Supporting Information

Maeda et al. 10.1073/pnas.1301674110

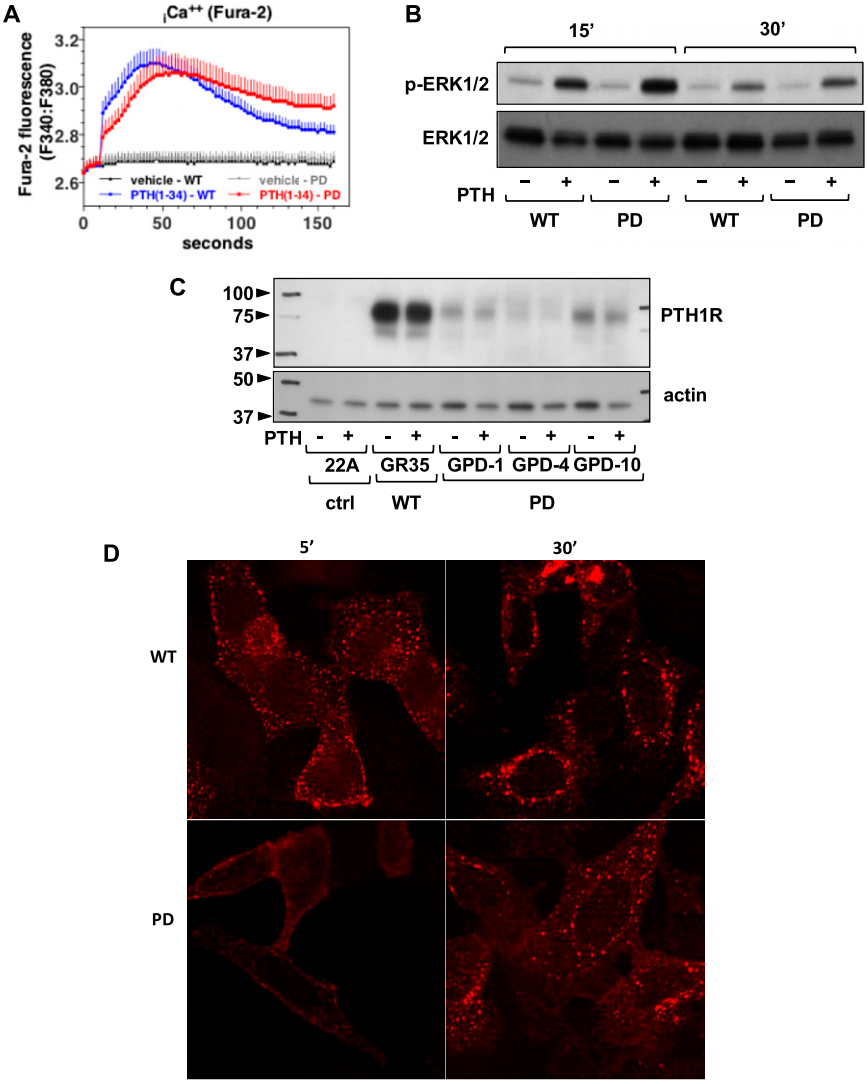

Fig. S1. Functional properties of the PD-PTHR1 in vitro. (*A*) Signaling via the PLC/IP3/iCa pathway was assessed in transiently transfected HEK-293 cells using Fura2-AM; ratiometric Fura2 fluorescence was recorded in cells treated with vehicle, or PTH(1-34) (100 nM). Data are means (±SEM) of six experiments each in quadruplicate. Fura-2 signaling parameters are reported in Dataset S4. (*B*) PTH(1-34)-induced ERK1/2 phosphorylation was assessed in HEK-293 cells transiently transfected to express either the WT-PTHR1 or PD-PTHR1; cells were treated with PTH(1-34) (100 nM) or buffer for 15 or 30 min, then analyzed by Western blot. (*C*) Ligand-induced internalization was assessed by confocal fluorescent microscopy in transiently transfected HEK-293 cells treated with the fluorescent (red) ligand TMR-PTH(1-34) (100 nM) for 5 or 30 min (400× magnification). (*D*) HEK-293-derived cell lines stably transfected to express either the rPTHR1-WT (GR-35) or rPTHR1-PD (GPD-1, GPD-4, and GPD-10), or control cells not PTHR1-transfected (GS-22A) were treated with vehicle or PTH(1-34) (100 nM) for 10 min, and then cell membranes were prepared and analyzed by Western blotting using a primary rabbit polyclonal antisera (PRB-620P, Covance Inc.) that recognizes an epitope in the Exon E2 region of the rat PTHR1. Detection was with a horse-radish peroxidase-conjugated secondary antibody directed towards rabbit IgG. Actin was analyzed to control for sample loading. Cell membranes were prepared as used in radioligand binding assays. Molecular weight markers (kDa) are indicated on the left.

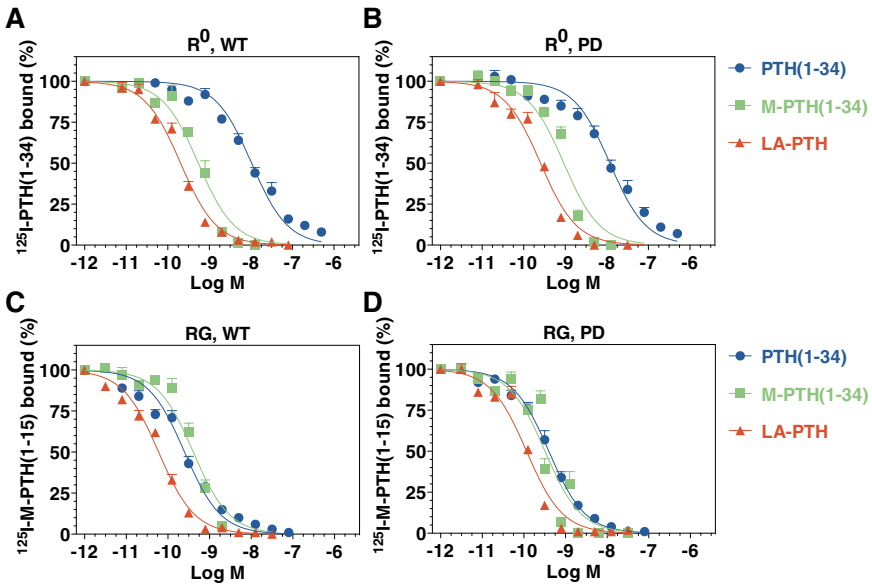

Fig. S2. Competition binding analysis of PTH(1–34) and modified PTH analogs. Competition binding assays were performed using membranes prepared from transiently transfected COS-7 cells; R^0 assays used ^{125}I-PTH(1–34) as tracer radioligand and the reactions contained GTPγS (1×10^{-5} M); RG assays used ^{125}I-M-PTH (1–15) tracer radioligand and membranes prepared from COS-7 cells cotransfected to express a high-affinity Gαs mutant. (*A*) Binding of PTH(1–34), M-PTH (1–34), and LA-PTH to the R^0 conformation of the WT-PTHR1. (*B*) Binding of the same analogs to the R^0 conformation of PD-PTHR1. (*C*) Binding to the RG conformation of the WT-PTHR1. (*D*) Binding to the RG conformation of the PD-PTHR1. Data are means (±SEM) of six [PTH(1–34) only] or three experiments, each performed in duplicate (Dataset S1).

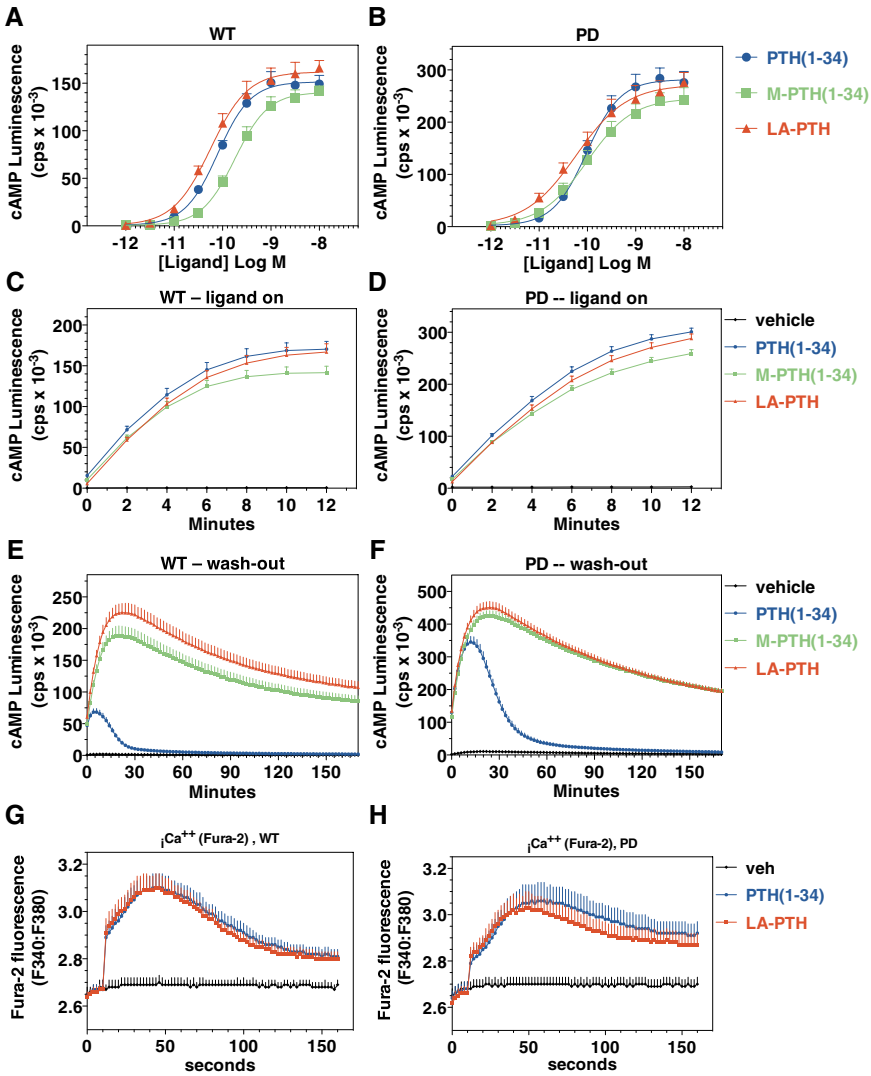

Fig. S3. cAMP and intracellular calcium signaling via the WT-PTHR1 and PD-PTHR1. Assays of cAMP accumulation were performed using HEK-293-derived cell lines stably transfected to express the luciferase-based cAMP glosensor protein along with either the WT-PTHR1 (GR-35 cells) or PD-PTHR1 (GPD-4 cells). (*A*) Dose–response analysis of PTH(1–34), M-PTH(1–34) and LA-PTH in cells expressing the WT-PTHR1. (*B*) Ligand dose–response analysis in cells expressing the PD-PTHR1. In the assays of *A* and *B*, luminescence was recorded 14 min after ligand addition. (*C*) Accumulation of cAMP in the presence of vehicle, PTH(1–34) (0.3 nM), M-PTH(1–34) (0.3 nM), or LA-PTH (0.1 nM) in cells expressing the WT-PTHR1. (*D*) Accumulation of cAMP in the presence of vehicle or the indicated ligands in cells expressing the PD-PTHR1. In *C* and *D*, the ligands were applied to the cells at $t = 0$ and luminescence was recorded for 12 min thereafter. (*E*) Accumulation of cAMP following ligand wash-out in cells expressing the WT-PTHR1. (*F*) Accumulation of cAMP following ligand wash-out in cells expressing the PD-PTHR1. In *E* and *F*, the cells of *C* and *D*, respectively, were removed from the plate reader at the end of the 12-min "ligand-on" phase, rinsed twice to remove unbound ligand, treated with fresh media containing luciferin, and luminescence was again recorded for an additional 180 min of "wash-out." Luminescence was recorded using a PerkinElmer Envision plate reader; cells were preloaded with luciferin for 30 min before ligand addition. Data are means (± SEM) of four experiments, each performed in duplicate (*A* and *B*) or quadruplicate (*C*–*F*). cAMP assay parameters are reported in Datasets S2 and S5. (*G*) Intracellular calcium signaling in cells expressing the WT-PTHR1. (*H*) Intracellular calcium signaling in cells expressing the PD-PTHR1. iCa assays used HEK-293 cells transiently transfected to express either the WT-PTHR1 or PD-PTHR1. The cells were preloaded with the calcium-sensitive dye Fura2-AM for 30 min, and then ratiometric Fura2-AM fluorescence (lex. = 340 nm vs. lex 380 nm; lem. = 515 nm) was measured using a PerkinElmer Envision plate reader; at $t = 10$ s, the cells were treated with vehicle, PTH(1-34), or LA-PTH, each ligand at a concentration of 100 nM. Data are means (±SEM) of six experiments, each performed in quadruplicate.

1. Binkowski BF, et al. (2011) A luminescent biosensor with increased dynamic range for intracellular cAMP. *ACS Chem Biol* 6(11):1193–1197.

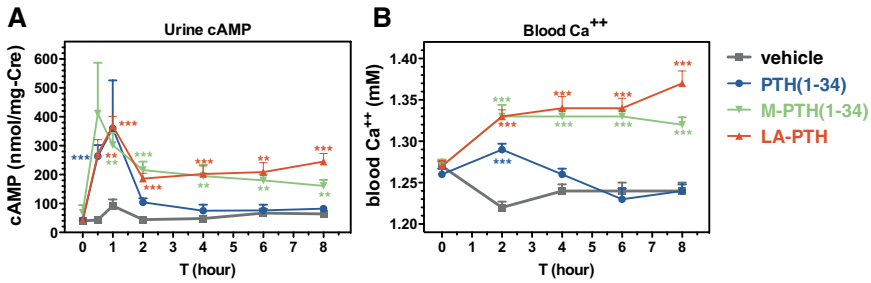

Fig. S4. Effect of PTH analogs on urine cAMP and blood Ca^{2+} in WT mice. (*A*) WT mice were injected with vehicle or the indicated ligands, each at a dose of 10 nmol/kg, and at times thereafter, and just before injection ($t = 0$) spot urine was collected and analyzed for cAMP. (*B*) Tail vein blood was collected from the same mice and analyzed for Ca^{2+}. Data are means± SEM; n = six mice per group; P vs. vehicle: *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

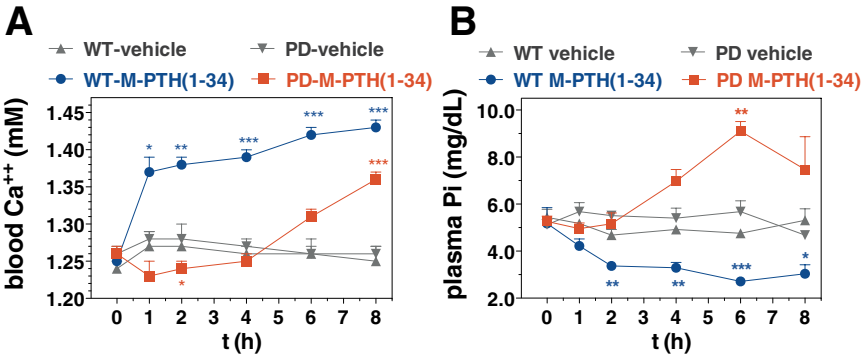

Fig. S5. Effects of M-PTH(1–34) on blood levels of Ca^{2+} and Pi in WT and PD mice. (*A*) WT or PD mice were injected s.c. with vehicle or M-PTH(1–34) (50 nmol/kg); tail vein blood was collected at the indicated times thereafter, or immediately before injection ($t = 0$), and assessed for the concentration of Ca^{2+}. (*B*) Mice were injected as in *A*; tail vein blood was collected and the plasma was assessed for the concentration of Pi. Data are means ± SEM; n = 3 or 4 mice per group; P vs. the corresponding vehicle: *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

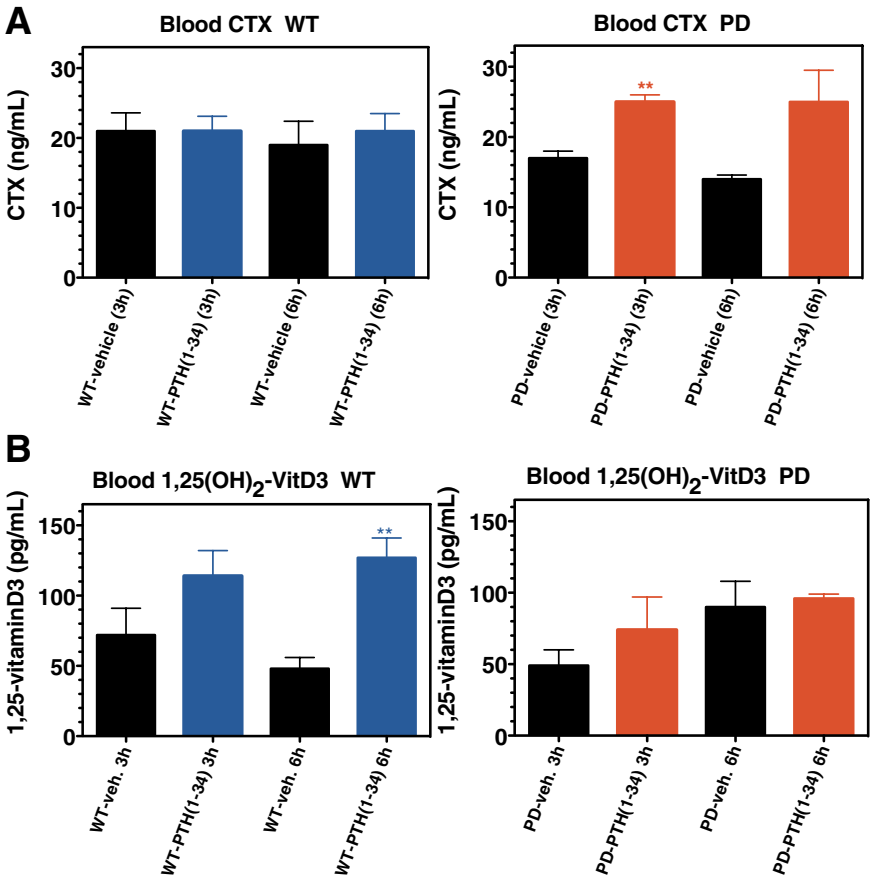

Fig. S6. Effects of PTH(1-34) on blood levels of CTX and 1,25$(OH)_2$-vitamin-D_3. (*A*) WT or PD mice were injected s.c. with vehicle or PTH(1-34) (50 nmol/kg) and tail vein blood was collected at 3 and 6 h after injection and assessed for concentrations of CTX. (*B*) Mice were treated as in *A*, and blood was assessed for 1,25$(OH)_2$-vitamin-D_3. Data are means ± SEM; $n = 3$ or 4 (CTX) or 4 [1,25$(OH)_2$-vitamin-D_3] mice per group; *P* vs. corresponding vehicle: $^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$. CTX, C-telopeptide.

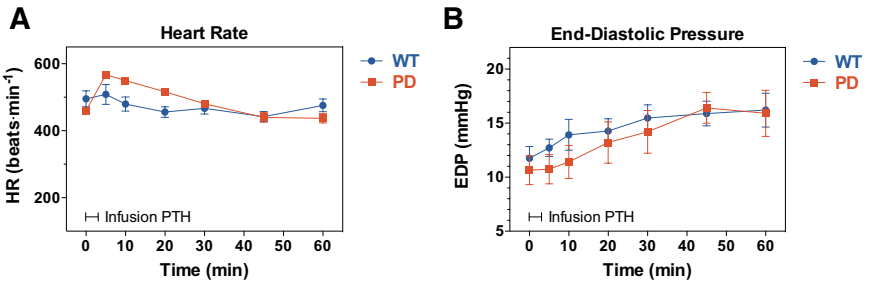

Fig. S7. Effects of PTH(1–34) infusion on heart rate and end diastolic pressure in WT and PD mice. As described in Fig. 7*C* legend, mice were anesthetized and infused into the right jugular vein with PTH (25 nmol·kg^{-1}·min^{-1}) for 2 min, and effects on cardiac function were measured using a pressure probe inserted into the left ventricle: (*A*) heart rate; (*B*) end-diastolic pressure; $t = 0$ indicates initial conditions immediately before PTH infusion and the bracket indicates time of PTH infusion. Data are means ± SEM; $n = 6$ mice per group.

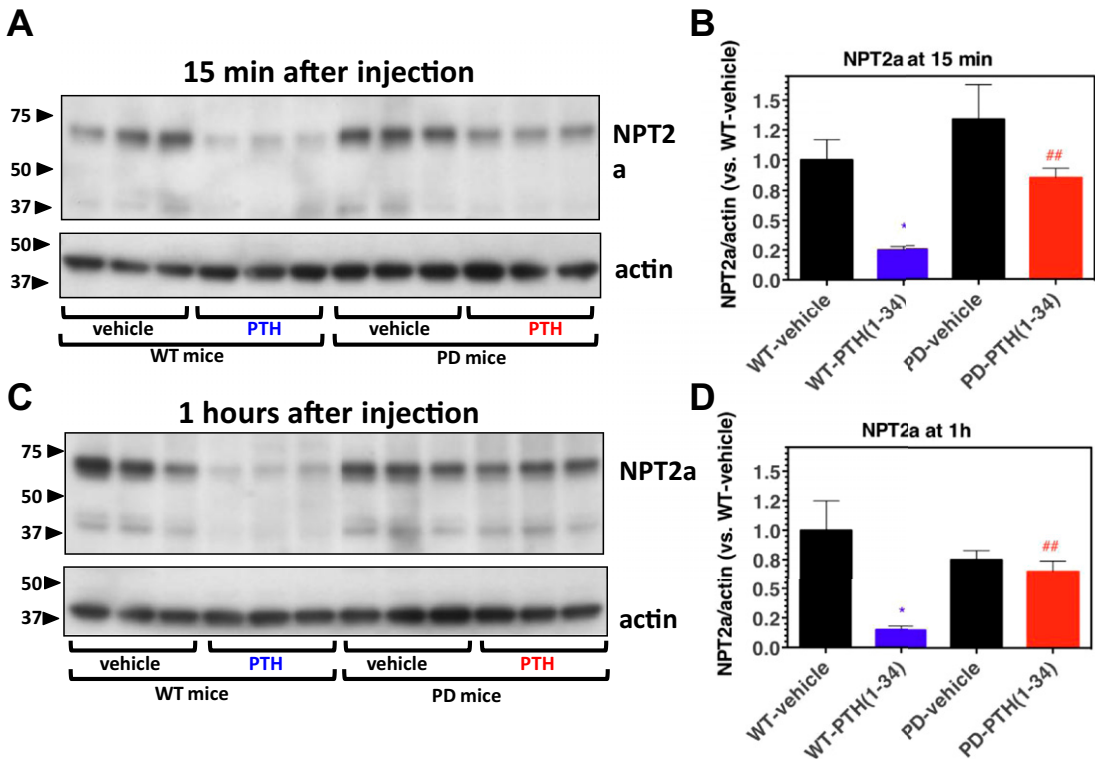

Fig. S8. Effects of PTH(1–34) on NPT2a protein levels in kidney brush-border membranes (BBMs). WT or PD mice were injected s.c. with vehicle or PTH(1–34) (50 nmol/kg), and BBMs were prepared from kidneys isolated 15 min (*A* and *B*) or 1 h (*C* and *D*) after injection and analyzed by Western blotting for the presence of NPT2a and actin. The graphs (*B*, *D*) show densitometric quantification of the ~70-kDa NPT2a band, normalized for each sample to the value obtained for the ~38-kDa actin band, and expressed as a percent of the mean corresponding value obtained with vehicle-injected WT mice. BBMs were prepared as described (1); 30 μg protein was loaded per lane; primary antibody for NPT2a was a gift from K. Miyamoto, Tokushima University, Japan; that for actin was from Santa Cruz (catalog no. sc-1616); secondary antibody was HRP-labeled goat anti-rabbit IgG (Cell Signaling Technology Inc. catalog no. 7074). Data are means ± SEM; $n = 3$ mice per group; *P* vs. corresponding vehicle: $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

1. Segawa H, et al. (2009) Type IIc sodium-dependent phosphate transporter regulates calcium metabolism. *J Am Soc Nephrol* 20(1):104–113.

PCR primers

Gene		Sequence	Length
GAPDH	Forward	cgtcccgtagacaaaatggt	20
GAPDH	Reverse	tcaatgaaggggtcgttgat	20
beta-actin (actb2)	Forward	ctaaggccaaccgtgaaaag	20
beta-actin (actb2)	Reverse	ccatcacaatgcctgtggta	20
CYP27B1	Forward	gctcctgcgacaagaaagtc	20
CYP27B1	Reverse	cctctgccattcttcaccat	20
CYP24A1	Forward	ccagcggctagagatcaaac	20
CYP24A1	Reverse	cacgggcttcatgagtttct	20
TRPV5	Forward	tctggagcttgtggtttcct	20
TRPV5	Reverse	cacccaggaggcagaaataa	20
RANKL	Forward	cgctctgttcctgtactttcg	21
RANKL	Reverse	agtcctgcaaatctgcgttt	20
OPG	Forward	gagtgtgaggaagggcgtta	20
OPG	Reverse	gcaaactgtgtttcgctctg	20

Fig. S9. Primers used for RT-PCR analysis.

Other Supporting Information Files

Dataset S1 (XLS)
Dataset S2 (XLS)
Dataset S3 (XLS)
Dataset S4 (XLS)
Dataset S5 (XLSX)