutants. Forty-eight hours following transfection of expression plasmids containing the sequences of OPG-wt and OPG-mutants in HEK-293 cells, supernatant media were collected and concentrated using a 30 kDa filter. The concentrated media were then separated in a SDS-PAGE and transferred to nitrocellulose membrane. Loading control was assessed by Coomassie Blue staining (A) and detection of OPG-wt and OPG mutants was performed using antibodies for IgG (B) and OPG (C).

human osteolytic prostate cancer cell line PC3, constitutively expressing firefly luciferase. Following confirmation of engraftment of the tumor cells by noninvasive imaging, human MSC, transfected with expression vectors encoding OPG^{wt} or OPG^{mut} (Y49R or F107A), were injected in the same tumor microenvironment within the tibia or systemically via tail vein. Mice were sacrificed on day 14 post MSC therapy and tibiae were isolated for micro-CT analysis. Results of this analysis confirmed significant bone destruction in the naïve group that received PC3 cells only. However, mice treated with either OPG^{wt} or OPG^{mut} (Y49R or F107A) demonstrated a significant increase in trabecular bone architecture and trabecular connectivity density as compared with the control group both by intratumoral and systemic administration of genetically engineered MSC, expressing OPG^{mut} thereby providing evidence that the developed OPG mutants Y49R and F107A were therapeutically effective *in vivo* against cancer-induced osteolytic bone damage (Fig. 5A and B).

Discussion

Osteoclasts are the primary cells for physiologic and pathologic bone resorption during bone remodeling, and RANKL is critically involved in the differentiation, activation, and survival of these cells upon binding to its specific receptor RANK and inducing

osteoclast differentiation of progenitor macrophages (7, 14). Increased expression of RANKL with certain malignancies, including breast cancer, prostate cancer, and multiple myeloma, causes activation of osteoclasts and remains an important mechanism in the formation of osteolytic bone lesions (15–17). The RANKL-RANK signaling axis, therefore, has become an important target for therapeutic intervention of osteolytic bone pathology. On the basis of the potent inhibitory actions of OPG on osteoclast differentiation and function, the therapeutic application of OPG in treatment of metastatic bone destruction has been recently studied (18–22). The outcome of phase I clinical trial using purified OPG in patients with multiple myeloma and breast cancer indicated that OPG was well tolerated and that a single dose suppressed levels of bone resorption markers, comparable to treatment with pamidronate (23). Thus, improving the biologic properties of OPG by uncoupling its TRAIL-binding ability will provide a crucial advancement in the utility of OPG for treatment of osteolytic malignancies. Results from the present study indicate that the two novel OPG variants, Y49R and F107A, indeed show a positive effect in bone remodeling following cancer-induced

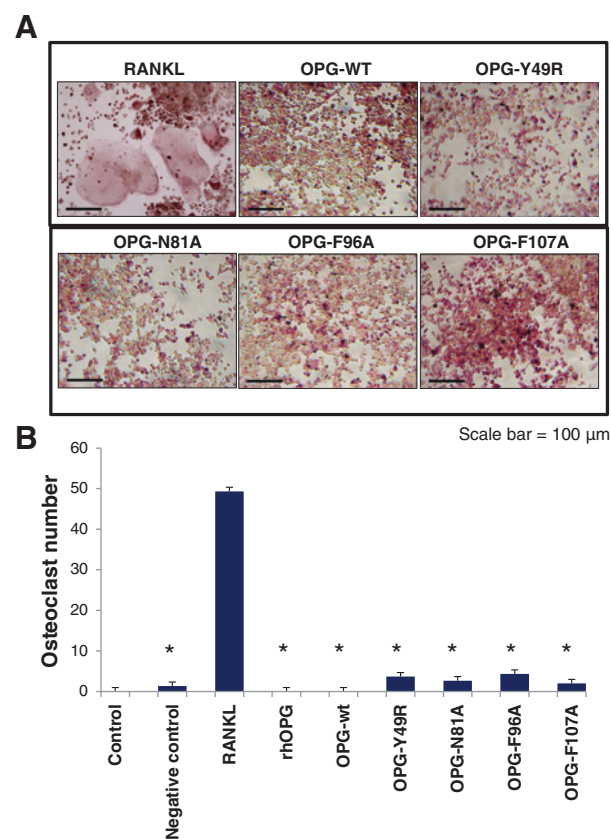
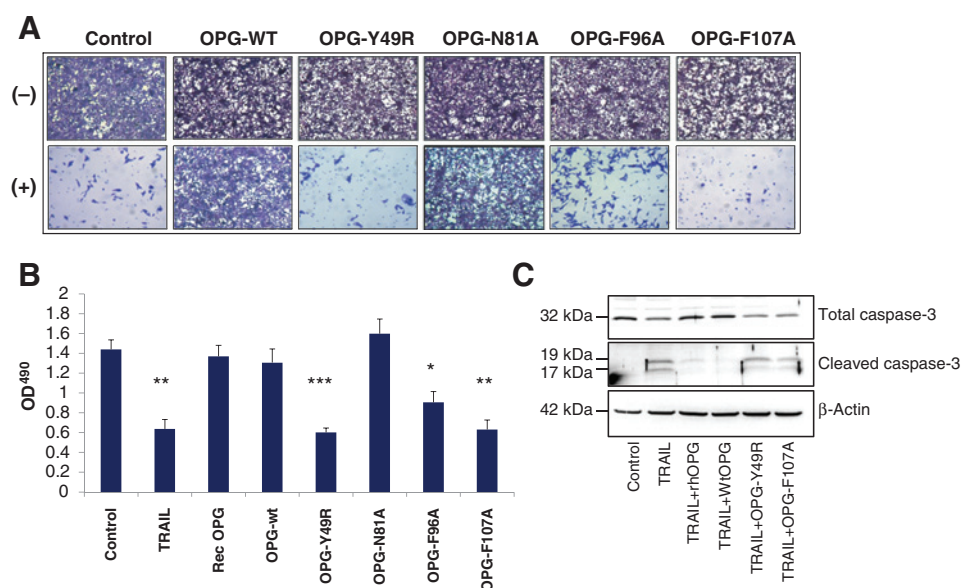


Figure 3. Osteoclast assay to determine retention of RANKL binding of OPG mutants. Primary macrophage cells were cultured in the presence of 200 ng of recombinant OPG, OPG-wt, or indicated OPG mutants in the presence or absence of 60 ng of recombinant RANKL. As a positive control, macrophage cells were cultured with RANKL alone. TRAP staining of macrophage cell cultures were performed 7 to 14 days after cells were cultured in the presence of OPG-wt or OPG-mutants and RANKL (A). Quantitative analysis based on the number of osteoclasts seen in 10 random fields (B); *, $P < 0.005$ compared with culture with RANKL only.

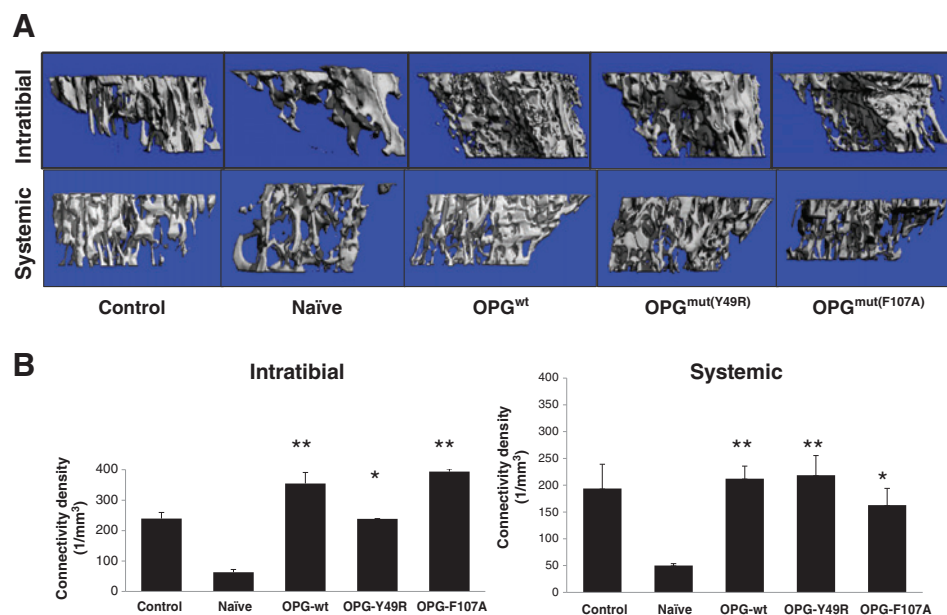
**Figure 4.**

Apoptosis assay to determine abrogation of TRAIL binding by OPG-mutants. MDA-MB-435 cells were cultured in the presence of 100 ng of TRAIL and 200 ng of OPG-wt or OPG-mutants. As a positive control, MDA-MB-435 cells were cultured with TRAIL alone. After 24 hours, MDA-MB-435 cells were either fixed in 3.7% paraformaldehyde and then stained with 0.05% Crystal violet for 30 minutes and viewed using a light microscope ($\times 100$; A) or cultured with 20 μ L of the solution 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) for 2 hours, and then measured at an absorbance of 490 nm (B; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$, compared with control). To determine downstream activity of TRAIL function, a human osteolytic cancer cell line, PC3, was cultured in a combination of TRAIL and OPG^{wt} or OPG^{mut} (Y49R or F107A) for 3 hours. Cells lysates were prepared by harvesting the cultures and Western blot analysis was performed for cleaved caspase-3 activity (C).

osteolytic damage without inhibiting TRAIL-induced apoptosis of cancer cells suggesting a strong therapeutic potential.

Osteoprotegerin also binds to TRAIL with similar affinity as with RANKL (24, 25), and therefore, OPG treatment raises the concern that it may affect the function of TRAIL. TRAIL is a cytokine expressed on the surface of tumor-infiltrating macrophages that induce apoptosis, specifically in malignant cells via

interactions with the death receptors DR4 and DR5 (26). Although a precise mechanism by which TRAIL specifically induces death of transformed cells is not known, a substantial role for TRAIL has been established as a critical effector molecule in tumor immunosurveillance (27–31), and TRAIL-deficient mice suffer from increased susceptibility to tumor initiation and metastases (32). These studies highlight the importance of TRAIL and

**Figure 5.**

Micro-CT assessment to determine the potential of OPG^{mut} in inhibiting tumor-induced osteolytic damage. Nude mice were injected intra-tibially with approximately 1×10^5 osteolytic prostate cancer cell line PC3. Twenty-four hours later, approximately 3×10^5 MSC overexpressing OPG^{wt}, OPG^{MY49R}, or OPG^{mF107A} were injected into the same tibia for intratumoral administration and by tail vein injection for systemic administration. Mice from both routes of MSC administration were sacrificed 14 days post MSC therapy and tibia were collected for micro-CT analysis to determine changes in the overall trabecular architecture (A) and connectivity density (B; *, $P < 0.05$; **, $P < 0.01$ compared with Naïve).

raise the concern that therapeutic administration of OPG might also diminish host immune defense mechanisms against malignant cells. Thus, the newly developed and characterized OPG mutants in this study will have the potential to overcome this limitation and concern.

Osteoprotegerin and RANK are members of the TNF-R superfamily, whereas RANKL and TRAIL are members of the TNF superfamily. The crystal structures of RANKL/OPG and TRAIL/DR5 complex have been resolved and the atomic models demonstrate conserved structural features of their respective superfamilies (33–37). Proteins in the TNF-R family adopt the elongated structures characterized by variable numbers of cysteine-rich domains (CRD) that form a scaffold of disulfide bridges (38) where the DR5 and OPG contains two and four CRDs, respectively. Monomers of both RANKL and TRAIL contain two antiparallel β -pleated sheets that form a β -sandwich as a core scaffold, which interact with adjacent subunits in a head-to-tail fashion to form a bell-shaped homotrimer (33–36). The elongated receptors fit into the grooves of adjacent protomers of the homotrimeric ligands, and this particular mode of interaction was demonstrated by the crystal structure of TRAIL/DR5 and RANKL/OPG (34, 35). Because of this conserved mode of interaction between the TNF and TNF-R family members, we hypothesized that OPG also shared a similar mode of interaction with TRAIL. Conservation in residues among family members is an indication of their functional importance; hence, it is plausible that the amino acid loop of TRAIL might also interact with the conserved residues in OPG in a similar manner. Because RANKL lacks this elongated loop structure, we predicted that the amino acid loop of TRAIL may provide a unique interaction with OPG and mutations on OPG residues that bind to TRAIL would generate OPG variants that will inhibit osteoclastogenesis but not TRAIL-mediated apoptosis of cancer cells. The crystal structure of OPG in complex with RANKL indicated that CRD2 and CRD3 of OPG play substantial roles in binding of OPG and RANKL, where the binding interface consists of two binding sites: site-1 identifying amino acid loop 50s (H47-L65) in CRD2 and site-2 identifying amino acid loop 90s (A90-L98) in CRD3 (39). Of the mutants generated and characterized for therapeutic bone remodeling, mutant Y49R falls within binding site-1 and mutant F96R falls within binding site-2. However, mutant F107A does not fall within these binding sites yet resulted in significant abolishment of TRAIL binding. It remains possible that conformational changes in the amino acid F107 to F107A may have altered the binding that may have been retained in full-length OPG protein as opposed to truncated OPG containing CRDs.

Results of the *in vitro* studies also confirmed that despite mutagenesis at amino acids, based on interactive domain analysis, abolishment of TRAIL-binding domains in three of the mutants did not affect binding of RANKL to the OPG mutants. In the osteoclast assay, all recombinant OPG proteins significantly inhibited RANKL-mediated osteoclastogenesis confirming functionality of the mutated proteins. When functionally testing for TRAIL-binding activity, the OPG-mutant N81A strongly inhibited TRAIL-mediated apoptosis, similar to wild-type OPG indicating that despite the amino acid substitution, the binding affinity of OPG to TRAIL was retained. However, OPG mutants Y49R, F96A, and F107A demonstrated a significant decrease in TRAIL binding, which resulted in a significant decrease in cell viability. Furthermore, results of the *in vivo* studies provided key evidence confirming functional activity of these two OPG mutants in a pre-clinical mouse model of a bone disseminated osteolytic tumor

demonstrated protection from bone destruction in both trabecular architecture and connectivity density.

Realizing the therapeutic potential of OPG in bone remodeling for tumor-induced osteolytic damage, much effort has gone to its clinical utility. In this study, we proposed the use of a novel OPG-mutant protein against cancer-induced osteolysis while potentially not interfering with TRAILs ability to induce apoptosis in cancer cells. Often times, epithelial carcinomas such as that of breast and prostate metastasize to the spine and long bones causing severe bone damage. With OPG being the native inhibitory protein of osteoclastogenesis, using this protein in conditions where there is severe osteolysis due to an imbalance in the RANKL/RANK/OPG signaling triad can potentially result in increased bone healing and low toxicity. In this regard, soluble RANK and antibodies targeting RANK have shown to be effective (40, 41), but toxicity remains an issue and therefore, direct site injection of OPG^{mut}, abolished in TRAIL binding by cell/gene therapy approach whereby low, yet sustained concentration of OPG can be systemically released to inhibit aggressive osteolytic damage. Moreover, the fact that the OPG mutants possess an altered TRAIL-binding domain would potentially allow endogenous TRAIL to target transformed cells. Furthermore, exogenous TRAIL therapy can be combined with the proposed OPG^{mut} therapy to diminish both osteolytic burden and tumor cell killing. In many advanced osteolytic malignancies where osteolytic lesions exist throughout the skeleton, a systemic approach will be more beneficial. In this context, it is noteworthy that used MSC is currently being tested in clinical studies (42).

Taken together, the current study demonstrates that RANKL-OPG-TRAIL molecular triad may be a valid target to develop novel therapy, particularly for tumor-associated bone destruction. The potential therapeutic application of the variant OPG that effectively inhibits osteoclastogenesis by retaining RANKL binding, while abolishing TRAIL binding, will be beneficial in malignant osteolytic bone pathologies encountered in breast cancer, prostate cancer, and multiple myeloma. The novel variants of OPG presented here may be used in therapeutic applications either as purified protein or by gene-based approaches through cell and gene therapy applications depending on the required duration of the therapy. Both OPG and TRAIL have been tested in preclinical settings and both proteins were well tolerated when used to treat pathologic conditions (43, 44). Furthermore, as studies have shown cells from human osteolytic malignancies such as U266, RPMI8226, MDA-MB-231 are sensitive to TRAIL (13, 45, 46) using OPG therapy in combination with TRAIL should result in the protection of bone from aggressive osteoclast damage and increase cancer cell death due to cytotoxic effects of TRAIL.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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