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Correlating RANK Ligand/RANK Binding Kinetics with Osteoclast Formation and Function

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Abstract

The interaction between Receptor Activator of NF- κ B Ligand (RANKL) and its receptor RANK is essential for the differentiation and bone resorbing capacity of the osteoclast. Osteoprotegerin (OPG), a soluble homodimer, acts as a decoy receptor for RANKL and thus inhibits osteoclastogenesis. An imbalance in the RANKL/RANK/OPG axis, with decreased OPG and/or increased RANKL, is associated with diseases that favor bone loss, including osteoporosis. Recently, we established a yeast surface display system and screened libraries of randomly mutated RANKL proteins to identify mutations that abolish binding to OPG while preserving recognition of RANK. These efforts yielded several RANKL variants possessing substantially higher affinity for RANK compared to their wild-type (WT) counterpart. Using recombinant RANKL mutant proteins, we find those with increased affinity for RANK produce more robust signaling in osteoclast lineage cells and have greater osteoclastogenic potential. Our results are the first to document gain of function RANKL mutations. They indicate that the physiological RANKL/RANK interaction is not optimized for maximal signaling and function, perhaps reflecting the need to maintain receptor specificity within the tumor necrosis factor superfamily (TNFSF). Instead, we find, a biphasic relationship exists between RANKL/RANK affinity and osteoclastogenic capacity. In our panel of RANKL variants, this relationship is driven entirely by manipulation of the kinetic off-rate. Our structure-based and yeast surface display-derived insights into manipulating this critical signaling axis may aid in the design of novel anti-resorptive therapies as well as provide a paradigm for design of other receptor-specific TNF superfamily ligand variants.

Keywords

osteoclast; signal transduction; kinetics; RANK; RANKL; TNF-Superfamily; bone; receptor

INTRODUCTION

The TNFSF is composed of at least 17 type II transmembrane ligands whose involvement in homeostasis and disease is wide-ranging (Hehlhans and Pfeffer 2005). Many of these cytokines target and/or are produced by cells of the immune system and participate in development and function of the hematopoietic lineage (Zauli and Secchiero 2006; Jiang, Chen and Schwarz 2008; Croft 2009; Mackay and Schneider 2009; Vujanovic 2011; Zhu and Fu 2011; Summers deLuca and Gommerman 2012; Upadhyay and Fu 2013). TNFSF cytokines act by binding to and stimulating TNF-receptor superfamily (TNFRSF) signaling (Cabal-Hierro and Lazo 2012; Li, Yin and Wu 2013). Additionally, there are several examples within TNFRSF of decoy receptors whose binding inhibits the activity of these cytokines (Bodmer, Schneider and Tschopp 2002). Due to its pathological significance, there are currently drugs which target TNFSF/TNFRSF signaling (Aggarwal, Gupta and Kim 2012; Croft, Benedict and Ware 2013).

Skeletal mass is regulated by the bone resorptive osteoclasts whose differentiation and function require RANKL, a member of the TNFSF (Novack and Teitelbaum 2008). By interacting with its receptor RANK, RANKL induces signaling to NF- κ B, MAP kinases, and NFATc1 among others (Blair, Robinson and Zaidi 2005; Asagiri and Takayanagi 2007). These crucial signaling events are blocked by OPG, a secreted decoy receptor that binds RANKL and prevents its interaction with RANK (Simonet, Lacey, Dunstan, Kelley, Chang, Luthy, Nguyen, Wooden, Bennett, Boone, Shimamoto, DeRose, Elliott, Colombero, Tan, Trail, Sullivan, Davy, Bucay, Renshaw-Gegg, Hughes, Hill, Pattison, Campbell, Boyle and et al. 1997; Yamaguchi, Kinoshita, Goto, Kobayashi, Tsuda, Morinaga and Higashio 1998). RANKL-mediated osteoclast activity, in excess of bone formation by osteoblasts, promotes pathological bone loss leading to diseases such as osteoporosis and inflammatory osteolysis (Hanada, Hanada and Penninger 2010). Therefore, understanding the molecular events initiating signal transduction remains key to the development of superior therapeutics targeting this signaling axis.

Most TNFSF members (including RANKL) exist as homotrimers in solution (Selmaj, Raine and Cross 1991; Wu and Hymowitz 2010) and it is generally assumed that the trimeric clustering of receptors is the primary initiating event for signal induction. We recently developed a novel inhibitor of RANKL-induced osteoclast formation and function relying on the manipulation of receptor oligomerization (Warren, Nelson, Decker, Zou, Fremont and Teitelbaum 2014). In the process of engineering a single-chain RANKL capable of antagonizing RANK signaling, we generated a panel of increased affinity RANKL mutants that allowed our construct to out-compete WT RANKL binding. In the present exercise, we explored the relationship of binding affinity to biological function subsequent to receptor ligation. We utilized a panel of RANKL mutants with RANK affinities increased by 2- to 500-fold. Increased RANKL affinity for RANK has a biphasic effect on osteoclastogenesis, reaching optimal activity at approximately 15-fold greater affinity than WT. These findings provide insights into the relationship between RANKL/RANK affinity and function, raising the possibility that high affinity cytokines may be useful in the design of TNFSF-based therapies.

MATERIALS AND METHODS

Production of mammalian RANKL protein

Constructs used for transient transfection of RANKL (Warren, Nelson, Decker, Zou, Fremont and Teitelbaum 2014) or OPG (Nelson, Warren, Wang, Teitelbaum and Fremont 2012) were previously described. DNA for transfection was prepared using an endotoxin-free DNA purification kit (Qiagen). For mammalian protein production, suspension adapted 293-Freestyle cells (Life Technologies) were maintained in serum-free Freestyle 293 expression medium (Life Technologies) according to the manufacturer's protocol. Cells were seeded at a density of $0.5 \times 10^6/\text{mL}$ and transfected the next day using the cationic lipid polyethylenimine (Aricescu, Lu and Jones 2006). Supernatant was harvested four and seven days after transfection, 0.22uM filtered and equilibrated by the addition of 1/10 volume $10 \times$ phosphate buffered saline (Gibco) and 10mM imidazole. The protein was captured on Ni-NTA Superflow resin (Qiagen) and washed using 10mM imidazole in PBS. Protein was eluted in steps from 25–500mM imidazole. Fractions containing purified protein were identified on coomassie stained SDS-PAGE. Positive fractions were pooled and concentrated using a disposable YM30 centricon (Millipore). All proteins were sterile filtered for use in cell culture.

Surface Plasmon Resonance (SPR)

All SPR experiments were performed on a Biacore T-100 (GE Healthcare) using CM5 sensor chips and HBS-EP buffer. To confirm receptor recruitment using RANKL variants, 100 response units (RU) of WT-RANKL or variant RANKL were coupled to individual lanes, leaving one reference flow cell uncoupled. Experiments to determine kinetic affinity constants of RANKL variants for RANK or OPG were performed and analyzed as previously described (Nelson, Warren, Wang, Teitelbaum and Fremont 2012).

Generation of osteoclasts from primary bone marrow macrophages

Long bones of eight week-old mice were flushed and the marrow subjected to red blood cell lysis. The remainder of the whole marrow was cultured on petri dishes maintained at 37°C with 6% CO_2 in alpha-mem containing 10% inactivated fetal bovine serum, 100 U/ml penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, (α -10 medium) supplemented with 1:10 CMG (conditioned medium supernatant containing recombinant M-CSF) (Takeshita, Kaji and Kudo 2000). Osteoclasts were differentiated in α -10 medium with 1:50 CMG and the relevant RANKL variant. Alternatively, osteoclasts were differentiated in the presence of 100 ng/mL of mutant RANKL with varying concentrations of monomeric OPG.

Detection of osteoclast formation

Cells were fixed in 4% paraformaldehyde in PBS for 15 minutes and stained for the tartrate resistant acid phosphatase (TRAP) using a kit (Sigma). Quantitative assessment of TRAP activity was performed using the fluorescent phosphatase substrate ELF-97 (Molecular Probes). Fixed cells were incubated with 100uM ELF-97 in 90mM citrate buffer pH 4.8, 80mM sodium tartrate for 15 minutes at room temperature. The reaction was stopped by the

addition of sodium hydroxide and fluorescence was visualized using the 345/530 excitation/emission filter on a Spectramax M2 plate reader.

Quantitative real-time PCR

To quantitate mRNA markers of osteoclast formation, RNA was isolated from cultured cells according to the manufacturer's protocol (Qiagen RNeasy miniprep kit). Equal amounts of RNA were used to perform reverse transcription (Bio-rad iScript) and quantitative real-time PCR was performed using Eva Ssofast qPCR kit (Bio-rad) using a 7500 fast machine (ABI). Cyclophilin was used as a housekeeping control gene. Data was analyzed according to the delta-delta Ct method and expressed relative to a control containing no RANKL addition (labeled BMM). Primers used were as follows: NFATc1 (Forward: 5'-CCCGTCACATTCTGGTCCAT-3', Reverse: 5'-CAAGTAACCGTGTAGCTGCACAA-3'), β 3 integrin (Forward: 5'-TTCGACTACGGCCAGATGATT-3', Reverse: 5'-GGAGAAAGACAGGTCCATCAAGT-3'), Cyclophilin (Forward: 5'-AGCATACAGGTCCTGGCATC-3', Reverse: 5'-TTCACCTTCCCAAAGACCAC-3').

Detection of Collagen Fragments from Culture Supernatant

Pre-osteoclasts were generated from primary bone marrow macrophages in the presence of wild-type RANKL and lifted with trypsin/EDTA. Equal numbers of pre-osteoclasts were plated on bovine bone slices in the presence of RANKL variants at 100 ng/mL for two additional days. The release of collagen peptides into the culture supernatant was detected by ELISA according to the manufacturer's protocol (Immunodiagnostic Systems).

RESULTS

RANKL mutants modify osteoclast formation

Recently, we reported affinity maturation of RANKL for its signaling receptor RANK, using yeast surface display (Warren, Nelson, Decker, Zou, Fremont and Teitelbaum 2014). We estimated the affinities of several RANKL mutants from titration curves generated on the surface of RANKL-displaying yeast cells. These mutants exhibit a 2- to 500-fold increase in RANK affinity relative to WT. All RANKL variants were identified on a background of two mutations (C220S, I246E) that enables increased protein production. These mutations do not affect the affinity of RANKL for RANK or its ability to generate osteoclasts (not shown). We further assessed the affinities for RANK and OPG of each RANKL variant using surface plasmon resonance (Table 1). As described previously (Warren, Nelson, Decker, Zou, Fremont and Teitelbaum 2014), a quadruple mutation (K194E/Q236H/F269Y/H270 or "KQFH") increases affinity 500-fold. Here we show that K194E alone also enhances RANK affinity, although less than 2-fold (Table 1). The single point mutant Q236H increases the affinity for RANK approximately 15-fold while the combination of K194E/Q236H ("KQ") increases binding to the receptor 20-fold, and K194E/Q236H/F269Y ("KQF"), 50-fold. Thus, we have established a panel of RANKL variants ranging from a slight to 500 fold increase in RANK affinity. Of note, the increases in affinity are driven almost entirely by changes in the kinetic off-rate with minimal variation in the on-rate. We also confirmed that

all proteins containing the Q236H mutation have reduced binding to OPG. Interestingly, the single point mutant, K194E, decreases binding to OPG approximately 4-fold.

To determine the osteoclastogenic capacity of our RANKL variants with increased affinity for RANK we cultured bone marrow macrophages with M-CSF and increasing concentrations of RANKL. Osteoclasts were identified by staining for tartrate resistant acid phosphatase (TRAP) activity (Figure 1A) and their abundance quantitated using a TRAP activity assay. These data were used to fit EC_{50} values for each RANKL variant (Figure 1B,C). A single RANKL point mutation, K194E, increases osteoclastogenesis two-fold. Q236H, possessing a 15-fold increase in affinity for RANK, displayed the most robust increase in osteoclastogenic activity (compare average WT EC_{50} = 9.78ng/mL versus Q236H EC_{50} = 1.46 ng/mL). Interestingly, further increases in affinity up to 50-fold higher than WT RANKL did not more efficiently promote osteoclastogenic potency. Moreover, KQFH RANKL, with a 500-fold increase in RANK affinity for RANK, did not generate osteoclasts as efficiently as Q236H, though it still outperforms WT cytokine. The non-linear relationship between efficiency of osteoclast formation and RANKL/RANK interaction holds when assessed using either dissociation constant (K_D , Figure 1D) or half-life ($t_{1/2}$, Figure 1E). In agreement with osteoclast formation as assessed by TRAP stain and TRAP activity, osteoclastogenic markers induced by RANKL variants show a similar affinity-dependent biphasic effect (Figure 1F,G). Hence, RANKL/RANK affinity exerts a biphasic effect of RANKL affinity for RANK on osteoclast formation, such that extremely high binding is less optimal than an intermediate increase.

Resistance of RANKL mutants to exogenously added OPG

During the development of a single-chain RANKL inhibitors possessing altered RANK affinities, we sought to decrease the capacity of the RANKL variants to bind the decoy receptor, OPG. To that end, the mutations we identified substantially decrease OPG affinity as assessed by yeast surface display and confirmed by SPR (Table 1). We asked if these mutants are resistant to the addition of exogenous OPG in osteoclastogenic conditions *in vitro*. Representative TRAP stained images of bone marrow macrophages cultured in the presence of increasing amounts of OPG and a constant amount of each RANKL variant clearly show that all mutations incorporating Q236H are markedly resistant to the effects of the decoy receptor (Figure 2). RANKL K194E-induced osteoclastogenesis was inhibited slightly at the highest doses of the decoy receptor, although not sufficient to permit the assessment of an IC_{50} value given the range of OPG concentrations used. Therefore, despite the similar binding footprints of OPG and RANK on RANKL (Nelson, Warren, Wang, Teitelbaum and Fremont 2012), it is possible to impose opposite effects on cellular function using receptor selective mutations.

Altered signaling to key osteoclastogenic mediators is enhanced by high affinity RANKL variants

To determine whether the osteoclastogenic effects of progressively increasing RANKL affinity for RANK reflects altered signaling, we stimulated bone marrow macrophages with RANKL variants. We chose to compare the moderately increased affinity variant Q236H, which most robustly generates osteoclasts, to our highest affinity variant, which is less

osteoclastogenic despite enhanced RANK binding. We then assessed two crucial signaling events in osteoclast differentiation, namely phosphorylation of NF- κ B and the MAP kinase, p38 (Figure 3A,B). The signaling induced by Q236H and KQFH RANKL is more robust than that of WT RANKL, and maximizes earlier. As with osteoclast formation, KQFH RANKL is not as potent as Q236H RANKL at phosphorylating these key osteoclastogenic molecules. Thus, like osteoclastogenesis, per se, the RANKL/RANK interaction optimizes signal transduction at intermediate, rather than extremely high, affinities.

Increased RANK affinity enhances RANKL-induced osteoclast function

RANKL not only promotes osteoclast formation, but also stimulates resorption of bone by the mature cell. To determine if osteoclast function is also modified by RANK/RANKL affinity, we cultured bone marrow macrophages on plastic in equal amounts of WT-RANKL for four days to generate pre-osteoclasts. We then lifted and plated an equal number of cells on bovine bone slices in low- or high-dose RANKL variants. After 24 hours, we assessed collagen fragments in the medium (Figure 4). Despite normalized osteoclast numbers, Q236H RANKL activates mature cells to resorb bone more than WT RANKL, indicating that this variant not only enhances differentiation but also functional activity.

DISCUSSION

Following the discovery of lymphotoxin- β and the TNF/TNFR1/TNFR2 ligand/receptor pairs almost thirty years ago, appreciation of the role of TNFSF members in human disease has continued to grow. These insights led to an increased interest in targeting cytokines or receptors to modulate autoimmunity, anti-tumor activity, and bone mass. Currently, such FDA-approved drugs are antibodies or receptor-Fc fusion proteins that act by sequestering the targeted cytokine (Aggarwal, Gupta and Kim 2012). However, several TNFSF members bind more than one receptor (Hehlhans and Pfeffer 2005) and there are circumstances in which inhibiting the actions of an individual receptor, while sparing others, would be desirable. Specifically, global sequestration of a TNFSF cytokine may enhance serious complications. For example, removal of TNF α is associated with an increased risk of severe infection and neoplasia. Such complications may reflect blunting of an immune-protective role of TNFR2 in septic shock (Peschon, Torrance, Stocking, Glaccum, Otten, Willis, Charrier, Morrissey, Ware and Mohler 1998; Ebach, Riehl and Stenson 2005), as well as LPS-, RANKL-, or TNF α -mediated bone resorption (Abu-Amer, Ross, Edwards and Teitelbaum 1997; Abu-Amer, Erdmann, Alexopoulou, Kollias, Ross and Teitelbaum 2000; Nagano, Alles, Mian, Shimoda, Morimoto, Tamura, Shimokawa, Akiyoshi, Ohya and Aoki 2011). Thus, antibodies specifically targeting TNFR1, while sparing TNFR2 may improve treatment of diseases such as rheumatoid arthritis and psoriasis (Kontermann, Munkel, Neumeyer, Muller, Branschadel, Scheurich and Pfizenmaier 2008).

Development of receptor-selective cytokine mutants presents as an alternative to antibody-mediated modulation of TNFSF/TNFRSF signaling pathways. Our present study elucidates the functional outcomes of differentially modulating RANKL affinity for its signaling receptor, RANK, and its decoy receptor OPG. We show that moderate increases in affinity for RANK lead to a substantial augmentation of osteoclast formation, signaling, and bone

resorption. Very high affinity RANKL mutants, however, are less efficient. To our knowledge, this is the first evidence that RANK signaling can be optimized by increasing affinity. On the other hand, the relationships between both RANK binding affinity and kinetic off-rate are not linearly related to osteoclast formation. Importantly, the differences in affinity are driven almost entirely by prolonged kinetic off-rates with very minimal variation in the on-rate (Table 1). This suggests that the main property that is optimized in our Q236H is not simply the affinity but rather the off-rate itself. Additionally, we provide the first evidence that a forward genetics approach can be employed to identify mutations in RANKL with enhanced binding to the signaling receptor while poorly recognizing the decoy receptor. Similar results have been obtained using known non-synonymous coding variants of the TNFSF member LIGHT (Cheung, Coppieters, Sanjo, Osborne, Norris, Coddington, Granger, Elewaut and Ware 2010), however no such polymorphisms have been described for RANKL.

The importance of optimized off-rate for RANKL signaling is consistent with previous studies exploring the association of T-cell receptor: peptide-MHC affinity and attendant biological events (Chervin, Stone, Holler, Bai, Chen, Eisen and Kranz 2009; Schmid, Irving, Posevitz, Hebeisen, Posevitz-Fejfar, Sarria, Gomez-Eerland, Thome, Schumacher, Romero, Speiser, Zoete, Michielin and Rufer 2010; Irving, Zoete, Hebeisen, Schmid, Baumgartner, Guillaume, Romero, Speiser, Luescher, Rufer and Michielin 2012). The observation that T-cell triggering occurs in an optimized peptide:MHC affinity window largely guided by variations in off-rate prompted a “serial engagement” model (Valitutti, Muller, Cella, Padovan and Lanzavecchia 1995). In this scenario, the relatively rapid dissociation of TCR from peptide: MHC facilitates sequential and monovalent triggering of many T-cell receptors. Relevant to our system, a non-linear relationship between TNF receptor binding and functional outcome has been previously suggested (Mukai, Shibata, Nakamura, Yoshioka, Abe, Nomura, Taniai, Ohta, Ikemizu, Nakagawa, Tsunoda, Kamada, Yamagata and Tsutsumi 2009). As higher order clustering has been implicated for the signal transduction of other TNFSF members (Holler, Tardivel, Kovacovics-Bankowski, Hertig, Gaide, Martinon, Tinel, Deperthes, Calderara, Schulthess, Engel, Schneider and Tschopp 2003; Siegel, Muppidi, Sarker, Lobito, Jen, Martin, Straus and Lenardo 2004; Henkler, Behrle, Dennehy, Wicovsky, Peters, Warnke, Pfizenmaier and Wajant 2005; Wu 2013), we posit that RANK signaling is facilitated by the rapid off-rate of WT RANKL, which acts to cluster individual receptors via serial engagement.

Despite the generally accepted dogma that recruitment of three receptors is required to initiate signaling downstream of TNFSF ligands, recent evidence using engineered single-chain versions of these molecules has suggested that recruitment of two receptors is sufficient. (Boschert, Krippner-Heidenreich, Branschadel, Tepperink, Aird and Scheurich 2010; Sudhamsu, Yin, Chiang, Starovasnik, Grogan and Hymowitz 2013). We recently demonstrated that a single-chain version of RANKL with our high-affinity variant (KQFH) at two receptor recruitment sites, combined with a mutation that prevents recruitment of the third receptor, cannot signal. This raises two distinct possibilities: the first is that, unlike the receptors for TNF α and LT $\alpha_1\beta_2$, RANK signaling requires that all three sites of RANKL be occupied prior to initiating signal transduction. Alternatively, RANK signaling downstream of two clustered receptors could actually reflect high order receptor clustering (as has been

implicated for other TNFR family members) (Holler, Tardivel, Kovacsovics-Bankowski, Hertig, Gaide, Martinon, Tinel, Deperthes, Calderara, Schulthess, Engel, Schneider and Tschopp 2003; Siegel, Muppidi, Sarker, Lobito, Jen, Martin, Straus and Lenardo 2004; Wu 2013) that is not permitted in the context of high-affinity, prolonged off-rate RANKL mutants. The latter scenario would buttress the argument that the ability of RANKL to dissociate from its receptor is crucial to its ability to initiate signaling.

Our results suggest that the use of forward-genetic approaches to identify novel mutations within the TNFSF enables generation of highly efficient agonists with predilection for one known binding partner over another. The supposition that selective inhibition and/or activation of TNF receptors may be beneficial is supported by the effect of TNFR2 agonism on the complications of experimental type I diabetes (Ban, Zhang, Wang, Kuhlreiber, Burger and Faustman 2008; Faustman and Davis 2010). An effective TNFR2 agonist that does not alter the TNFR1 pathway could provide a novel avenue for the treatment this or other autoimmune and cardiovascular diseases. This strategy could also be applicable to other TNFSF ligand/receptor pairs. Because of the non-linear relationship between RANKL/RANK affinity and function, the development of receptor modifiers within the protein family through non-biased, forward genetic approaches may necessitate the use of functional, rather than affinity-based, screens (Levin, Bates, Ring, Krieg, Lin, Su, Moraga, Raeber, Bowman, Novick, Pande, Fathman, Boyman and Garcia 2012).

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Figure 1.

Osteoclastogenic potential of RANKL variants. (A) The capacity of mutant RANKL proteins to generate osteoclasts from bone marrow macrophages was assessed by TRAP stain using increasing amounts of purified RANKL. (B) Titration curves of osteoclastogenesis were fit using a four-parameter dose-response curve. (C) EC₅₀ values calculated from the curve fits in (B). EC₅₀ values representing the ability of each RANKL variant to generate osteoclasts *in vitro* is plotted against either the binding constant K_D (D) or the kinetic half-life t_{1/2} (E). Assessment of osteoclastogenic markers by real-time

detection of NFATc1 (F) or β 3 integrin (G) mRNA levels. A RANKL concentration close to the EC₅₀ value of WT-RANKL (5 ng/mL) was used for all variants.

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Figure 2.

Ability of monomeric OPG to inhibit RANKL-induced osteoclast formation. (A) Varying amounts of monomeric OPG were added to cultures containing 100 ng/mL of each RANKL variants and osteoclasts were stained for TRAP activity. (B) Quantitation of TRAP activity in the presence or absence of OPG.

Figure 3. Signaling activation by RANKL variants. (A) Bone marrow macrophages were serum starved and stimulated with 100 ng/mL WT, Q236H, or KQFH RANKL. Phosphorylation of NF- κ B and p38 were assessed by western blot. (B) Densitometry of western blots depicted in (A).

Figure 4. Activation of mature osteoclasts by RANKL variants. After 4 days in culture with WT RANKL, osteoclasts were plated in equal numbers on bovine bone slices and stimulated with WT, Q236H, or KQFH. The release of collagen fragments (CTx) was measured after 24 hours.

Table 1
Kinetic affinity parameters of RANKL variants binding to monomeric RANK and OPG
Kinetic affinity parameters of RANKL mutants binding to monomeric RANK or OPG were determined using SPR

RANKL:	194	236	269	270	RANK		OPG	
					K_D (uM)	K_{on} ($M^{-1}s^{-1}$)	K_{off} (s^{-1})	K_D (uM)
WT*	K	Q	F	H	1.403	1.51×10^5	0.208	0.031
K194E	E	Q	F	H	0.899	1.13×10^5	0.101	0.119
Q236H	K	H	F	H	0.081	3.00×10^5	0.023	1.095
KQ	E	H	F	H	0.067	1.74×10^5	0.017	3.087
KQF	E	H	Y	H	0.029	1.67×10^5	0.005	7.022
KQFH*	E	H	Y	Y	0.003	3.07×10^5	0.001	N.D.

* Previously reported (Warren, Nelson, Decker, Zou, Fremont and Teitelbaum 2014).
Values represent the averages of three independent experiments.