Mutations in TNFRSF11A, affecting the signal peptide of RANK, cause familial expansile osteolysis

Mutations in *TNFRSF11A*, affecting the signal peptide of RANK, cause familial expansile osteolysis

Anne E. Hughes[1], Stuart H. Ralston[2], John Marken[3], Christine Bell[2], Heather MacPherson[2], Richard G.H. Wallace[4], Wim van Hul[5], Michael P. Whyte[6], Kyoshi Nakatsuka[7], Louis Hovy[8] & Dirk M. Anderson[3]

Familial expansile osteolysis[1,2] (FEO, MIM 174810) is a rare, autosomal dominant bone disorder characterized by focal areas of increased bone remodelling. The osteolytic lesions, which develop usually in the long bones during early adulthood, show increased osteoblast and osteoclast activity. Our previous linkage studies mapped the gene responsible for FEO to an interval of less than 5 cM between *D18S64* and *D18S51* on chromosome 18q21.2–21.3 in a large Northern Irish family[3,4]. The gene encoding receptor activator of nuclear factor-κ B (RANK; ref. 5), *TNFRSF11A*, maps to this region. RANK is essential in osteoclast formation[6,7]. We identified two heterozygous insertion mutations in exon 1 of *TNFRSF11A* in affected members of four families with FEO or familial Paget disease of bone (PDB). One was a duplication of 18 bases and the other a duplication of 27 bases, both of which affected the signal peptide region of the RANK molecule. Expression of recombinant forms of the mutant RANK proteins revealed perturbations in expression levels and lack of normal cleavage of the signal peptide. Both mutations caused an increase in RANK-mediated nuclear factor-κ B (NF-κB) signalling *in vitro*, consistent with the presence of an activating mutation.

We studied the genetic basis of FEO in members of the large Northern Irish FEO family in which linkage to markers on 18q has been established. We also recruited a smaller American FEO family with affected individuals in four generations, and one affected male from a German family with three affected males in two generations who were diagnosed with osteolytic expansile PDB (ref. 8). Genetic linkage analysis in the American FEO fam-

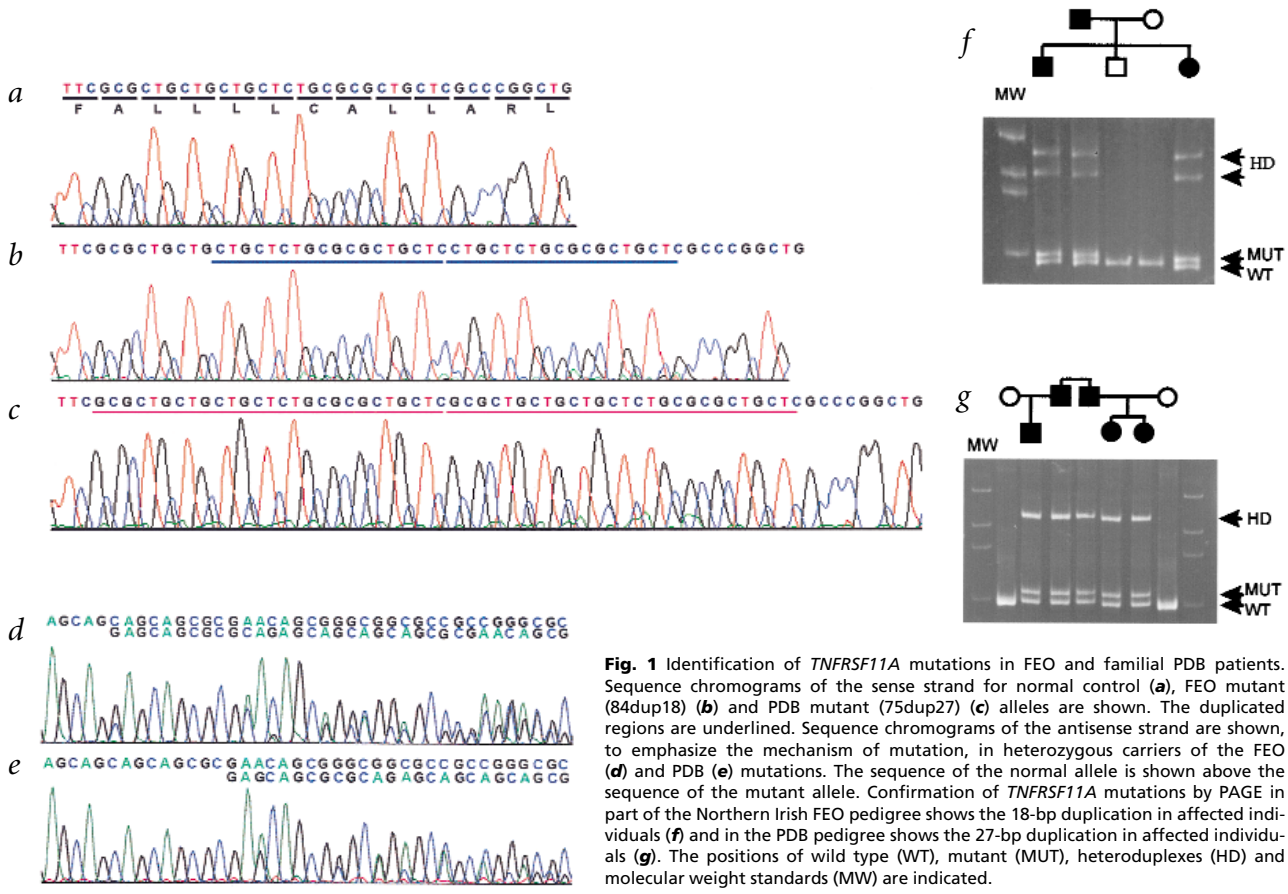

Fig. 1 Identification of *TNFRSF11A* mutations in FEO and familial PDB patients. Sequence chromograms of the sense strand for normal control (***a***), FEO mutant (84dup18) (***b***) and PDB mutant (75dup27) (***c***) alleles are shown. The duplicated regions are underlined. Sequence chromograms of the antisense strand are shown, to emphasize the mechanism of mutation, in heterozygous carriers of the FEO (***d***) and PDB (***e***) mutations. The sequence of the normal allele is shown above the sequence of the mutant allele. Confirmation of *TNFRSF11A* mutations by PAGE in part of the Northern Irish FEO pedigree shows the 18-bp duplication in affected individuals (***f***) and in the PDB pedigree shows the 27-bp duplication in affected individuals (***g***). The positions of wild type (WT), mutant (MUT), heteroduplexes (HD) and molecular weight standards (MW) are indicated.

[1]*Department of Medical Genetics, The Queen's University of Belfast, Belfast, UK.* [2]*Department of Medicine and Therapeutics, University of Aberdeen, Aberdeen, UK.* [3]*Department of Molecular Biology, Immunex Corporation, Seattle, Washington, USA.* [4]*Department of Orthopaedic Surgery, Musgrave Park Hospital, Belfast, UK.* [5]*Department of Medical Genetics, University of Antwerp, Antwerp, Belgium.* [6]*Division of Bone and Mineral Diseases, Washington University School of Medicine, St. Louis, Missouri, USA.* [7]*Second Department of Internal Medicine, Osaka City Hospital, Osaka, Japan.* [8]*Orthopädische Universitatsklinik, Frankfurt/Main, Germany. Correspondence should be addressed to A.E.H. (e-mail: A.Hughes@qub.ac.uk).*

Table 1 • Distribution of genotypes and alleles of *TNFRSF11A* polymorphism IVS6+79A/G

Genotype	Sporadic PDB	Controls
AA	17	15
AG	29	33
GG	21	15
Alleles		
A	63 (0.47)	63 (0.50)
G	71 (0.53)	63 (0.50)

ily confirmed linkage to the *FEO* locus on 18q (data not shown), although their disease allele was transmitted on a different haplotype. Accordingly, the Northern Irish and American FEO families are unrelated. *TNFRSF11A* is a candidate for causing FEO because it maps to the *FEO/PDB2* region, is highly expressed in bone and has an important role in osteoclast differentiation and activity. The nucleotide sequence of *TNFRSF11A* mRNA was known at the start of our study. We characterized the intron-exon organization and the promoter region of *TNFRSF11A* by direct sequencing of genomic clones and PCR products and by vectorette PCR on PACs containing genomic DNA. A series of four GC boxes, which are recognized by the SP1 transcription factor, are present within the proximal 250 bases of the transcription start point. This region also contains several near-consensus GC-box sequences. There are no CAAT or TATA boxes. We identified several single-base polymorphisms in intron 6. Analysis of their segregation within the Northern Irish FEO kindred supported localization of *TNFRSF11A* between markers *D18S383* and *D18S51*. Analysis of clones in the Genebridge 4 radiation hybrid panel suggested a location in the region of *D18S60* between *WI-9823* and *SGC33768*. *TNFRSF11A* is therefore located within the defined critical region for FEO.

We screened affected and unaffected members of the Northern Irish and American FEO families for mutations in all exons, intron-exon junctions, 5′ and 3′ UTRs, and the promoter region of *TNFRSF11A* by direct sequencing of PCR products. We identified an identical tandem duplication of bases 84–101 in exon 1 of all affected individuals tested (*TNFRSF11A* 84dup18; Fig. 1*a*,*b*,*d*). This duplication segregated with the disease in both families. We confirmed the insertion in affected individuals by the presence of an allele of increased size, which was visible after polyacrylamide gel electrophoresis (PAGE; Fig. 1*f*). We did not detect insertions in unaffected members of the FEO families, 90 individuals with sporadic PDB or 158 controls (data not shown). We found an identical *TNFRSF11A* 84dup18 duplication in DNA from the only available affected member of the German family. We then performed mutation screening in members of four PDB families with evidence of possible linkage to 18q (ref. 9), which revealed a slightly larger duplication involving bases 75–101 (*TNFRSF11A* 75dup27; Fig. 1*c*,*e*) in exon 1 that co-segregated with the disease in one PDB family (Fig. 1*g*). We did not find *TNFRSF11A* mutations in the other three PDB families, or in cDNA prepared from affected bone of five patients with sporadic PDB. Both *TNFRSF11A* duplications share an identical 3′ endpoint at base 101 and are likely to have arisen by reverse slippage during DNA replication.

We detected several *TNFRSF11A* polymorphisms that are not of functional significance. These include –1G/A, close to the start position of transcription, and 30T/C, within the 5′-UTR, 9 bases before the initiation codon. We found an IVS1+5G/A polymorphism in the 5′ splice site of intron 1. The rarer AG/gtaaa variant segregated with the Northern Irish FEO duplication, whereas we found the identical American mutation on a homozygous AG/gtaag background. This implies that these FEO mutations arose independently. The similarity of the phenotypes in both families suggests that splicing is unlikely to be affected by the IVS1+5G/A polymorphism. We identified a variant, V/A192, in exon 6, which encodes a predicted cysteine-rich pseudorepeat in the extracellular region of RANK. Valine (which shows homology at this position with mouse Rank) was more common than alanine at this site. V/A192 was in strong linkage disequilibrium with three intronic polymorphisms: IVS5-17T/C, IVS6+79A/G and IVS6+166A/G. Intron 6 contained another cluster of polymorphisms at its 3′ end: IVS6-151A/G, IVS6-245A/G and IVS6-258A/C.

We typed DNA from 67 Northern-Irish patients with sporadic PDB and 63 age-matched controls for *TNFRSF11A* polymorphism IVS6+79A/G (Table 1). The distribution of genotypes in cases and controls did not deviate from Hardy-Weinberg equilibrium. We found no significant association between this polymorphism and sporadic PDB (χ^2=0.13; P=0.72 with 1 d.f.), and no associations when polymorphisms in the promoter region of *TNFRSF11A* were typed in a smaller sample (data not shown). This shows that *TNFRSF11A* has, at most, a minor gene effect in the pathogenesis of sporadic PDB, a finding supported by other studies (A. Sparks *et al*., manuscript submitted).

We used mammalian expression plasmids encoding full-length wild-type *TNFRSF11A* (pWTRANK), FEO mutant RANK (pFEORANK) and PDB mutant RANK (pPDBRANK) in transient transfection and labelling experiments to assess the relative expression levels of the three different forms of RANK. Lower levels of recombinant receptor were expressed by pFEORANK (~25% of wild type) and pPDBRANK (50% of wild type) compared with pWTRANK transfectants (Fig. 2). In addition, the mutant RANK proteins expressed were larger than wild-type RANK (Fig. 2), and were of a size consistent with the lack of normal cleavage of the signal peptide. These results suggest that both mutations result in the inability of RANK protein to traffic normally to the cell surface.

We co-transfected plasmids pWTRANK, pFEORANK and pPDBRANK into mammalian cells with an NF-κB–responsive luciferase reporter plasmid to assess whether the duplications in the RANK signal peptide found in FEO or familial PDB affect the functional capacity of the mutant receptor. Cell lysates contained up to a twofold higher level of luciferase activity when transfected

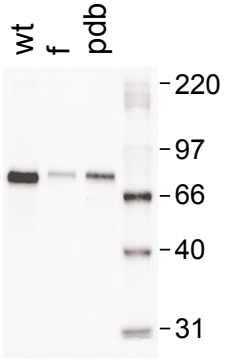

Fig. 2 Mutations result in decreased expression levels of RANK and lack of signal peptide cleavage. Metabolically labelled cell lysates from 293EBNA cells transfected with pWTRANK (w), pFEORANK (f) and pPDBRANK (pdb) were immunoprecipitated with anti-RANK monoclonal antibody M331 and resolved by PAGE. The relative amounts of immunoprecipitated protein were the same using four different anti-human RANK monoclonal antibodies (data not shown). The larger apparent sizes of FEORANK and PDBRANK compared with wild-type RANK (~3 kD larger) are consistent with lack of cleavage of the 30-aa (FEO) and 33-aa (PDB) signal peptides predicted by the GCG program SIGNALPEP. The sizes of protein molecular weight standards (in kD) are shown at right.

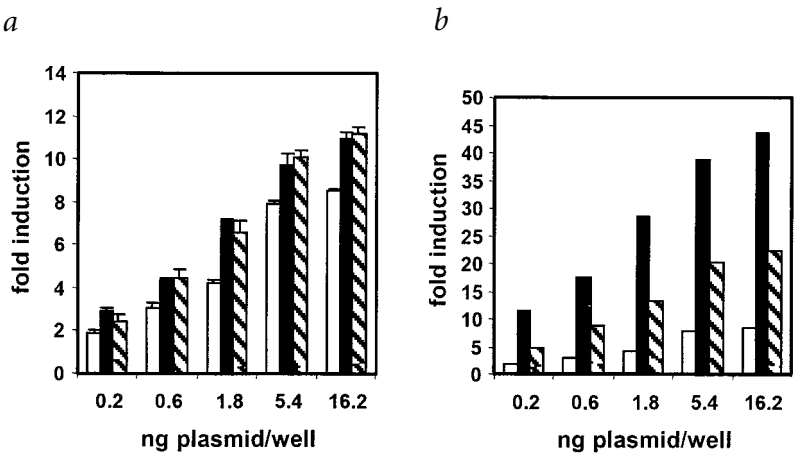

Fig. 3 The FEO and PDB mutations result in increased constitutive RANK signalling. ***a***, RANK activation of an NF-κB–responsive luciferase reporter was assessed in 293EBNA cells transfected with increasing amounts of pWTRANK, pFEORANK or pPDBRANK plasmid DNA. Two days post-transfection, cell lysates were collected and assayed for luciferase activity. Luciferase activity is presented as increase relative to control lysates from cells transfected with luciferase reporter plasmid alone. Values were normalized to levels of β-galactosidase. Reporter activity is shown from lysates from cells transfected with pWTRANK (open bars), pFEORANK (filled bars) and pPDBRANK (crosshatched bars). Data are means±standard deviation, n=3 from one representative experiment of three which gave comparable results. ***b***, The data shown in (*a*) adjusted for the relative expression levels of FEO RANK (0.25×) and PDB RANK (0.5×); wild type=1×, based on quantitation of the RANK protein bands shown in Fig. 2. Quantitation was performed on a STORM Phosphorimager using the ImageQuant program (data not shown).

with pFEORANK or pPDBRANK compared with pWTRANK (Fig. 3*a*). These differences were amplified when the reporter activities were recalculated on the basis of the relative receptor expression levels (Fig. 3*b*). These results show that the FEO and PDB mutations result in increased constitutive activation of RANK.

FEO and familial PDB are similar disorders. They are distinguished clinically by the distribution of osteolytic lesions, with sparing of the axial skeleton in FEO. The Northern Irish and American FEO families share the same *TNFRSF11A* 84dup18 mutation and have very similar clinical features. Deafness is the earliest manifestation of the disease and often occurs before the age of ten. There is early loss of adult dentition due mainly to resorption in the cervical region of the teeth. Expansile osteolytic lesions present from the late teenage years until late middle age. Affected individuals usually develop one or two lesions, which are most prevalent in the tibia, ulna, humerus and femur. Amputation has been performed for symptom control in both families. No member of either family has had involvement of the skull or pelvis, which are common sites for PDB lesions. The small German family, which has the same 84dup18 mutation, appears to have more severe disease. The proband has large deformities affecting both hands, both tibiae and both femorae. His father died as a result of Paget sarcoma associated with a lesion in the femur and also had lesions affecting both arms and the skull, and his brother has similar, though less pronounced, skeletal deformities. The family with PDB that carries the *TNFRSF11A* 75dup27 mutation is of Japanese origin. Most affected individuals presented in their teens and early twenties with bone pain or deformity. Unlike the FEO families, affected individuals have involvement of the axial skeleton with lesions in the spine, pelvis and mandible as well as at sites associated with lesions in FEO. All affected patients have dental problems and several have hearing impairment. These four families have similar activating mutations in *TNFRSF11A*. The inter- and intra-familial phenotypic variation may reflect subtle differences in the effects of the two mutations, differing genetic backgrounds or variation in expression of potential cytokine triggers of osteolysis.

The *TNFRSF11A* mutations that we have described produce short in-frame insertions within the signal peptide region of RANK. These insertions result in lower levels of recombinantly expressed RANK protein, a lack of normal cleavage of the RANK signal peptide and increased RANK-mediated activation of NF-κB. The duplications occur in the 'h' or hydrophobic core region of the RANK signal peptide[10]. The length of this region is critical for proper signal peptidase cleavage[11]. A possible mechanism to explain our findings is that lack of proper RANK signal peptide cleavage results in higher intracellular accumulation of defective RANK translation products in compartments of the secretion pathway. This accumulation then leads to a higher incidence of receptor self-association, and thus, increased constitutive RANK signal transduction. In addition to RANK, several other members of the TNF-receptor superfamily transmit signals using the same downstream pathways. The fine-tuning mechanisms for regulating their wide-ranging roles in cellular proliferation, differentiation, survival and apoptosis remain to be elucidated. All of these may be critical for maintaining normal bone metabolism. Our results highlight the importance of regulatory factors in the RANK-mediated osteoclastogenesis pathway in diseases of bone metabolism.

Methods

Genomic organization of *TNFRSF11A*. We identified the position of each intron within *TNFRSF11A* and obtained end sequence using several methods, including DNA sequencing of PCR products generated with primer pairs designed at intervals within the cDNA and vectorette PCR on genomic clones. We selected PACs 108M4 and 131H23 from the RPCI1 PAC library[12] by hybridization of *TNFRSF11A* cDNA to high-density gridded filters (supplied by the UK HGMP Resource Centre). We made vectorette libraries of pooled DNA from both PACs using *Alu*I, *Rsa*I, *Stu*I, *Nru*I, *Sma*I and *Hae*III (ref. 13).

Genomic DNA amplification. We amplified exon 1 of *TNFRSF11A* from genomic DNA using forward primer 5′–TGGGGTGCGGCAAGG CAGGAG–3′ and reverse primer 5′–AAGGCGGAGGAGCCAGGATGC–3′. We performed PCR in reactions (20 μl) using *Taq* DNA polymerase (0.5 U; Qiagen), 1×buffer and solution Q, dNTPs (200 μM), primers (0.3 μM) and DNA (50 ng). PCR conditions were as follows: initial denaturation at 97 °C for 3 min followed by 35 cycles of 97 °C for 1 min; annealing at 65 °C in the first 10 cycles, then 63 °C for 1 min and extension at 72 °C for 1 min. We amplified polymorphism IVS6+79A/G by amplification of IVS6+14 to IVS6+300 using forward primer 5′–GAGCCTGTTGGTTGATAGACGTG–3′ and FAM-labelled reverse primer 5′–ACTTGTGACCACCATCATCC-CAC–3′ as described above (annealing at 60 °C/55 °C). This polymorphism was typed using the BESS T-Scan system (Epicentre). Primers and conditions for amplification of all other exons are available on request.

Mutation detection and analysis. We purified PCR products on Wizard columns (Promega) and sequenced with an ABI377 instrument using BigDye terminator cycle sequencing chemistry. We included 5% DMSO in sequencing reactions for exon 1. Mutations were confirmed by size variation after electrophoresis on 5% polyacrylamide gels run in 1×TBE buffer and stained with ethidium bromide. We excised individual alleles and re-amplified by PCR before sequencing as above.

Wild-type and mutant *TNFRSF11A* expression vector construction. We used two sets of synthetic oligonucleotide linker pairs to recreate the 5′ coding region of wild-type human *TNFRSF11A* (WTRANK) and to create the corresponding regions of FEO *TNFRSF11A* (FEORANK) and PDB

TNFRSF11A (PDBRANK). For WTRANK, we synthesized four oligonucleotides: 1, 5′–TCGACGCCATGGCCCCGCGCGCCCGGCGGCGCCGCCCGCTGTTCG–3′; 2, 5′–CGCTGCTGCTGCTCTGCGCGCTGCTCGCCCGGCTGCA–3′; 3, 5′–GCCGGGCGAGCAGCGCGCAGAGCAGCAGCAGCGCGAACAGC–3′; 4, 5′–GGGCGGCGCCGCCGGGCGCGCGGGGCCATGGCG–3′. These oligonucleotide sets encode the 5′ coding region of *TNFRSF11A* from the initiator codon through the *Pst*I site (nt 39–112). For FEORANK, we replaced oligonucleotides 2 and 3 with 5′–CGCTGCTGCTGCTCTGCGCGCTGCTCGCCCGGCTGCA–3′ (5) and 5′–GCCGGGCGAGCAGCGCGCAGAGCAGGAGCAGCGCGCAGAGCAGCAGCAGCGCGAACAGC–3′ (6). For PDBRANK, we replaced oligonucleotides 2 and 3 with 5′–CGCTGCTGCTGCTCTGCGCGCTGCTCGCGCTGCTGCTG–3′(7), 5′–GCGAGCAGCGCGCAGAGCAGCAGCAGCGCGAACAGC–3′ (8), 5′–CTCTGCGCGCTGCTCGCCCGGCTGCA–3′ (9) and 5′–GCCGGGCGAGCAGCGCGCAGAGCAGCAGCAGC–3′ (10). Oligonucleotide pairs were phosphorylated with ATP and T4 polynucleotide kinase and allowed to anneal following heat inactivation of the kinase. We ligated oligonucleotide pairs 1/4 and 2/3 to the downstream coding fragment of *TNFRSF11A* (*Pst*I-*Not*I fragment from pcdna3:huRANK; ref. 5) and a mammalian expression vector backbone to generate pWTRANK. Alternatively, oligonucleotide pairs 1/4 and 5/6 were ligated to downstream *TNFRSF11A* and vector DNA fragments to generate pFEORANK, and oligonucleotide pairs 1/4, 7/8 and 9/10 were ligated to downstream *TNFRSF11A* and vector DNA fragments to generate pPDBRANK. We verified all constructs by dye termination sequencing of both DNA strands.

Cell transfection and reporter assay. An NF-κB–responsive reporter plasmid using a promoter with three NF-κB–binding sites fused to a luciferase reporter has been described[14]. We transfected the reporter plasmid (40 ng/well) alone or in combination with varying amounts of pWTRANK, pFEORANK or pPDBRANK plasmids into 293EBNA cells (Invitrogen) using the DEAE-dextran transfection method. A plasmid expressing β-galactosidase (25 ng/well) was included as an internal transfection efficiency control. Total amounts of plasmid DNA for each transfection were equalized by the addition of empty vector. We collected cell lysates from transfected cultures after 48 h incubation using reporter lysis buffer (Promega). We measured luciferase activity in cell lysates using luciferase substrate (Promega) and an EG&G/Berthold Luminometer. Relative luciferase activity was normalized to the β-galactosidase activity in each sample.

Metabolic labelling. We transfected subconfluent 293EBNA cells in 6-well plates with pWTRANK, pFEORANK or pPDBRANK (1 μg/well). Cells were metabolically labelled 48 h following transfection for 15 min with [^{35}S]-cysteine/methionine (50 μCi/ml; Promix, Amersham) in cysteine-free, methionine-free DMEM (Gibco). Cells were washed twice with PBS, collected with PBS/0.1% Triton X-100 and clarified cell lysates immunoprecipitated with anti-human RANK antibody M331 (ref. 5; 10 μg/ml) and protein A– and G–Sepharose (Pharmacia). Immune complexes were resolved by PAGE and radiolabelled proteins detected by autoradiography.

GenBank accession numbers. Human *TNFRSF11A* mRNA, AF018253 mouse *Tnfrsf11a*, mRNA AF019046.

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