GPR98/*Gpr98* Gene Is Involved in the Regulation of Human and Mouse Bone Mineral Density

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Context: Genetic factors are important in the development of osteoporosis.

Objective: The aim of this study was to search for novel genes that regulate bone mineral density (BMD).

Design: We performed a search for 57,244 single-nucleotide polymorphisms (SNP) associated with BMD using SNP arrays and a replication study.

Setting and Patients: Baseline examinations were conducted in Japanese postmenopausal women. The mean (SD) age of the subjects was 66.5 (8.4) yr. We chose five SNP associated with BMD as those having lower combined *P* values between the first-stage (n = 251) and the second-stage (n = 499) analyses than the value determined by Bonferroni's correction. We also analyzed the bone-related phenotypes in knockout mice of a candidate gene.

Results: We focused on an SNP of G protein-coupled receptor 98 (*GPR98*) gene that showed a significant *P* value after the multiple-comparison tests in Japanese postmenopausal women. The subjects with one or two risk SNP (GG and AG genotype groups) had an increased risk of fractures (AA *vs.* GG + AG; $P = 0.043$). Femoral BMD was significantly lower in 12-wk-old *Gpr98*-knockout mice than in wild-type mice. A three-point bending test revealed that this morphological phenotype did in fact correlate with mechanical fragility in *Gpr98*-knockout mice. Compared with primary wild-type osteoblasts, primary *Gpr98*-deficient osteoblasts had increased *Rankl* expression and induced activity for osteoclastogenesis and osteoclastic function.

Conclusions: Genetic analyses in both human and mouse models uncovered the importance of the *GPR98* gene in the regulation of bone metabolism. **(*J Clin Endocrinol Metab* 97: E565–E574, 2012)**

Osteoporosis is a common skeletal disease characterized by low bone mineral density (BMD) and microarchitectural deterioration of bone tissue, leading to decreased skeletal strength and increased susceptibility to fracture (1). Osteoporosis and osteoporotic fractures are known to reduce the quality of life of the elderly and have recently become a concern in developing countries as well as in developed countries (2).

BMD is a complex quantitative trait with normal distribution and is thought to be 50–90% under genetic con-

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Abbreviations: BMD, Bone mineral density; BV, bone volume; DXA, dual-energy x-ray absorptiometry; GPR98b, G protein-coupled receptor 98b; GWA, genome-wide association; KO, knockout; LD, linkage disequilibrium; MAF, minor allele frequency; M-CSF, macrophage colony-stimulating factor; micro-CT, microcomputed tomography; QQ, quantile-quantile; qRT-PCR, quantitative RT-PCR; RANKL, receptor activator of nuclear-factor-κB ligand; SNP, single-nucleotide polymorphism; TRACP, tartrate-resistant acid phosphatase; TV, tissue volume; WT, wild type.

trol based on twin and family studies (3–6). Modest associations have been found between BMD variations in postmenopausal women and polymorphisms in some genes (7, 8), including those encoding the vitamin D receptor (*VDR*) (9), estrogen receptor α (10), collagen type Iα1 (11), low-density lipoprotein receptor-related protein 5 (*LRP5*) (12–14), and secreted frizzled-related protein 4 (*sFRP4*) (15). Although the genetic background of osteoporosis has been studied for many years, the main susceptibility genes and the molecular mechanisms underlying this disease remain largely unknown (16). The identification of novel candidate genes that contribute to osteoporosis susceptibility will impact the diagnosis and treatment of this disorder. Rapid technological advances have made it feasible to pursue large-scale genome-wide association (GWA) studies (17, 18). GWA is an unbiased approach that involves scanning the entire human genome to identify novel genes/genome regions with modest effects on complex human diseases/traits. A number of GWA studies have found novel single-nucleotide polymorphisms (SNP) associated with complex diseases/traits, including osteoporosis and BMD in Caucasian (19–22) and Korean (23) populations. These SNP were mapped as close to or within *LRP5* (20), *TNFRSF11A* (21), *TNFRSF11B* (20), *sFRP4* (23), *ADAMTS18* (22), and *TGFBR3* (22).

We have previously performed a large-scale analysis of SNP in 251 Japanese postmenopausal women using the Affymetrix GeneChip Human Mapping 50K Hind array (first-stage analysis) (24) to identify common genetic variants associated with BMD. We chose 13 SNP in the first-stage analysis; these SNP have *P* values lower than the thresholds determined by quantile-quantile (QQ) plots of *P* values from single SNP analyses of dominant and recessive models of inheritance of total-body BMD ($P < 6.99 \times 10^{-6}$ and $P < 1.60 \times 10^{-5}$, respectively) (24).

In the present study, we performed a replication analysis of the association between the 13 SNP and BMD in another population as a second-stage analysis. We discovered that five SNP had significant associations with a low BMD phenotype, with lower combined *P* values between the first- and the second-stage analyses than the *P* values determined by Bonferroni's correction. By analyzing associations between the array SNP and the deviations in BMD determined by dual-energy x-ray absorptiometry (DXA), we determined that a common variant in the 3′-flanking region of the *GPR98* gene, rs10514346, is a candidate BMD-related polymorphism. The association of rs10514346 with BMD was replicated in an *in silico* analysis of data from the Framingham Heart Study (19). We also have shown that *Gpr98*-knockout (KO) mice display low BMD as well as bone fragility. Thus, genetic analyses in both human and mouse models revealed that *GPR98*/*Gpr98* is a novel candidate gene associated with osteoporosis susceptibility.

Subjects and Methods

Study population

This is a prospective observational study conducted between 1993 and 2006 (24). Baseline examinations were conducted on unrelated ambulatory postmenopausal volunteers living in the central area of Japan. Numbers of the subjects were 251 postmenopausal women in the first-stage analysis and 499 postmenopausal women in the second-stage replication study. The exclusion criteria were endocrine disorders such as hyperthyroidism, hyperparathyroidism, adrenal disease, or diabetes mellitus with insulin treatment, renal disease, a history of extensive gastrointestinal surgery, and use of medications known to affect bone metabolism. The mean (SD) age of the subjects was 66.5 (8.4) yr. Basic characteristics of the human subjects are shown in Supplemental Table 1 (published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). There were no smokers in the group. All women provided written informed consent before the study. This study was approved by the ethics committees of the University of Tokyo Hospital and the participating clinical institutes.

We also tested the association between the candidate SNP and all fractures using data from 675 Japanese postmenopausal women. Subjects who had sustained fractures from major trauma were excluded from the analysis. The period of follow-up for each participant was calculated as the time from inclusion in the study to the time of first fracture, death, or loss to follow-up or to the end of 2006, whichever occurred first. All of the subjects in the present study except those whose first fracture event was observed within 1 yr were followed up for more than 1 yr [mean observational period (SD), 5.1 (3.4) yr]. The termination of the observation was at the end of 2006; *i.e.* the maximum observation time was 13 yr.

Measurement of human BMD

DXA scans of the total body were performed to determine human BMD (grams per square centimeter; DPX-L machine; GE Medical Systems Lunar Corp., Madison, WI). In our population, total-body BMD was strongly associated with age ($r^2 = 0.08$; $P < 0.0001$) and body weight ($r^2 = 0.17$; $P < 0.0001$). We calculated the Z scores of total-body BMD (total Z) by using a linear model optimized by the Akaike information criterion (25) to adjust for the effects of confounding factors, including age and body weight, on BMD variation. The following formula was used to determine total Z: total Z = total-body BMD (grams per square centimeter) − 0.872 + [0.00396 × age (years)] − [0.00708 × weight (kilograms)].

Large-scale association analysis

A two-stage replication strategy was used for this study. In the first stage (discovery stage), we did a large-scale association to select SNP based on *P* values using 251 Japanese postmenopausal women (Supplemental Fig. 1) (24). We used the Affymetrix 50K Hind SNP GeneChip (57,244 SNP) to examine genetic association of SNP with total-body BMD in the according to the manufacturer's protocol, as previously described (24).

In brief, we selected autosomal SNP with genotypic call rates of 95% or higher, a minor allele frequency (MAF) of at least 10%, and Hardy-Weinberg equilibrium of at least 0.0001 and chose 15,662 SNP among 57,244 SNP for analysis that met these criteria. We analyzed the association between total-body BMD and SNP under the assumption of the dominant and recessive models for a minor allele in each SNP, by using the quantitative trait loci estimation model as previously described (24). The significance levels for the statistical tests under the assumptions of the dominant model and the recessive model were determined based on the log *P* value of QQ plots (24). In the present study, as the second-stage replication analysis, the associations between BMD and 13 SNP selected in the first stage were analyzed using 499 postmenopausal women having no overlap with the subjects in the first stage. The 13 SNP, including rs10514346, which is located in the 3′-flanking region of the *GPR98* gene, were genotyped using the TaqMan PCR method using Assays-on-Demand SNP Genotyping Products and protocols (Applied Biosystems, Foster City, CA). Among 13 SNP, we identified five SNP that were associated with a low BMD phenotype, with significant combined *P* values under the significance levels for the first and second stages. The significance level was set at 0.05/15,662 (3.19×10^{-6}) using Bonferroni's correction, which was a multiple-comparison method.

Linkage disequilibrium (LD) analysis

We genotyped rs10514346 and 14 additional SNP present close to it in and near the *GPR98* gene in Japanese postmenopausal women. To evaluate the state of LD among several SNP (r^2), we used Haploview software (MIT Broad Institute, Cambridge, MA) to analyze and visualize LD (26).

Animals

Generation and characterization of the *Gpr98*/*Vlgr1*-KO mice were previously described (27). Briefly, to generate *Gpr98*-KO mice, we disrupted the region including exons 2–4. The embryonic stem cells obtained were injected into embryonic d-3 blastocysts taken from C57BL/6J mice and transferred into the uteri of pseudopregnant ICR females. Chimeric mice were mated with C57BL/6J mice to generate heterozygous mutants. C57BL/6J mice and ICR mice were obtained from SLC (Hamamatsu, Japan). *Gpr98*-KO mice were maintained in a C57BL/6J background. The *Gpr98* heterozygous mice were crossed to obtain *Gpr98*-KO and wild-type (WT) mice. Twelve-week-old male *Gpr98*-KO and WT mice were used in the experiments. Genotyping was performed by PCR analysis based on previously described methods (27). All the mice were housed under specific pathogen-free conditions (22 C, 12 h light, 12 h dark, and 50% humidity) with free access to food pellets and tap water. The femurs were harvested, and soft tissues were removed for further measurement of BMD and microcomputed tomographic and bone biomechanical analysis. All experiments were conducted in accordance with the guidelines for animal experiments of the University of Tokyo.

Measurement of mouse BMD

The BMD of the right femurs of mice was measured by DXA using the Lunar PIXImus2 densitometer (GE Medical Systems). The whole right leg was fixed with 4% paraformaldehyde in 0.1 M phosphate buffer and placed in a specimen tray. After calibration, duplicate cycles of four scans were obtained. We measured femoral BMD using DXA in the lateral projection of the femur. Projectional DXA measurement to calculate areal BMD included cortical and trabecular bone regions.

Quantitative microcomputed tomography (micro-CT)

Quantitative micro-CT scanning of the cortical and trabecular bone of the femurs of *Gpr98*-KO and WT mice was performed. It was analyzed by the micro-CT system (μCT-40; Scanco Medical, Bassersdorf, Switzerland), as previously reported (28). A three-dimensional analysis was performed to calculate morphometrical indices including bone volume fraction [bone volume (BV)/tissue volume (TV)] at the femoral metaphysis and cortical thickness at the femoral midshaft.

Biomechanical analysis of femoral bones

The mechanical properties of the diaphyses of femurs were evaluated using a three-point bending test. The load was applied midway between two supports placed 8 mm apart. The femur was positioned so that the loading point was at the center of the femoral diaphysis and bending occurred along the mediolateral axis. The bending test was performed in a saline bath at 37 C. Load-displacement curves were recorded at a crosshead speed of 5 mm/min using a material testing machine MZ500S (Maruto Co., Ltd., Tokyo, Japan). The maximum load and stiffness were analyzed using CTRwin software (System Supply Co., Ltd., Kanagawa, Japan).

Quantitative RT-PCR (qRT-PCR) in mouse primary osteoblasts

Primary cultures of calvarial osteoblasts were prepared using the sequential collagenase/Dispase digestion method (29). In brief, calvaria were removed from newborn pups derived from WT and *Gpr98*-KO mice; the calvaria were denuded of soft tissue and digested with 1 mg/ml collagenase and 2 mg/ml Dispase in PBS for 15 min at 37 C with gentle agitation. The procedure was repeated twice, and cells from the second digestion were collected and grown to confluence in α-MEM supplemented with antibiotics and 10% fetal calf serum. After 5 d in culture, we collected the mRNA. The mRNA expression levels of murine *Gpr98*, the receptor activator of nuclear-factor-κB ligand [RANKL (*Rankl*)], osteopontin (*Opn*), bone sialoprotein (*Bsp*), IL-6 (*Il6*), osteoprotegerin (*Opg*), alkaline phosphatase (*Alp*), colony-stimulating factor 1 (*Csf1*), tumor necrosis factor-α (*Tnf*α), IGF-I (*Igf-I*), β-catenin, and c-Fos in mouse primary osteoblasts were evaluated by qRT-PCR using a Prism 7000 System (Applied Biosystems) and SYBR Green I fluorescence as previously described (24). cDNA was synthesized from 1 μg total RNA using the First Strand cDNA Synthesis Kit (GE Healthcare Life Sciences). The relative levels of mouse *Gpr98*, *Rankl*, *Opn*, *Bsp*, *Il6*, *Opg*, *Alp*, *Csf1*, *Tnf*α, *Igf-I*, *β-catenin*, and *c-Fos* mRNA normalized to that of a reference gene (hypoxanthine-guanine phosphoribosyl transferase, *Hprt1*) were determined using the comparative cycles-at-threshold-fluorescence (Ct) method. Sequences of the PCR primers are as follows: mouse *Gpr98* (forward 5′-TACTGCCATTGTGTCGCTGAG-3′, reverse 5′-TGCTATGTACACTGTCCTG-3′), mouse *Rankl* (forward 5′-CCAGCATCAAAATCCCAAGTTC-3′, reverse 5′-TGCCCGACCAGTTTTTCG-3′), mouse *Opn* (forward 5′-CCCTCGATGTCATCCCTGTT-3′, reverse 5′-CTGCCCTTTCCGTTGTTGTC-3′), mouse *Bsp* (forward 5′-CCAGGAGAGTGCCGATCAGT-3′, reverse 5′-GAT

GTTCCAGGCTGGCTTTG-3′), mouse *Il6* (forward 5′-ACCACGGCCTTCCCTACTTC-3′, reverse 5′-CTGTTGGGAGTGGTATCCTCTGT-3′), mouse *Opg* (forward 5′-GCCTGGGACCAAAGTGAATG-3′, reverse 5′-CTTGTGAGCTGTGTCTCCGTTT-3′), mouse *Alp* (forward 5′-GCTGATCATTCCCACGTTTT-3′, reverse 5′-CTGGGCCTGGTAGTTGTTGT-3′), mouse *Csf1* (forward 5′-AGTATTGCCAAGGAGGTGTCAG-3′, reverse 5′-ATCTGGCATGAAGTCTCCATTT-3′), mouse *Tnfα* (*forward* 5′-GACGTGGAAGTGGCAGAAGAG-3′, reverse 5′-TGCCACAAGCAGGAATGAGA-3′), mouse *Igf-I* (forward 5′-CTGGACCAGAGACCCTTTGC-3′, reverse 5′-GGACGGGGACTTCTGAGTCTT-3′), mouse *β-catenin* (forward 5′-TGCTGAAGGTGCTGTCTGTC-3′, reverse 5′-GCTGCACTAGAGTCCCAAGG-3′), and mouse *c-fos* (forward 5′-AATCCGAAGGGAACGGAATAA-3′, reverse 5′-TCCGCTTGGAGTGTATCTGTCA-3′).

Bone marrow cell culture

Bone marrow cells from long bones of 12-wk-old WT mice were cultured in α-MEM containing 10% fetal calf serum and 5 ng/ml macrophage colony-stimulating factor (M-CSF) (PeproTech, Rocky Hill, NJ) for 16 h. Nonadherent cells were harvested and cultured for three more days in the presence of 30 ng/ml M-CSF. Floating cells were removed, and adherent cells were used as osteoclast precursors. The cells were further cocultured with primary osteoblasts derived from WT or *Gpr98*-KO mice in medium supplemented with 30 ng/ml M-CSF and 100 ng/ml RANKL (PeproTech) for 7 d. Cells were fixed with 4% formaldehyde in PBS for 10 min. The fixed cells were washed three times with PBS and stained for tartrate-resistant acid phosphatase (TRACP) according to the manufacturer's instructions (Sigma Chemical Co., St. Louis, MO). The numbers of $TRACP^+$ multinucleated cells (three or more nuclei in each cell) were counted under the microscope. For pit formation assay, bone marrow cells were inoculated on calcium phosphate-coated 24-well plates at a density of 1×10^5 cells per well and cocultured with primary osteoblasts derived from WT or *Gpr98*-KO mice in 1 ml growth medium containing 100 ng/ml RANKL. After 7 d, the plates were washed with PBS and treated with 5% sodium hypochlorite for 5 min. After washing the plates with tap water and then drying them, 20 different regions in each well were photographed by microscopy, and the pit areas were measured with Image J software (National Institutes of Health, Bethesda, MD). The results were presented as mean values ± SE. Statistical analysis was performed based on Student's *t* test.

Statistical analysis for mouse data

Data from mouse samples are expressed as the mean ± SE. Differences between the mean values were analyzed using the unpaired Student's *t* test.

Results

Large-scale association of SNP with total-body BMD and fracture

A two-stage replication study was conducted as shown in Supplemental Fig. 1. First, we used the Affymetrix 50K Hind SNP GeneChip (57,244 SNP) to examine the genetic association of SNP with total body BMD in 251 subjects as previously described (24). For the analysis, we chose 15,662 SNP with genotypic call rates of at least 95%, a MAF of at least 10%, and Hardy-Weinberg equilibrium of at least 0.0001 among 57,244 SNP (Supplemental Fig. 1). In the first stage, we selected 13 SNP from the Affymetrix 50K SNP array, five for a dominant model using a significance level of 6.99×10^{-6} and eight for a recessive model using a significance level of 1.60×10^{-5} (24). In the present study, we successfully genotyped the selected 13 SNP as a second-stage analysis in an additional 499 postmenopausal women who were not included in the first-stage analysis. Among these SNP, we identified five SNP (rs165222, rs229042, rs10514346, rs1370005, and rs10494622) that were associated with a low BMD phenotype, with significant combined *P* values (1.05×10^{-8} to 3.66×10^{-7}) for the first and second stages (Table 1). The significance level was set at 3.19×10^{-6}, which was calculated as 0.05/15,662, using Bonferroni's correction (Supplemental Fig. 1). Recently, a large-scale association with bone mass based on data from the Framingham Heart

TABLE 1. Second-stage screening of SNP for association with total-body BMD

SNP information, dbSNPID	Gene region	Chromosome	Alleles		Association test		*In silico* database	
			Allele	MAF	*P* value[a]	Threshold	Femoral neck BMD[b]	Lumbar spine BMD[b]
rs165222		2q24.3	A→G	0.480	1.05×10^{8}	0.066	0.15	0.12
rs229042	*ADAMTS1*	21q21.3	T→C	0.452	2.88×10^{8}	0.067	0.53	0.23
rs10514346	*GPR98*	5q14.3	A→G	0.277	1.88×10^{7}	0.079	0.020	0.040
rs1370005	*WDSOF1*	8q22.3	T→G	0.357	2.36×10^{7}	0.185	0.98	0.58
rs10494622		1q31.1	C→T	0.348	3.66×10^{7}	0.113	0.43	0.89

Thirteen SNP selected by QQ plots of the first-stage screening results were secondarily screened in 499 Japanese postmenopausal women. Five SNP, indicated by dbSHPID (database of short nucleotide sequence variations, ID; http://www.ncbi.nlm.nih.gov/SHP) with significant combined *P* values ($<3.19 \times 10^{-6}$; set using Bonferroni's correction) for the first- and second-stage analyses were identified as associated with low BMD.

[a] Combined first-stage and second-stage *P* values were described.

[b] Database from Framingham Heart Study (http://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/analysis.cgi?study_id=phs000007.v1.p1&phv=&phd=&pha=1754&phsf=&phvf=&phdf=&phaf=3).

Study was reported (19). The Affymetrix 100K array set was used in that report. Therefore, we reviewed the Framingham Heart Study database for the five selected SNP. Among the five SNP, only SNP rs10514346 was associated with both femoral neck and lumbar spine BMD in postmenopausal women (Table 1; $P = 0.02$ and 0.04, respectively). Interestingly, SNP rs10514345, which is located about 60 kb from rs10514346, had the lower *P* value (femoral neck BMD, $P = 0.0009$; L2–L4 BMD, $P = 0.0000215$) in the study. The rs10514345 SNP is located in intron 76, and rs10514346 is located in the 3′-flanking region of the *GPR98* gene. We therefore considered this gene to be a good candidate for further investigation. Association of *GPR98* polymorphisms with BMD in the Framingham Heart Study revealed that SNP rs10514345 and rs10514346 were associated with BMD. Therefore, we also genotyped an additional 14 SNP close to rs10514346 that were in or near the *GPR98* gene (Supplemental Fig. 2). LD analysis revealed that these SNP were not in LD in the Japanese population.

Because the minor G allele of the rs10514346 SNP was more frequently observed in the lower BMD group, it is possible that the polymorphism near the *GPR98* gene is associated with incident fractures. We investigated the association using the time-to-event method (Kaplan-Meier estimates and Cox proportional hazards model). Figure 1 shows Kaplan-Meier estimates for the incident fracture rate divided by the *GPR98* genotype over the observation time. The GG and AG genotype groups combined showed an apparently higher rate and earlier onset of incident fractures than the AA genotype group (log-rank test, $P = 0.043$; Fig. 1). These data suggest that the G allele of the rs10514346 SNP gene may be a risk factor for incident fracture because it results in lower BMD. To confirm this possibility, we also analyzed the effect of rs10514346 SNP on future fractures using the Cox proportional hazards model. The hazard ratio for the combined GG + AG genotype group was 1.34 ($P = 0.044$). The GG genotype was not significantly different from the AG genotype in the fracture analysis, although the mean fracture rates were lower with the GG genotype than with the AG genotype. We also analyzed the background and biochemical data by genotype (Supplemental Table 2). Body weight, body height, and body mass index were not statistically different among the genotypes. Total-body BMD was significantly lower in the GG-genotype group than in the AA-genotype group. The levels of serum osteocalcin, a bone formation marker, and urine deoxypyridinoline, a bone resorption marker, were significantly higher in the GG-genotype group than in the AA-genotype group, suggesting that high bone turnover conditions were more common in the GG group (Supplemental Table 1). These data indicated that the rs10514346 SNP was associated with low BMD as well as high bone turnover and risk of incident fractures.

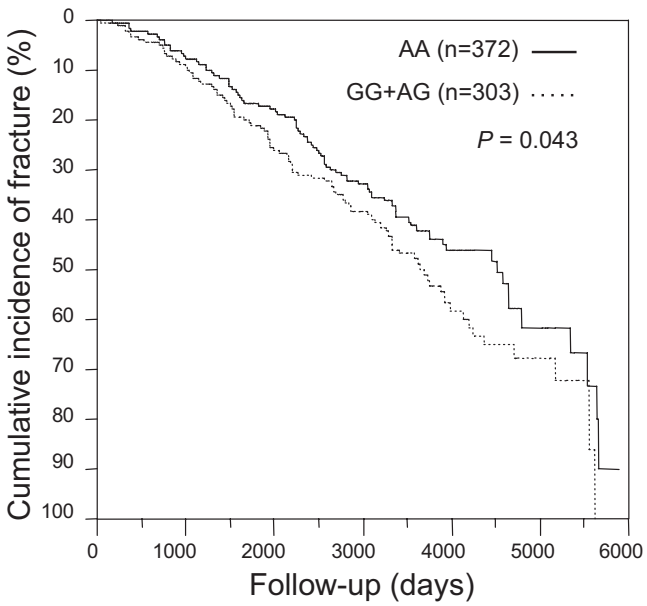

FIG. 1. Association between rs10514346 genotypes and cumulative incidence of fracture. The *GPR98* polymorphism was divided into two categories: the A allele and the G allele. The presence of the G allele enhanced the risk for future fracture (log-rank test, $P < 0.05$).

Decreased BMD in *Gpr98*-KO mice

To examine the direct association between the *GPR98* gene and bone tissue, we used *Gpr98*-KO mice. As previously described, *Gpr98*-KO mice were viable and fertile, breeding resulted in a normal Mendelian distribution, and body weight was normal (27). We first evaluated the gross appearance of the entire hind limb of 3-month-old *Gpr98*-KO mice by radiography. The shapes of the leg bones of *Gpr98*-KO were grossly normal, although the radiographs suggested a slight decrease in bone density in the femurs of *Gpr98*-KO mice relative to that of WT mice (Fig. 2A). The BMD of the femur was quantified using DXA. Notably, the BMD was significantly decreased in *Gpr98*-KO mice compared with that of WT mice (WT, 0.0645 ± 0.0008 g/cm^2; *Gpr98*-KO, 0.0593 ± 0.0012 g/cm^2, $P < 0.005$; Fig. 2B). We also analyzed the body weight and length, and the length of the femoral bone, in these mice (Supplemental Table 3). These data were not statistically different between *Gpr98*-KO mice and WT mice. Body weight data were consistent with our previous report (27).

Microstructural differences in trabecular bones of *Gpr98*-KO mice

Next, the three-dimensional bone microstructure was evaluated using micro-CT analysis. These studies revealed

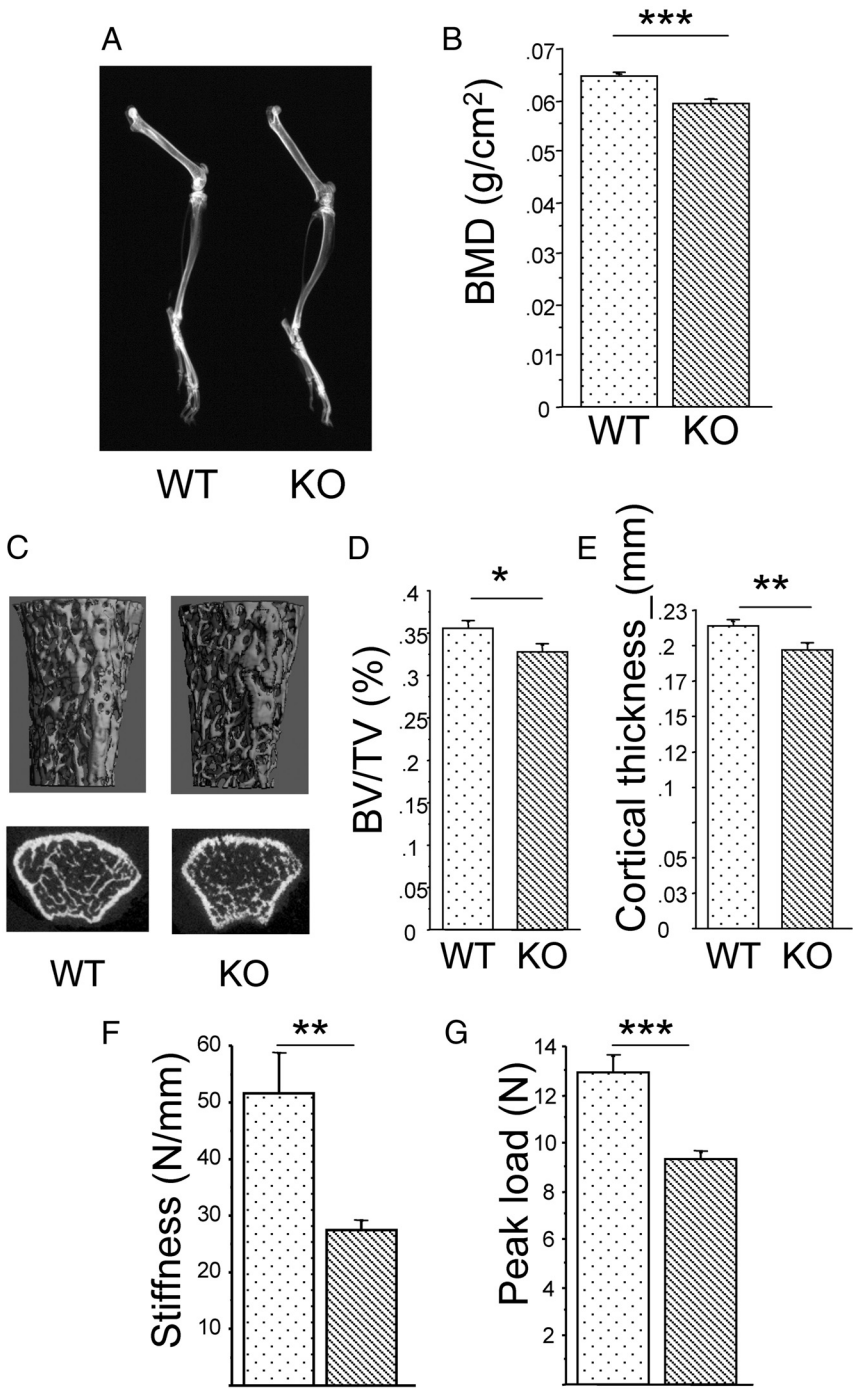

FIG. 2. Low BMD and increased mechanical fragility of bone in *Gpr98*-KO mice. A, Representative radiographs of right legs of WT and *Gpr98*-KO male mice are shown. No apparent gross abnormality was observed in *Gpr98*-KO. B, BMD of the right femurs of WT (n = 6) and *Gpr98*-KO (n = 5) male mice are shown. ***, $P < 0.005$. C, Representative three-dimensional micro-CT images of the femoral cortical and trabecular bones; *left panels*, WT male mice; *right panels*, *Gpr98*-KO male mice. D, Microstructural parameters (BV/TV) of femoral trabecular bones at the femoral metaphysis of WT (n = 6) and *Gpr98*-KO (n = 5) male mice derived from micro-CT analysis. E, Cortical thickness at the femoral midshaft of WT and *Gpr98*-KO male mice derived from micro-CT analysis. **, $P < 0.01$; *, $P < 0.05$. F, Stiffness of the femoral diaphyses of WT (n = 6) and *Gpr98*-KO (n = 5) mice calculated using a three-point bending test. G, Peak loads of the femoral diaphyses of WT (n = 6) and *Gpr98*-KO (n = 5) male mice were calculated using a three-point bending test. **, $P < 0.01$; ***, $P < 0.001$.

remarkable bone loss in the femoral cortical and trabecular bone of 3-month-old *Gpr98*-KO mice (Fig. 2C). Calculation of the standard three-dimensional parameters of the trabecular bone revealed a significant and substantial decrease in the BV/TV at the femoral metaphysis of *Gpr98*-KO mice compared with that of WT mice (Fig. 2D; WT, 14.85 ± 1.01%; *Gpr98*-KO, 5.92 ± 0.79%, $P < 0.05$). The cortical thickness at the femoral midshaft was also decreased significantly in *Gpr98*-KO mice, indicating the decreased thickness of cortical bone (Fig. 2E).

Differences in mechanical fragility of cortical bone of *Gpr98*-KO mice

Having demonstrated a reduction in cortical and trabecular bone structure, we next evaluated whether the morphological phenotypes of the femoral cortical and trabecular bones in *Gpr98*-KO mice were actually associated with mechanical fragility. To analyze mechanical fragility, we performed the three-point bending test. Both of the calculated mechanical parameters, stiffness and maximum load, were decreased in *Gpr98*-KO mice (Fig. 2, F and G), indicating mechanical weakness of the femurs of these mice.

Gpr98 deficiency enhances the expression of *Rankl* mRNA in primary osteoblasts and osteoclastogenesis

To search for the molecular mechanism of *Gpr98*-related bone loss, we used qRT-PCR to analyze differences in mRNA expression between primary WT and *Gpr98*-KO osteoblasts. We confirmed that *Gpr98* expression was detected in primary osteoblasts derived from WT but not *Gpr98*-KO calvaria (Fig. 3A). *Rankl* expression was increased and osteopontin expression decreased in primary *Gpr98*-KO osteoblasts compared with those of WT osteoblasts (Fig. 3, B and C). We also analyzed other osteoclastogenic factors

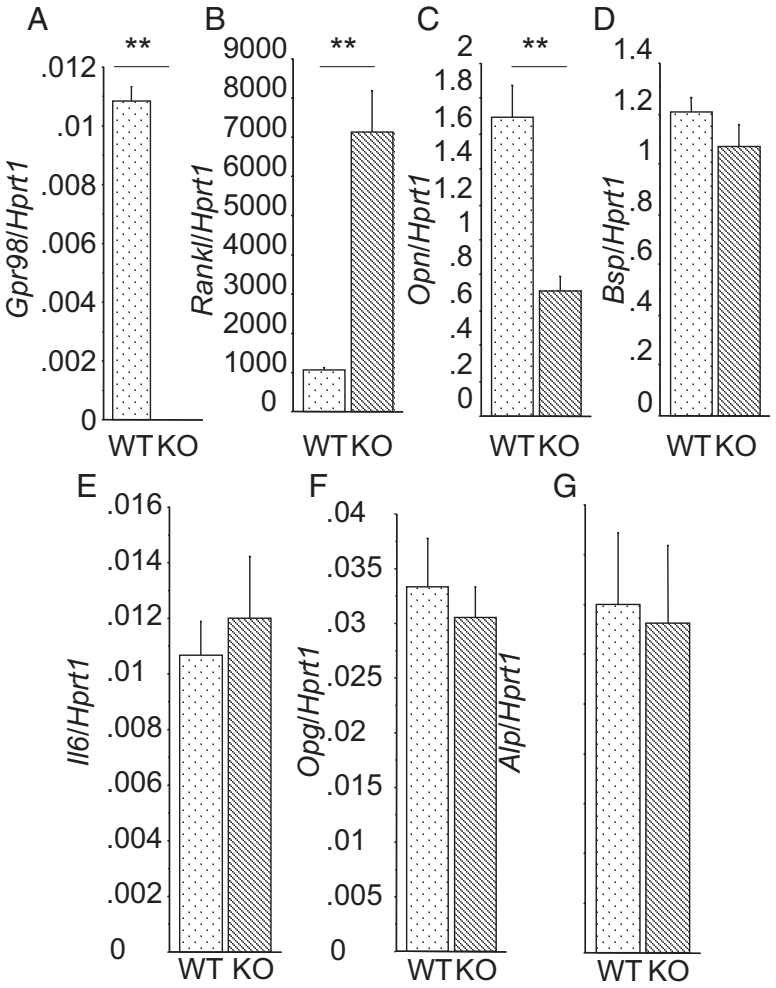

FIG. 3. Effect of *Gpr98* deficiency on differentiation markers of primary cultured osteoblasts. A, Murine *Gpr98* mRNA expression in both primary WT and *Gpr98*-KO osteoblasts was analyzed quantitatively by RT-PCR. B–D, mRNA expression levels of receptor activator of nuclear factor κB ligand (*Rankl*, B), osteopontin (*Opn*, C), bone sialoprotein (*Bsp*, D), *Il6* (E), osteoprotegerin (*Opg*, F), and alkaline phosphatase (*Alp*, G) in both primary WT and *Gpr98*-KO osteoblasts were analyzed quantitatively by RT-PCR. The relative mRNA levels normalized to the level of the reference gene hypoxanthine-guanine phosphoribosyl transferase (*Hprt1*) were determined using the comparative Ct (cycles at threshold fluorescence) method. **, $P < 0.01$.

and cytokines in primary *Gpr98*-KO osteoblasts and found that expression of these factors was not statistically different from expression in primary WT osteoblasts (Supplemental Fig. 3). Next, we evaluated the abilities of primary WT and *Gpr98*-KO osteoblasts to induce osteoclastogenesis. Bone marrow cells derived from WT mice were cocultured with primary WT or *Gpr98*-KO osteoblasts. The cells were cultured and treated with M-CSF (30 ng/ml) and RANKL (100 ng/ml). After 7 d, osteoclastic cells were identified by multinuclearity and TRACP staining (Fig. 4A *left and right panels*). The number of osteoclastic cells was found to be significantly higher in the *Gpr98*-KO cultures than in the WT controls (Fig. 4B). We further examined *Gpr98* deficiency in osteoblasts affects the function of osteoclasts. For the analysis of osteoclasts, bone marrow cells cocultured with primary WT or *Gpr98*-KO osteoblasts were cultured on calcium phosphate-coated plates, and resorption pits were quantified. The areas of the pits formed by the cells cocultured with primary *Gpr98*-KO osteoblasts were significantly larger than those formed by the cells cocultured with primary WT osteoblasts (Fig. 4C). Taken together, these results indicate that *Gpr98*-deficient osteoblasts had an induced activity for osteoclastogenesis and that induced osteoclastic cells have *bona fide* bone resorptive activity.

Discussion

Rapid advancements have made it feasible to pursue powerful large-scale association studies (17, 18). Large-scale association studies are an unbiased approach that involves scanning the entire human genome to identify novel genes/genome regions with modest effects on complex human diseases/traits. A number of large-scale association studies have revealed novel findings for complex diseases such as obesity, type 2 diabetes, inflammatory bowel disease, and prostate cancer (17, 18); a GWA study of BMD, osteoporosis, and osteoporotic fracture has also been reported (19–23). Recently, we used the Affymetrix GeneChip Human Mapping 50K Hind SNP array to genotype 57,244 SNP in Japanese postmenopausal women to identify common genetic variants associated with BMD (24). In the present study, we added a second-stage analysis resulting in identification of rs10514346, which is located in the *GPR98* gene, as a novel candidate SNP associated with BMD in Japanese postmenopausal women. A 100K large-scale association study on bone-related quantitative traits in the Framingham Heart Study was previously reported (19). Analyzing this open dataset *in silico*, we found that rs10514346 was associated with both femoral neck and lumbar spine BMD in Caucasian women. The reproducibility in a different race of the association of a *GPR98* SNP with BMD further encourages our assumption that this gene contributes to the osteopenia. In addition, the present findings are consistent with a genome-wide scan for the quantitative trait locus in the regulation of femoral neck BMD that includes rs10514346 on 5q14.3 (30). Moreover, another group reported that the rs10514345 SNP in the *GPR98* gene was associated with total-body BMD (31). Although we could

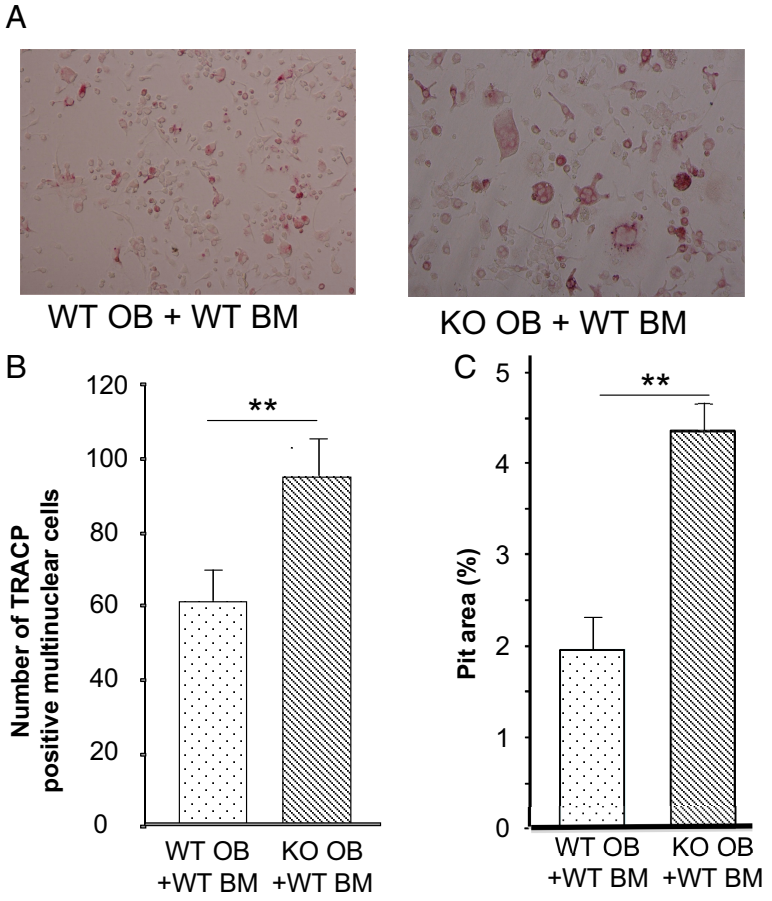

FIG. 4. *Gpr98* deficiency in primary osteoblasts (OB) enhances osteoclastogenesis and osteoclastic resorption in a coculture with bone marrow (BM) cells. A, Osteoclastogenesis in bone marrow cells cocultured with primary osteoblasts derived from WT or *Gpr98*-KO mice. Representative images of TRACP staining to evaluate the osteoclastogenesis of bone marrow cells are shown. B, The numbers of TRACP-positive multinucleated cells (three or more nuclei for each cell) were counted. The bone marrow cells derived from WT mice were cocultured with primary WT or *Gpr98*-KO osteoblasts (WT OB + WT BM or KO OB + WT BM) in a 24-well plate in the presence of M-CSF and RANKL. Cells were stained with TRACP after 7 d in culture. **, $P < 0.01$. C, Bone marrow cells cocultured with primary WT or *Gpr98*-KO osteoblasts were cultured on calcium phosphate-coated plates, and resorption pit areas were measured. **, $P < 0.01$.

not identify a haplotype block in and near the *GPR98* gene, the SNP or linked SNP may influence protein expression or the function of *GPR98* in bone homeostasis.

We provide important new information defining not only an association in humans between a *GPR98* SNP and osteopenia but also an important role for *Gpr98* in mouse bone metabolism. Our preliminary data also show that homozygous *Gpr98*-KO mice (n = 5) had significantly lower BMD than heterozygotes (WT *vs.* heterozygotes *vs.* *Gpr98*-KO was 0.064 + 0.002 *vs.* 0.062 + 0.002 *vs.* 0.059 g/cm^2 + 0.002; $P = 0.0047$ by the Kruskal-Wallis test). These data suggest that low BMD resulted in a gene dose-dependent manner. The current results also demonstrate that systemic *Gpr98* deficiency results in low BMD with mechanical fragility, thus confirming the importance of this G protein-coupled receptor as a regulator of bone homeostasis. Concordant with reduced BMD, decreased trabeculae and cortical thickness were observed in *Gpr98*-KO mice. These data suggest the presence of fragile bone, which leads to mechanical fragility. Our observations of the femoral bones of *Gpr98*-KO mice by the three-point bending test indicated mechanical weakness. These data were in agreement with our clinical fracture data. In the present study, we have shown that the rs10514346 SNP was associated with both low BMD and a high risk of fracture. These human and mouse data suggest that the *GPR98*/*Gpr 98* gene regulates BMD and bone fragility.

Despite these discoveries, the function of *GPR98* in the bone remains unclear. The qRT-PCR analysis showed that *Gpr98* was expressed in the primary osteoblasts. Then, we noticed that *Rankl* expression in primary osteoblasts derived from *Gpr98*-KO mice was increased compared with that in cells derived from WT mice. RANKL is a member of the TNF receptor superfamily, which is essential for osteoclastogenesis (32). It binds to its receptor, an activator of nuclear factor-κB, on the surface of osteoclast precursors and enhances their differentiation, survival, and fusion and also activates mature osteoclasts and inhibits their apoptosis. The activation of RANKL induces high-turnover osteoporosis. The qRT-PCR data suggest that the osteoclastic cells may be functionally activated by the high expression of *Rankl* by primary *Gpr98*-KO osteoblasts, which results in osteopenia observed in *Gpr98*-KO mice. Actually, our study showed increased multinuclear osteoclastogenesis in bone marrow cells cocultured with primary *Gpr98*-KO osteoblasts relative to that in bone marrow cells cocultured with primary WT osteoblasts. Using pit formation assay, we also revealed that osteoclastic cells induced by primary *Gpr98*-KO osteoblasts are functionally activated. The osteoclasts activated by the high levels of Rankl produced by osteoblasts in *Gpr98*-KO mice may lead to a high-turnover state of the bone of those mice with low BMD. However, our results indicate that RANKL must be added to the culture for osteoclastogenesis to occur. We cocultured without the addition of RANKL but observed osteoclastogenesis only in the presence of RANKL. Thus, these results suggest that either the sensitivity to RANKL or to another factor is responsible for the increased osteoclastogenesis. Our clinical data in humans showed that the rs10514346 SNP was associated with high turnover as evidenced by increased bone resorption and formation markers.

The *GPR98* gene is also called the very large G protein-coupled receptor-1 gene (*Vlgr1*) (33). The longest gene product, G protein-coupled receptor 98b (GPR98b), is 6307 amino acids (6298 amino acids in mice) in length, with a very large ectodomain containing 35 calcium exchanger β-repeats and a pentraxin homology domain (34). Mutations in the *GPR98* gene implicate G protein signaling in the pathogenesis of Usher syndrome type IIc, which is an autosomal recessive genetic disorder; the phenotype is a moderate-to-severe sensorineural hearing loss and progressive retinitis pigmentosa (35). *In situ* hybridization studies on mouse embryo sections have shown that high-level expression of *Gpr98* is restricted to the central nervous system and eye (36). *Gpr98*(*Vlgr1*)-mutant or -KO mice show susceptibility to audiogenic seizures (27, 36, 37). These data suggest that *GPR98*/*Gpr98* has a fundamental role in the development of the central nervous system. Recently, the discovery that neuronal control of bone remodeling is mediated by leptin and neuromedin U has shed light on a new regulatory mechanism for bone remodeling in which bone mass may be regulated by a variety of neuropeptides and their receptors (38–40). The GPR98 protein belongs to a 33-member subgroup of the large N-terminal family B seven-transmembrane receptors. Of the 33 large N-terminal family B seven-transmembrane members, 32 are orphan receptors, and only a few have demonstrated specific G protein signaling. Many of these putative G protein-coupled receptors are expressed in the brain, and several have apparent functions in development. These data suggest that *Gpr98* could play an important role in bone homeostasis through changes in its expression or function in the central nervous system, besides the direct effect on osteoblasts that we have revealed in the present study. In particular, the identification of the ligands of *GPR98*/*Gpr98* could be an important step in elucidating the mechanism by which osteopenia is caused by a mutated *GPR98*/*Gpr98* gene in the central nervous system or bone tissue.

Taken together, the present results suggest that the *GPR98*/*Gpr98* signaling pathway would be critical in the regulation of BMD and bone fragility. In conclusion, we have shown an association between the SNP in the *GPR98* gene and BMD in Japanese postmenopausal women. Therefore, *GPR98* genotyping may be beneficial in the prevention and management of osteoporosis. The present findings regarding the correlation of *GPR98* polymorphism with BMD provide a promising new direction for the clinical management of osteoporosis that could lead to the development of new diagnostic markers as well as therapeutic options based on this molecular target.

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