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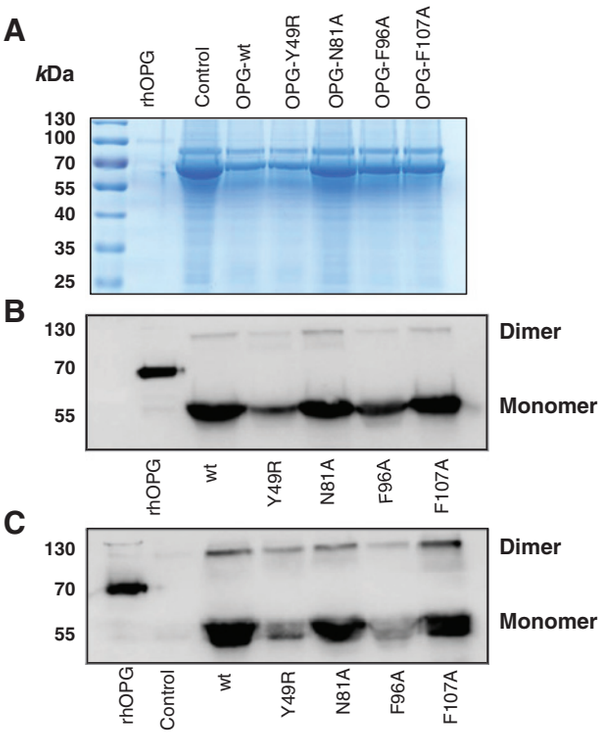

Figure 2.
Immunoblot analysis of purified OPG mutants. Forty-eight hours following transfection of expression plasmids containing the sequences of OPG-wt and OPG-mutants in HEK-293 cells, supernatant media were collected and concentrated using a 30 kDa filter. The concentrated media were then separated 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

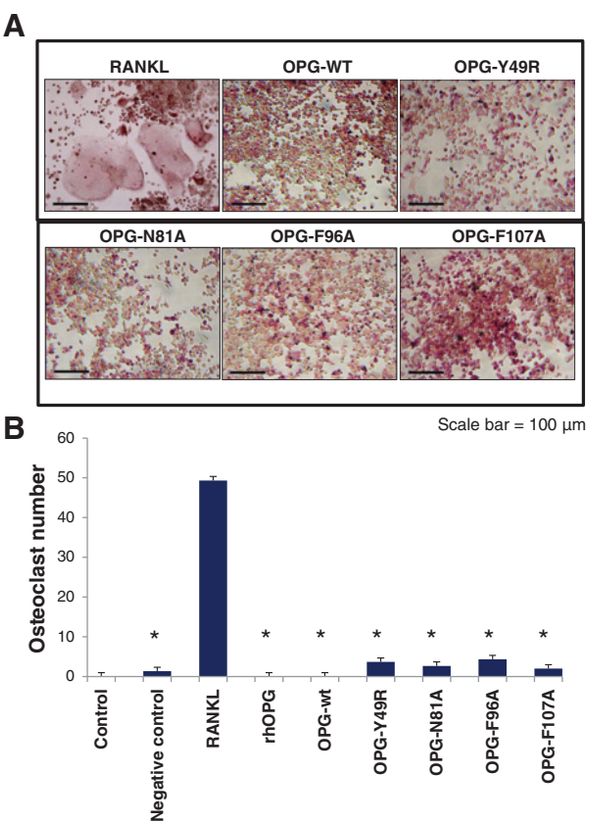

Figure 3.
Osteoclast assay to determine retention of RANKL binding of OPG mutants. Primary macrophage cells were cultured in the presence of 200 ng of recombinant OPG, OPG-wt, or indicated OPG mutants in the presence or absence of 60 ng of recombinant RANKL. As a positive control, macrophage cells were 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 osteoclast seen in 10 random fields (B; *, $P < 0.005$ compared with culture with RANKL only).

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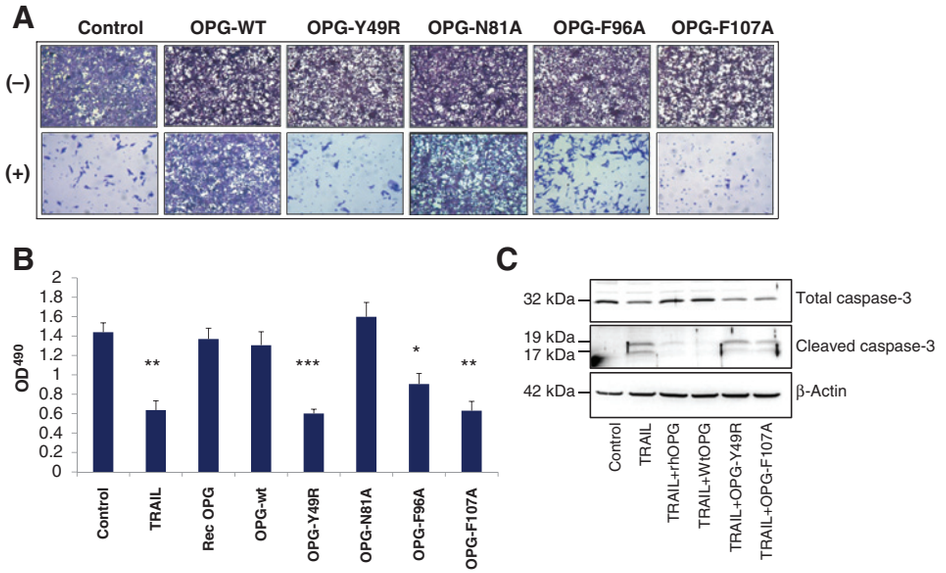

Figure 4.
Apoptosis assay to determine abrogation of TRAIL binding by OPG-mutants. MDA-MB-435 cells were cultured in the presence of 100 ng of TRAIL and 200 ng of OPG-wt or OPG-mutants. As a positive control, MDA-MB-435 cells were cultured with TRAIL alone. After 24 hours, MDA-MB-435 cells were either fixed in 3.7% paraformaldehyde and then stained with 0.05% Crystal violet for 30 minutes and viewed using a light microscope (×100; A) or cultured with 20 μL 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

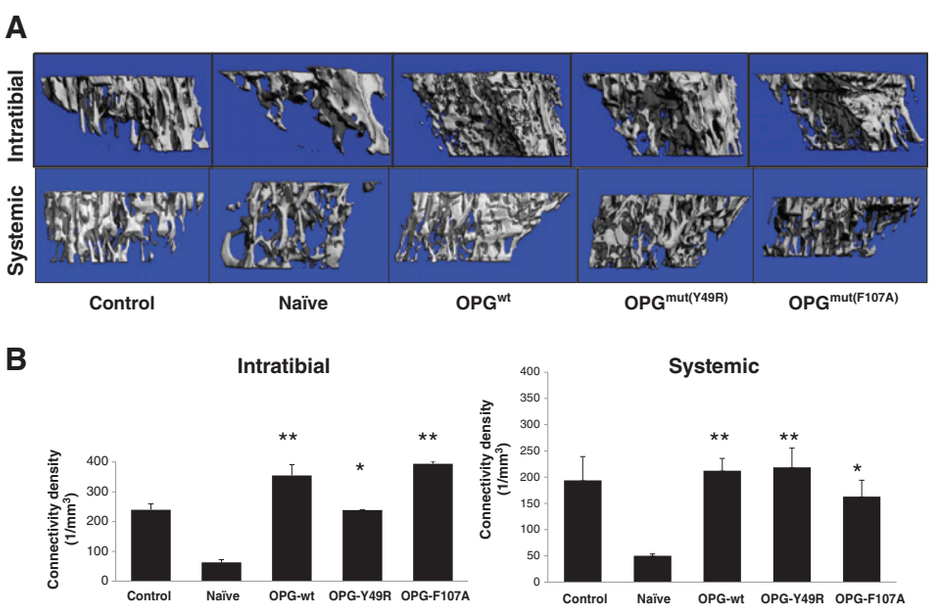

Figure 5.
Micro-CT assessment to determine the potential of OPG^{mut} in inhibiting tumor-induced osteolytic damage. Nude mice were injected intra-tibially with approximately 1×10^5 osteolytic prostate cancer cell line PC3. Twenty-four hours later, approximately 3×10^5 MSC overexpressing OPG^{wt}, OPG^{mY49R}, 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).

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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 indentifying 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 preclinical 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.

Authors' Contributions

Conception and design: J.T. Higgs, J.S. Jarboe, S. Ponnazhagan

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.T. Higgs, J.S. Jarboe, JH Lee, C. Deivanayagam, S. Ponnazhagan

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.T. Higgs, J.S. Jarboe, JH Lee, S. Ponnazhagan

Writing, review, and/or revision of the manuscript: J.T. Higgs, JH Lee, S. Ponnazhagan

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Ponnazhagan
Study supervision: C. Deivanayagam, S. Ponnazhagan

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References

1. Khosla S. Minireview: the OPG/RANKL/RANK system. Endocrinology 2001;142:5050–5.
2. Maginn EN, Browne PV, Hayden P, Vandenberghe E, MacDonagh B, Evans P, et al. PBOX-15, a novel microtubule targeting agent, induces apoptosis, upregulates death receptors, and potentiates TRAIL-mediated apoptosis in multiple myeloma cells. Br J Cancer 2011;104:281–9.
3. Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr Rev 1999; 20:345–57.
4. Hofbauer LC, Khosla S, Dunstan CR, Lacey DL, Spelsberg TC, Riggs BL. Estrogen stimulates gene expression and protein production of osteoprotegerin in human osteoblastic cells. Endocrinology 1999;140:4367–70.
5. Hofbauer LC, Gori F, Riggs BL, Lacey DL, Dunstan CR, Spelsberg TC, et al. Stimulation of osteoprotegerin ligand and inhibition of osteoprotegerin production by glucocorticoids in human osteoblastic lineage cells: potential paracrine mechanisms of glucocorticoid-induced osteoporosis. Endocrinology 1999;140:4382–9.
6. Itonaga I, Fujikawa Y, Sabokbar A, Murray DW, Athanasou NA. Rheumatoid arthritis synovial macrophage-osteoclast differentiation is osteoprotegerin ligand-dependent. J Pathol 2000;192:97–104.
7. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, et al. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci U S A 1998;95:3597–602.
8. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Lüthy R, et al. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell 1997;89:309–19.
9. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, et al. UCSF Chimera–a visualization system for exploratory research and analysis. J Comput Chem 2004;25:1605–12.
10. Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. Acta Crystallogr D Biol Crystallogr 2004;60:2126–32.
11. Chanda D, Kumar S, Ponnazhagan S. Therapeutic potential of adult bone marrow-derived mesenchymal stem cells in diseases of the skeleton. J Cell Biochem 2010;111:249–57.
12. Szafran AA, Folks K, Warram J, Chanda D, Wang D, Zinn KR. Death receptor 5 agonist TRA8 in combination with the bisphosphonate zoledronic acid attenuated the growth of breast cancer metastasis. Cancer Biol Ther 2009;8:1109–16.
13. Zhu L, Huang X, Choi KY, Ma Y, Zhang F, Liu G, et al. Real-time monitoring of caspase cascade activation in living cells. J Control Release 2012;163: 55–62.
14. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, et al. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 1998;93:165–76.
15. Kitazawa S, Kitazawa R. RANK ligand is a prerequisite for cancer-associated osteolytic lesions. J Pathol 2002;198:228–36.
16. Thomas RJ, Guise TA, Yin JJ, Elliott J, Horwood NJ, Martin TJ, et al. Breast cancer cells interact with osteoblasts to support osteoclast formation. Endocrinology 1999;140:4451–58.
17. Wittrant Y, Theoleyre S, Chipoy C, Padrines M, Blanchard F, Heymann D, et al. RANKL/RANK/OPG: new therapeutic targets in bone tumours and associated osteolysis. Biochim Biophys Acta 2004;1704:49–57.
18. Corey E, Brown LG, Kiefer JA, Quinn JE, Pitts TE, Blair JM, et al. Osteoprotegerin in prostate cancer bone metastasis. Cancer Res 2005;65:1710–8.
19. Croucher PI, Shipman CM, Lippitt J, Perry M, Asosingh K, Hijzen A, et al. Osteoprotegerin inhibits the development of osteolytic bone disease in multiple myeloma. Blood 2001;98:3534–40.
20. Morony S, Capparelli C, Sarosi I, Lacey DL, Dunstan CR, Kostenuik PJ. Osteoprotegerin inhibits osteolysis and decreases skeletal tumor burden in syngeneic and nude mouse models of experimental bone metastasis. Cancer Res 2001;61:4432–6.
21. Vanderkerken K, De Leenheer E, Shipman C, Asosingh K, Willems A, Van Camp B, et al. Recombinant osteoprotegerin decreases tumor burden and increases survival in a murine model of multiple myeloma. Cancer Res 2003;63:287–9.
22. Zhang J, Dai J, Qi Y, Lin DL, Smith P, Strayhorn C, et al. Osteoprotegerin inhibits prostate cancer-induced osteoclastogenesis and prevents prostate tumor growth in the bone. J Clin Invest 2001;107:1235–44.
23. Body JJ, Greipp P, Coleman RE, Facon T, Geurs F, Fermand JP, et al. A phase I study of AMGN-0007, a recombinant osteoprotegerin construct, in patients with multiple myeloma or breast carcinoma related bone metastases. Cancer 2003;97:887–92.
24. Emery JG, McDonnell P, Burke MB, Deen KC, Lyn S, Silverman C, et al. Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. J Biol Chem 1998;273:14363–7.
25. Vitovski S, Phillips JS, Sayers J, Croucher PI. Investigating the interaction between osteoprotegerin and receptor activator of NF-kappaB or tumor necrosis factor-related apoptosis-inducing ligand: evidence for a pivotal role for osteoprotegerin in regulating two distinct pathways. J Biol Chem 2007;282:31601–9.
26. Griffith TS, Wiley SR, Kubin MZ, Sedger LM, Maliszewski CR, Fanger NA. Monocyte-mediated tumoricidal activity via the tumor necrosis factor-related cytokine, TRAIL. J Exp Med 1999;189:1343–54.
27. Smyth MJ, Cretney E, Takeda K, Wiltrout RH, Sedger LM, Kayagaki N, et al. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) contributes to interferon gamma-dependent natural killer cell protection from tumor metastasis. J Exp Med 2001;193:661–70.
28. Takeda K, Hayakawa Y, Smyth MJ, Kayagaki N, Yamaguchi N, Kakuta S, et al. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. Nat Med 2001;7:94–100.
29. Takeda K, Smyth MJ, Cretney E, Hayakawa Y, Yamaguchi N, Yagita H, et al. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in NK cell-mediated and IFN-gamma-dependent suppression of subcutaneous tumor growth. Cell Immunol 2001;214:194–200.
30. Zerafa N, Westwood JA, Cretney E, Mitchell S, Waring P, Iezzi M, et al. Cutting edge: TRAIL deficiency accelerates hematological malignancies. J Immunol 2005;175:5586–90.
31. Takeda K, Smyth MJ, Cretney E, Hayakawa Y, Kayagaki N, Yagita H, et al. Critical role for tumor necrosis factor-related apoptosis-inducing ligand in immune surveillance against tumor development. J Exp Med 2002;195: 161–9.
32. Cretney E, Takeda K, Yagita H, Glaccum M, Peschon JJ, Smyth MJ. Increased susceptibility to tumor initiation and metastasis in TNF-related apoptosis-inducing ligand-deficient mice. J Immunol 2002;168: 1356–61.
33. Cha SS, Kim MS, Choi YH, Sung BJ, Shin NK, Shin HC, et al. 2.8 A resolution crystal structure of human TRAIL, a cytokine with selective antitumor activity. Immunity 1999;11:253–61.
34. Cha SS, Sung BJ, Kim YA, Song YL, Kim HJ, Kim S, et al. Crystal structure of TRAIL-DR5 complex identifies a critical role of the unique frame insertion in conferring recognition specificity. J Biol Chem 2000;275: 31171–7.
35. Hymowitz SG, Christinger HW, Fuh G, Ultsch M, O'Connell M, Kelley RF, et al. Triggering cell death: the crystal structure of Apo2L/TRAIL in a complex with death receptor 5. Mol Cell 1999;4:563–71.

Published OnlineFirst January 30, 2015; DOI: 10.1158/1541-7786.MCR-14-0492

36. Lam J, Nelson CA, Ross FP, Teitelbaum SL, Fremont DH. Crystal structure of the TRANCE/RANKL cytokine reveals determinants of receptor-ligand specificity. J Clin Invest 2001;108:971–9.
37. Mongkolsapaya J, Grimes JM, Chen N, Xu XN, Stuart DI, Jones EY, et al. Structure of the TRAIL-DR5 complex reveals mechanisms conferring specificity in apoptotic initiation. Nat Struct Biol 1999;6:1048–53.
38. Naismith JH, Devine TQ, Kohno T, Sprang SR. Structures of the extracellular domain of the type I tumor necrosis factor receptor. Structure 1996;4:1251–62.
39. Nelson CA, Warren JT, Wang MW, Teitelbaum SL, Fremont DH. RANKL Employs Distinct Binding Modes to Engage RANK and the Osteoprotegerin Decoy Receptor. Structure 2012;20:1971–82.
40. Cummings SR, San Martin J, McClung MR, Siris ES, Eastell R, Reid IR, et al. Denosumab for prevention of fractures in postmenopausal women with osteoporosis. N Engl J Med 2009;361:756–65.
41. Zhou Z, Chen C, Zhang J, Ji X, Liu L, Zhang G, et al. Safety of denosumab in postmenopausal women with osteoporosis or low bone mineral density: a meta-analysis. Int J Clin Exp Pathol 2014;7:2113–22.
42. Gopal K, Amirhamed HA, Kamarul T. Advances of human bone marrow-derived mesenchymal stem cells in the treatment of cartilage defects: a systematic review. Exp Biol Med 2014;239:663–9.
43. Zierhut ML, Gastonguay MR, Martin SW, Vicini P, Bekker PJ, Holloway D, et al. Population PK-PD model for Fc-osteoprotegerin in healthy postmenopausal women. J Pharmacokinet Pharmacodyn 2008;35:379–9.
44. Doi T, Murakami H, Ohtsu A, Fuse N, Yoshino T, Yamamoto N, et al. Phase 1 study of conatumumab, a pro-apoptotic death receptor 5 agonist antibody, in Japanese patients with advanced solid tumors. Cancer Chemother Pharmacol 2011;68:733–7.
45. Shipman CM, Croucher PI. Osteoprotegerin is a soluble decoy receptor for tumor necrosis factor-related apoptosis-inducing ligand/Apo2 ligand and can function as a paracrine survival factor for human myeloma cells. Cancer Res 2003;63:912–6.
46. Labrinidis A, Diamond P, Martin S, Hay S, Liapis V, Zinonos I, et al. Apo2L/TRAIL inhibits tumor growth and bone destruction in a murine model of multiple myeloma. Clin Cancer Res 2009;15:1998–2009.