

A Single Nucleotide Polymorphism in Osteonectin 3' Untranslated Region Regulates Bone Volume and Is Targeted by miR-433

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ABSTRACT

Osteonectin/SPARC is one of the most abundant noncollagenous extracellular matrix proteins in bone, regulating collagen fiber assembly and promoting osteoblast differentiation. Osteonectin-null and haploinsufficient mice have low-turnover osteopenia, indicating that osteonectin contributes to normal bone formation. In male idiopathic osteoporosis patients, osteonectin 3' untranslated region (UTR) single-nucleotide polymorphism (SNP) haplotypes that differed only at SNP1599 (rs1054204) were previously associated with bone mass. Haplotype A (containing SNP1599G) was more frequent in severely affected patients, whereas haplotype B (containing SNP1599C) was more frequent in less affected patients and healthy controls. We hypothesized that SNP1599 contributes to variability in bone mass by modulating osteonectin levels. Osteonectin 3' UTR reporter constructs demonstrated that haplotype A has a repressive effect on gene expression compared with B. We found that SNP1599G contributed to a miR-433 binding site, and miR-433 inhibitor relieved repression of the haplotype A, but not B, 3' UTR reporter construct. We tested our hypothesis *in vivo*, using a knock-in approach to replace the mouse osteonectin 3' UTR with human haplotype A or B 3' UTR. Compared with haplotype A mice, bone osteonectin levels were higher in haplotype B mice. B mice displayed higher bone formation rate and gained more trabecular bone with age. When parathyroid hormone was administered intermittently, haplotype B mice gained more cortical bone area than A mice. Cultured marrow stromal cells from B mice deposited more mineralized matrix and had higher osteocalcin mRNA compared with A mice, demonstrating a cell-autonomous effect on differentiation. Altogether, SNP1599 differentially regulates osteonectin expression and contributes to variability in bone mass, by a mechanism that may involve differential targeting by miR-433. This work validates the findings of the previous candidate gene study, and it assigns a physiological function to a common osteonectin allele, providing support for its role in the complex trait of skeletal phenotype. © 2014 American Society for Bone and Mineral Research.

KEY WORDS: OSTEOPOROSIS; MOLECULAR PATHWAYS-REMODELING; GENETIC ANIMAL MODELS; NONCOLLAGENOUS PROTEINS; HUMAN ASSOCIATION STUDIES

Introduction

Osteoporosis is a prevalent disorder characterized by low bone mineral density (BMD), deterioration of bone micro-architecture, and increased incidence of fracture,⁽¹⁾ and genetic factors account for 60% to 80% of total variability in BMD.⁽²⁾ Understanding the genetic determinants underlying bone mass may improve prognosis and provide novel targets for therapeutic intervention. In this regard, genome-wide association studies (GWAS) and candidate gene studies have associated allelic variants of genes such as estrogen receptor (ER)- α , transforming growth factor (TGF)- β , osteoprotegerin, and type I collagen A1

with bone mass.⁽³⁻⁶⁾ However, only a few studies have demonstrated a mechanism whereby a polymorphism could contribute to bone mass phenotype.

Indeed, a previous GWAS study in premenopausal women linked variations in BMD to genomic regions including 5q33-35.⁽⁷⁾ Although candidate genes in the 5q33-35 interval were not identified, this region contains the gene for osteonectin/*Sparc* (secreted protein acidic and rich in cysteine), one of the most abundant noncollagenous extracellular matrix proteins in bone. In osteoblasts, osteonectin promotes commitment, differentiation, and survival. Osteonectin also suppresses adipogenic differentiation of mesenchymal precursor cells. *In vivo*, osteonectin-null and

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haploinsufficient mice develop low-turnover osteopenia, characterized by reduced osteoblast and osteoclast number and surface, and low bone formation rate.^(8–12) Moreover, osteonectin-null mice accumulate less bone in response to intermittent administration of parathyroid hormone (PTH), the best bone-anabolic treatment clinically available at this time.⁽¹²⁾

Based on these findings, we had previously performed a candidate gene study to determine whether three single-nucleotide polymorphisms (SNPs) in the 3' untranslated region (UTR) of osteonectin (Fig. 1A) were associated with bone mass in a cohort of men with low-turnover idiopathic osteoporosis, a disorder primarily attributed to genetic determinants.⁽¹³⁾ Briefly, this cohort consisted of middle-aged white men with a BMD T-score of less than -2.0 at the lumbar spine, who lacked known secondary causes for osteoporosis. The control subjects were age and body mass index matched to the patients, and had BMD T-scores of more than 1.0 at the lumbar spine. As a group, the idiopathic osteoporosis patients had mean serum PTH and IGF1 levels in the low normal range. Their indices of bone formation were significantly reduced, although eroded surface was not different between patients and their matched controls.^(14,15) In the osteoporotic cohort, prevalence of fragility fracture was 23%.⁽¹³⁾

In this cohort, one of the two most common osteonectin 3' UTR haplotypes that we identified, haplotype A, was found at a higher frequency in the most severely affected osteoporotic patients, whereas the second most common haplotype, B, was found at a higher frequency in the healthy controls. In addition, haplotype B was associated with higher BMD in the patient population.⁽¹³⁾ Osteonectin 3' UTR haplotype A contained SNPs at cDNA bases 1046C_1599G_1970T, whereas haplotype B consisted of SNPs 1046C_1599C_1970T (Fig. 1A). Because these BMD-associated haplotypes differed only at cDNA base 1599, we hypothesized that SNP 1599G/C (rs1054204) may impact osteonectin expression and affect bone mass.

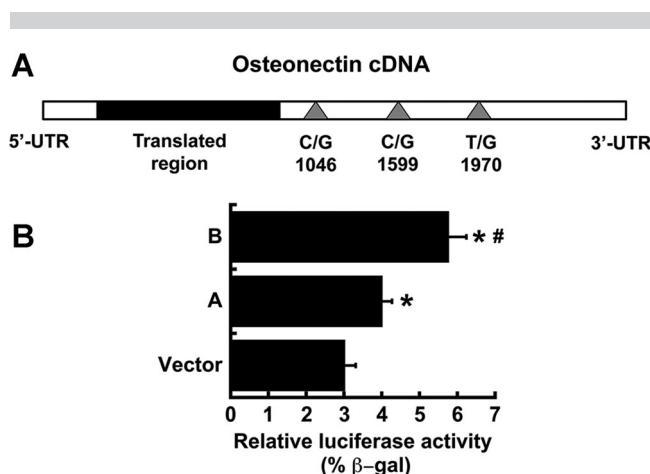


Fig. 1. Osteonectin 3' UTR SNP 1599 regulates 3' UTR function. (A) Schematic representation of the human osteonectin cDNA and the characterized SNPs at bases 1046, 1599, and 1970 (gray triangles). (B) Human ON 3' UTR (cDNA bases 1018–2123) representing haplotypes A and B were cloned into pMIR-REPORT luciferase vector. Luciferase activity was quantified in hFOB1.19 cells and normalized to β-galactosidase activity (* $p < 0.05$ different from vector; # $p < 0.05$ different from haplotype A, $n = 6$).

The 3' UTR represents a powerful regulatory region, with the potential to modulate mRNA stability, translation, and localization.⁽¹⁶⁾ Polymorphisms in the 3' UTR have the potential to alter the secondary structure of the mRNA, as well as its interaction with trans-acting factors, such as microRNAs (miRNAs, miRs). miRNAs are small, endogenous noncoding RNAs that, for the most part, decrease the stability and/or translation of protein-encoding mRNAs.

Recent studies have associated mutations or SNPs in miRNA binding with skeletal disorders. For example, a SNP in the 3' UTR of *Fgf2* (fibroblast growth factor 2), that abrogates miR-146a and -146b binding sites, was linked to low BMD in osteoporotic patients.⁽¹⁷⁾ Another study attributed a mutation in the binding site for miR-433 in the 3' UTR of *Hdac6* (histone deacetylase 6) to the pathogenesis of X-linked chondrodysplasia.⁽¹⁸⁾

In this study, we show that human osteonectin SNP1599 differentially regulates gene expression and contributes to a miR-433 binding site. Specifically, 1599G, found in haplotype A, has a repressive effect on gene expression and osteoblastic differentiation compared with 1599C, which is found in haplotype B. Moreover, using novel knock-in mouse models, we demonstrate that compared with mice carrying human osteonectin haplotype A 3' UTR (SNP 1599G), mice with the haplotype B 3' UTR knock-in (SNP 1599C) have higher levels of osteonectin in bone, higher bone formation rate, increased trabecular bone volume with age, and a greater increase in cortical bone volume in response to the bone-anabolic PTH treatment. These data substantiate the relationship between osteonectin 3' UTR SNP 1599 and skeletal phenotype, validate our initial observations in the cohort of idiopathic osteoporosis patients, and suggest that SNP 1599 affects bone mass by modulating osteonectin expression.

Materials and Methods

Generation of osteonectin 3' UTR haplotype constructs

Human osteonectin 3' UTR (cDNA bases 1018–2123) fragments representing haplotypes A and B found in idiopathic osteoporosis patients were PCR amplified from genomic DNA and cloned downstream of the luciferase gene in pMIR-REPORT vector (Life Technologies, Carlsbad, CA, USA). Details of cloning are described in the Supplemental Information.

Cell culture, transfection, and luciferase activity

Human fetal osteoblastic 1.19 cell line (hFOB1.19) was purchased from American Type Culture Collection (Manassas, VA, USA; ATCC number CRL-11372) and were grown at 33.5°C and differentiated into osteoblast cultures at 39.5°C in the presence of differentiation cocktail: 100 μg/mL ascorbic acid, 10⁻⁸ M menadione (vitamin K), 5 mM β-glycerolphosphate (β-GP), and 10⁻⁷ M 1-25(OH)₂-vitamin D₃ (all from Sigma, St. Louis, MO, USA).^(19,20) Bone marrow stromal cells (BMSCs) harvested from long bones of mice were differentiated in presence of 5 mM β-glycerolphosphate and 50 μg/mL ascorbic acid.^(8,11)

Haplotype A and B 3' UTR luciferase constructs were transfected into hFOB1.19 cells using Fugene6 (Roche, Indianapolis, IN, USA). For cotransfection of miRNA inhibitors or mimics and luciferase constructs into hFOB1.19 cells, X-tremeGENE reagent (X-tremeGENE; Roche) was used. Details of cell culture and transfection are described in the Supplemental Information.

Generation of osteonectin knock-in mice

A knock-in strategy was used to replace the mouse osteonectin 3' UTR with human haplotype A or haplotype B 3' UTR. These mouse models were generated at the Gene Targeting and Transgenic Facility at University of Connecticut Health Center (UCHC). Osteonectin haplotype A (ON^{+A}) and haplotype B (ON^{+B}) mice were generated using 129 embryonic stem cells and were back-crossed into C57BL/6J. Mice heterozygous for A and B alleles (ON^{A/B}) were bred to create homozygous A and B mice (ON^{A/A} and ON^{B/B}).

Quantitative RT-PCR analysis

RNA was extracted using the miRNeasy Mini Kit (Qiagen, Valencia, CA, USA). miR-433 levels were determined using the TaqMan MicroRNA assay (Life Technologies) and normalized to RNU48 for human tissue or sno202 RNA for mouse tissue. Osteonectin, alkaline phosphatase, osteocalcin, and bone sialoprotein (Ibsp) mRNA levels were determined using MMLV-Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and iQ SYBR Green Supermix (BioRad, Hercules, CA, USA) and normalized to 18sRNA. Primer sequences and details of quantitative RT-PCR are described in the Supplemental Information.

Western blot analysis

Equal amounts of long-bone extract were subjected to Western blot analysis for osteonectin and β -actin. Rabbit anti-bovine osteonectin primary antibody (BON-1; gift of L Fisher, NIDCR, NIH) (1:4000), rabbit anti- β -actin primary antibody (Abcam, Cambridge, MA, USA; 1:1000), and goat anti-rabbit-horseradish peroxidase conjugated secondary antibody (1:20,000) were used. Experimental details are described in the Supplemental Information.

Osteoblast differentiation, mineralization, and proliferation analysis

BMSCs harvested from long bones were cultured in osteoblast differentiation medium. Cultures were stained with either 1% alizarin red S pH 6.45 (Sigma) or 0.05% crystal violet (Fisher Scientific, Pittsburgh, PA, USA). Alizarin red stain was quantified at absorbance 405 nm, whereas crystal violet stain was measured at 570 nm, as described in the Supplemental Information. Cell proliferation was assessed using MTS CellTiter 96 Aqueous One Solution cell proliferation assay kit (Promega, Madison, WI, USA), according to the manufacturer's instructions.

Morphological analysis

To study bone-anabolic response, 10-week-old male mice were injected subcutaneously with 40 μ g/kg/d rhPTH (1-34) (PTH) (Bachem, Torrance, CA, USA) or vehicle alone (2% heat-inactivated mouse serum in acidified saline), 5 days per week for 4 weeks.⁽¹²⁾ Details of μ CT imaging and histomorphometry of femurs are described in the Supplemental Information. All procedures were approved by the Institutional Animal Care and Use Committee at UCHC.

Statistics

All quantified data are expressed as a mean \pm SEM. Statistical significance was determined by one-way ANOVA with Bonferroni post hoc test or Student's *t* test. Allele frequencies were evaluated using the Freeman-Halton extension of Fisher's exact test.

Results

SNP 1599 modulates osteonectin 3' UTR function and represses haplotype A 3' UTR

To determine the role of SNP 1599 in regulating the osteonectin 3' UTR, we cloned the 1-kb osteonectin 3' UTR, representing human haplotype A or B, into a luciferase reporter construct. In these reporter constructs, the cloned sequence functioned as 3' UTR for the luciferase gene, the transcription of which was constitutively driven by a strong promoter. The constructs were transiently transfected into hFOB1.19 cells, a conditionally immortalized human osteoblastic cell line. We found that the haplotype A 3' UTR construct had lower luciferase activity compared with haplotype B, suggesting that haplotype A, which was found at a higher frequency in the most severely affected osteoporosis patients, had a more repressive effect on gene expression (Fig. 1B). These data indicate that SNP 1599 contributed to differences in osteonectin 3' UTR function.

SNP 1599 introduces a novel miR-433 binding site in haplotype A 3' UTR

To determine whether miRNAs may mediate the differential regulation of osteonectin by SNP 1599G/C, we used RNAhybrid (<http://bibiserv.techfak.uni-bielefeld.de/bibi/Tools.html>) and miRbase v15.0 to assemble a panel of candidate miRNAs with the potential to interact with the region containing SNP 1599.⁽²¹⁾ The list of candidate miRNAs was further refined, based on whether SNP 1599 would facilitate or disrupt the interaction of the miRNA with the osteonectin 3' UTR. We observed the potential for differential interaction of miR-433-3p, miR-493-5p, and miR-374a-3p in presence of 1599G (haplotype A) compared with 1599C (haplotype B) (Table 1). Moreover, these miRNAs are expressed in hFOB1.19 cells.

To test the hypothesis that the candidate miRNAs may differentially target haplotypes A and B in vitro, hFOB1.19 cells were transiently cotransfected with either haplotype A or B 3' UTR reporter constructs and inhibitor for miR-433-3p, miR-493-5p, or miR-374a-3p. In the presence of miR-433 inhibitor, luciferase activity of the haplotype A construct was significantly increased compared with the nontargeting control, whereas activity of the haplotype B construct was not affected. In contrast, inhibitor for miR-374a-3p or miR-493-5p did not significantly increase luciferase expression from either haplotype A or B constructs (Fig. 2A). miR-433 targeting of haplotype A 3' UTR was also evaluated in hFOB1.19 cells using miR-433 mimic and a scramble mimic control. In the presence of miR-433 mimic, haplotype A 3' UTR luciferase activity was inhibited compared with the control, whereas haplotype B 3' UTR activity was not significantly affected (Fig. 2B).

These results suggest that SNP 1599 introduces a novel miR-433 binding site in haplotype A 3' UTR, which may contribute to differential regulation of osteonectin. However, our data do not preclude the possibility that other miRNAs or trans-acting factors could also demonstrate differential regulation of the haplotype A and B 3' UTRs.

miR-433 expression decreases during osteoblastic differentiation

Because miR-433 could differentially regulate the human osteonectin 3' UTR, we determined whether the expression of this miRNA was altered during osteoblastic differentiation, using

Table 1. RNAhybrid Analysis of Putative miR-433, -493, and -374 Binding Sites in Osteonectin 3' UTR^a

miRNA	Haplotype A – SNP1599G					Haplotype B – SNP1599C				
433-3p	target 5'A	GAAAGAUUCU	G	A3'		target 5'A	GAAAGAUUCU	GUC	A 3'	
	ACUGA		GGGGCU	UGUUAUGA		ACUGA		GGGGCU	UUAUGA	
	UGGCU		CCUCGG	GUAGUACU		UGGCU		CCUCGG	AGUACU	
	miRNA 3'UC			A 5'		miRNA 3'UC		GU	A 5'	
493-5p	target 5'	A	AUU	GGG	U	A 3'				
	GAAAG	CU	GCUGUG	UAUGA						
	CUUUC	GA	UGGUAC	AUGUU						
	miRNA 3'	UUA	G		5'					
374a-3p	target 5'	C	G	C	A 3'					
	U	GGG	UGUGU	UUAUGA						
	A	UCC	ACA	AAUAAU						
	miRNA 3'	GUGAAU	G	A	5'					

^aSNP 1599 G/C is underlined in the seed binding region of haplotype A or B 3' UTR.

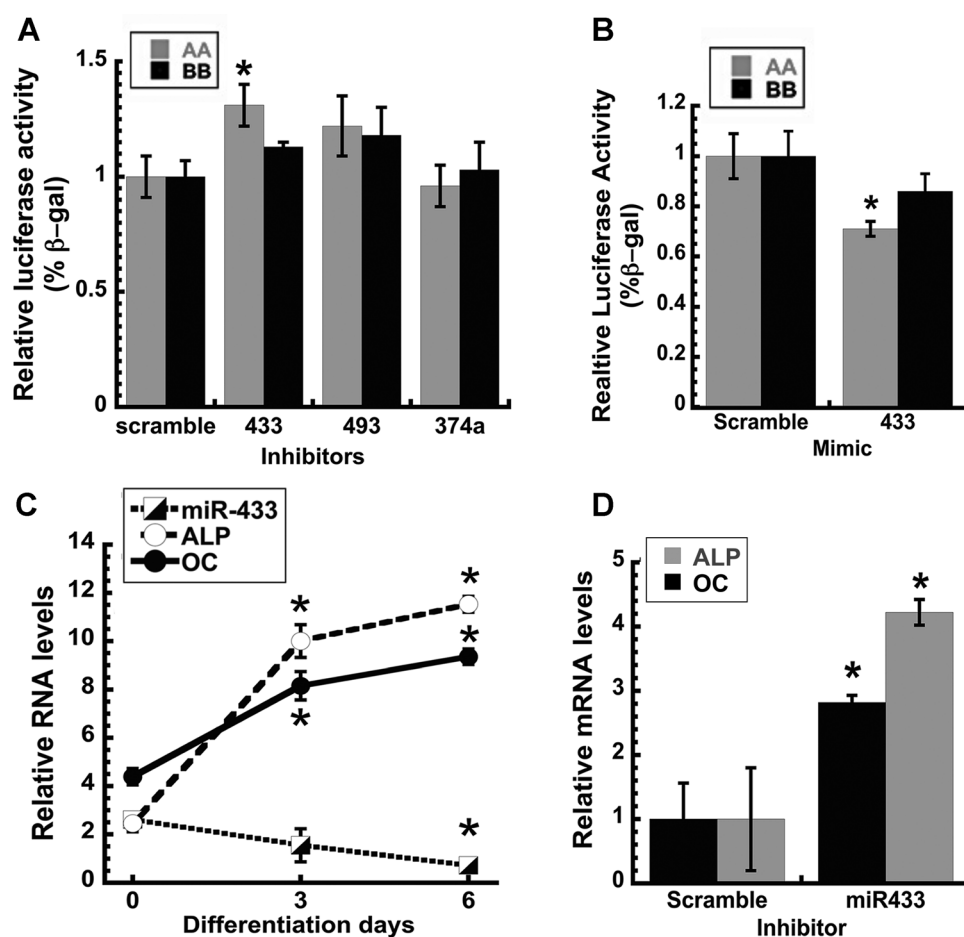


Fig. 2. miR-433 represses haplotype A 3' UTR and decreases during osteoblastic differentiation in vitro. Luciferase activity of haplotype A and B pMIR-report constructs in hFOB1.19 cells cotransfected with specific miRNA inhibitors, mimic or scramble control. Inhibitors for miR-433, -493, and -374a (A) and miR-433 mimic or scramble control mimic (B) were tested; luciferase activity was normalized to β-galactosidase activity (**p* < 0.05 different from scramble control, *n* = 6). (C) miR-433, alkaline phosphatase (ALP), and osteocalcin (OC) RNA in hFOB 1.19 cells at confluence (0) and during osteoblastic differentiation. miR-433 normalized to RNU48; ALP and OC mRNA normalized to 18s RNA (**p* < 0.05 different from confluence, *n* = 3). (D) ALP and OC RNA in hFOB1.19 cells transfected with miR-433 or scramble inhibitor and cultured in osteoblast differentiation medium for 3 days (**p* < 0.05 different from scramble inhibitor, *n* = 3).

hFOB1.19 cells as a model.^(19,20) Osteoblastic differentiation was induced using a vitamin D-containing cocktail, and after 3 or 6 days, mRNAs for the early osteoblastic marker, alkaline phosphatase (ALP), and the mature osteoblast marker, osteocalcin (OC), were dramatically increased. In contrast, expression of miR-433 decreased during differentiation, such that miR-433 levels were lowest when osteoblastic differentiation markers were highest (Fig. 2C). A similar phenomenon was reported by others when miR-433 levels were evaluated in a BMP2-treated murine preosteoblast cell line.⁽²²⁾

To determine the role of miR-433 in human osteoblastic differentiation, hFOB1.19 cells were transfected with either a miR-433 inhibitor or a scramble control inhibitor and subjected to osteoblastic differentiation for 3 days. Here, miR-433 inhibitor increased mRNA for the osteoblast markers ALP and OC compared with the scramble control, confirming that miR-433 is a negative regulator of osteoblast maturation in vitro (Fig. 2D).

SNP 1599 affects osteonectin expression in vivo

Many mature miRNAs display sequence conservation across species, and the sequence of mature miR-433 is identical between mouse and human (miRBase). Whereas selected regions of the mouse and human osteonectin 3' UTR are highly conserved, such as the miR-29 binding sites in the proximal portion of the 3' UTR,⁽²³⁾ mouse and human osteonectin are not well conserved in the region containing human SNP1599 (UCSD Genome Browser). Therefore, to examine the impact of SNP 1599 on osteonectin expression in bone in vivo, we used a knock-in strategy to replace the mouse osteonectin 3' UTR with the 1-kb human osteonectin 3' UTR, representing either haplotype A ($ON^{A/A}$) or haplotype B ($ON^{B/B}$) (Supplemental Fig. S1). In relation to other mouse strains, C57Bl/6 mice have a low bone mass phenotype.^(24,25) We chose to examine the function of the human osteonectin 3' UTR haplotypes in the C57Bl/6 genetic background because our candidate gene study revealed an association between osteonectin haplotype and bone mass only in the idiopathic osteoporosis patient group. We reasoned that the effect of the osteonectin 3' UTR haplotype might be most apparent in a low bone mass background, where other gene variants may not be sufficient to rescue a low bone mass phenotype. Moreover, we chose to limit our present analysis to males, to further mimic our previous study in male idiopathic osteoporosis patients.

Osteonectin transcript and protein levels in femur of homozygous haplotype A and B ($ON^{A/A}$ and $ON^{B/B}$) male mice were examined. Western blot analysis of protein extracts demonstrated two- to three-fold lower osteonectin levels in $ON^{A/A}$ femur compared with $ON^{B/B}$, indicating that SNP 1599 contributed to differential osteonectin accumulation in bone in vivo (Fig. 3B). At the same time, qRT-PCR revealed a ~two fold increase in osteonectin mRNA in femur of $ON^{A/A}$ mice compared with $ON^{B/B}$ (Fig. 3A), whereas miR-433 levels were similar (Supplemental Fig. S2). This apparent discrepancy may be related to the fact that the protein data represent the accumulation of osteonectin in bone tissue, whereas the RNA data represent a window of gene expression at 6 to 8 weeks of age.

SNP 1599 affects changes in bone mass with age

Using μ CT, we analyzed the skeletal phenotype of male $ON^{A/A}$ and $ON^{B/B}$ mice at 10 and 20 weeks of age (Supplemental Fig. S3). The femurs of 10-week-old mice $ON^{A/A}$ and $ON^{B/B}$ displayed similar trabecular and cortical bone parameters (Table 2). At 20 weeks of age, trabecular bone volume and trabecular thickness were

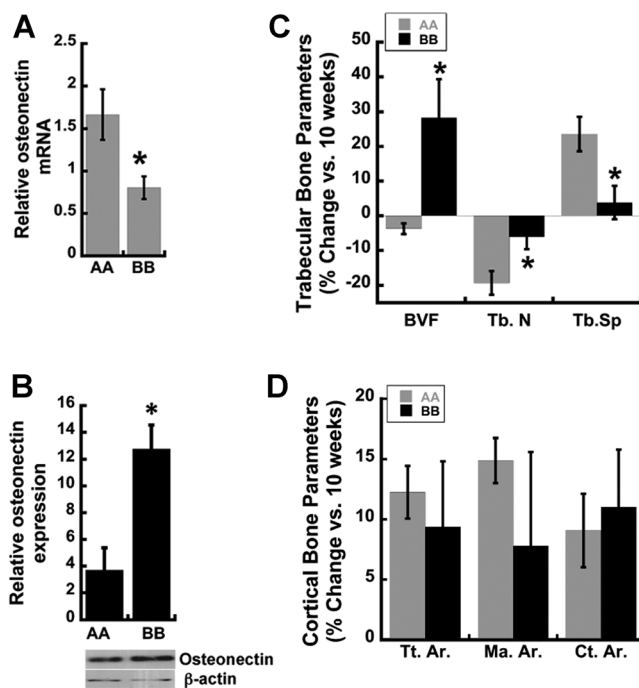


Fig. 3. Osteonectin levels and gain in trabecular bone is higher in haplotype B knock-in mice. (A) Osteonectin mRNA was quantified by qRT-PCR using RNA isolated from the femurs of 6- to 8-week-old $ON^{A/A}$ and $ON^{B/B}$ mice. Osteonectin mRNA levels were normalized to 18s RNA ($n = 4$ mice/haplotype, $*p < 0.05$ different from haplotype A). (B) Western blotting for osteonectin and β -actin in lysates from long bones of $ON^{A/A}$ and $ON^{B/B}$ mice ($*p < 0.05$ different from haplotype A, $n = 3$ mice/group). (C, D) μ CT analysis of changes in femoral bone from 10 to 20 weeks of age in $ON^{A/A}$ and $ON^{B/B}$ mice. BVF = trabecular bone volume; Tb.N. = trabecular bone number; Tb.Sp. = trabecular bone spacing; Tt.Ar = total (periosteal) area; Ma.Ar = marrow area; Ct.Ar = cortical area. $*p < 0.05$ different from haplotype A, $n = 4-9$ mice/group.

significantly higher in $ON^{B/B}$ mice compared with $ON^{A/A}$ (Table 2). When the percentage change in trabecular bone parameters between 10 and 20 weeks was examined, the differences between the two genotypes became more apparent (Fig. 3C). For example, between 10 and 20 weeks of age, $ON^{B/B}$ mice realized a 20% to 25% gain in trabecular bone volume fraction (BVF), whereas $ON^{A/A}$ mice did not. Trabecular number (Tb.N) decreased with age in both the genotypes, and the decrease was significantly less in $ON^{B/B}$ mice. Trabecular spacing (Tb.Sp.) increased between 10 and 20 weeks of age in $ON^{A/A}$ mice but not in $ON^{B/B}$ animals (Fig. 3C). In contrast, cortical bone area (Ct.Ar.) increased from 10 to 20 weeks of age in both genotypes to a similar extent (Fig. 3D). This was not unexpected because the osteonectin-null and haploinsufficient mice display primarily defects in trabecular bone volume.⁽¹⁰⁾ Overall, these data suggest that human osteonectin 3' UTR haplotypes A and B differentially affect the trabecular bone compartment and that $ON^{B/B}$ gained more trabecular bone with age than $ON^{A/A}$ mice.

SNP 1599 modifies the bone anabolic effect of intermittent PTH

Because intermittent administration of PTH is the best bone anabolic therapy currently available, we studied the response of

Table 2. Micro-CT Analysis of Trabecular and Cortical Bone Parameters in Femur of 10-Week-Old and 20-Week-Old Male ON^{A/A} and ON^{B/B} Mice

	AA		BB	
	10 weeks	20 weeks	10 weeks	20 weeks
Trabecular parameters				
BVF (%)	12.60 ± 0.01	12.20 ± 0.00	11.70 ± 0.02	14.80 ± 0.01*
Tb.Th. (μm)	45.48 ± 3.72	46.97 ± 1.23	53.06 ± 2.76	55.24 ± 1.89*
Tb.N. (/mm)	5.20 ± 0.33	4.20 ± 0.18	4.47 ± 0.30	4.23 ± 0.14
Tb.Sp. (μm)	192.25 ± 11.97	237.56 ± 9.59	225.06 ± 17.56	232.34 ± 9.46
Cortical parameters				
Tt.Ar. (mm ²)	1.96 ± 0.10	2.24 ± 0.04	1.89 ± 0.03	2.06 ± 0.11
Ma.Ar. (mm ²)	1.08 ± 0.04	1.24 ± 0.02	1.00 ± 0.03	1.08 ± 0.08
Ct.Ar. (mm ²)	0.88 ± 0.06	0.96 ± 0.03	0.88 ± 0.02	0.98 ± 0.04

BVF = trabecular bone volume fraction; Tb.Th. = trabecular thickness; Tb.N. = trabecular bone number; Tb.Sp. = trabecular bone spacing; Tt.Ar. = total (periosteal) area; Ma.Ar. = marrow area; Ct.Ar. = cortical area.

10 weeks: *n* = 4–5 mice per group; 20 weeks: *n* = 8–9 mice per group. Mean ± SEM.

**p* < 0.05 different from haplotype A.

ON^{A/A} and ON^{B/B} mice to this treatment. Ten-week-old male mice were injected daily with 40 μg/kg PTH (1-34) or vehicle, 5 days per week, for 4 weeks. μCT analysis showed that after 4 weeks of treatment, PTH significantly increased cortical bone area in both ON^{A/A} and ON^{B/B} mice; however, the gain in cortical bone in ON^{B/B} mice was nearly twice that observed in ON^{A/A} mice (Fig. 4A, Table 3). The PTH-mediated increase in the total cross-sectional area (Tt.Ar.) was greater in ON^{B/B} mice compared with ON^{A/A} mice, providing a mechanism for increased cortical bone area. PTH-mediated changes in marrow area (Ma.Ar.) did not reach significance (*p* = 0.07) in either genotype (Fig. 4A and Table 3). These data indicate that the bone anabolic effect of intermittent PTH was greater in ON^{B/B} mice compared with ON^{A/A}.

We also assessed changes in the trabecular bone at the femoral metaphysis of PTH- or vehicle-injected mice using μCT. Although the PTH-mediated increase in trabecular bone volume was not statistically significant, PTH did increase trabecular thickness and number to a similar extent in mice of both genotypes (Fig. 4B, Table 3). Histomorphometry was used to evaluate bone remodeling parameters and bone formation rate in femoral trabecular region of vehicle- and PTH-injected ON^{A/A} and ON^{B/B} mice.⁽¹²⁾ Interestingly, osteoblast number was lower in vehicle-treated ON^{B/B} mice compared with ON^{A/A}, whereas bone formation rate (BFR) was higher in the vehicle-treated ON^{B/B} mice (Fig. 4C, D, Table 4, Supplemental Fig. S4). These data suggest greater bone-forming activity, per cell, in ON^{B/B} mice. With PTH treatment, osteoblast number and bone formation rate in both ON^{A/A} and ON^{B/B} mice were significantly increased, such that they were equivalent. Differences in osteoclast number and eroded surface were not found between ON^{A/A} and ON^{B/B} mice, in the presence or absence of PTH treatment (Table 4). Altogether, these data indicate that ON^{A/A} mice have decreased bone formation compared with ON^{B/B} mice, providing an explanation for the failure of ON^{A/A} mice to increase trabecular bone volume from 10 to 20 weeks of age (Fig. 4D).

To determine whether PTH might have a direct effect on miR-433 expression, wild-type mouse BMSCs were cultured to confluence, serum-deprived, and treated with PTH or vehicle for 3, 6, 12, or 24 hours (Supplemental Fig. S6). However, PTH treatment did not regulate miR-433 levels at any time point

tested, suggesting that the actions of PTH on bone in vivo may not be related to direct effects on miR-433.

Cell autonomous effect of SNP 1599 on osteoblastic differentiation and mineralized matrix deposition

To determine whether the effect of SNP 1599 on bone formation was cell autonomous, we monitored osteoblastic differentiation markers in BMSCs from ON^{A/A} and ON^{B/B} mice cultured for up to 2 weeks. After 1 week of culture in osteoblast differentiation medium, ON^{B/B} cells displayed significantly more osteocalcin and bone sialoprotein mRNA compared with ON^{A/A} cultures (Fig. 4E, F). After 2 weeks of culture, bone sialoprotein mRNA levels were no longer significantly different between genotypes, whereas osteocalcin mRNA remained elevated in ON^{B/B} cultures.

We also assessed mineralized matrix deposition in BMSCs undergoing osteoblastic differentiation in vitro. Mineralized matrix deposition was quantified using alizarin red staining, whereas differences in cell density were monitored by crystal violet staining (Supplemental Fig. S5). The stains were then solubilized and quantified, and alizarin red staining was normalized to crystal violet (Fig. 4G–I). Although both cultures were plated at the same density, crystal violet staining was significantly greater in ON^{A/A} compared with ON^{B/B} cultures (Fig. 4H). Crystal violet staining peaked at week 2 in ON^{A/A} cultures and at week 3 in ON^{B/B} cultures. However, despite the lower cell number, ON^{B/B} cultures showed a greater alizarin red staining at weeks 3 and 4 of differentiation (Fig. 4G). After normalizing alizarin red staining by crystal violet, the difference in mineralized matrix deposition between ON^{A/A} and ON^{B/B} cultures became more apparent (Fig. 4I). This suggests that ON^{B/B} osteoblasts deposited significantly more mineralized matrix per cell compared with ON^{A/A} cultures, and supports the in vivo observations (Fig. 4C, D).

To determine whether there were inherent differences in the growth rate of ON^{A/A} and ON^{B/B} BMSCs, MTS assay was used to monitor the growth of subconfluent cultures. We found that the growth rate for ON^{A/A} and ON^{B/B} stromal cells did not differ (Supplemental Fig. S5A). Similarly, previous in vitro studies showed that osteonectin-null osteoblasts displayed decreased osteoblast maturation and mineralized matrix deposition but no

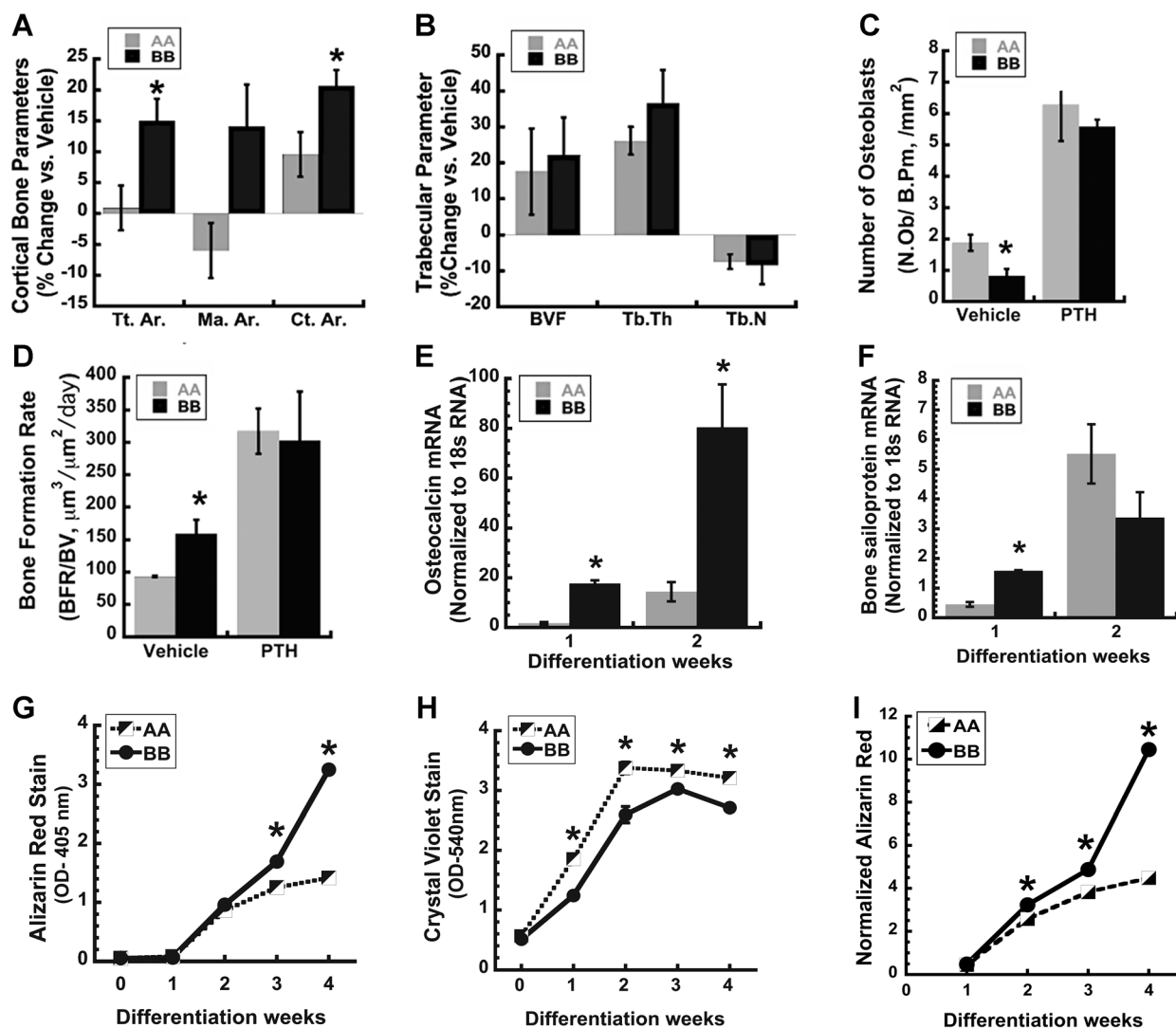


Fig. 4. PTH-induced gain of cortical bone area is greater in haplotype B knock-in mice, and BMSCs from haplotype B mice have enhanced osteoblastic differentiation in vitro. μ CT and histomorphometric analysis of femoral trabecular bone from 14-week-old $ON^{A/A}$ and $ON^{B/B}$ mice that had been injected for 4 weeks with 40 μ g/kg/d PTH or vehicle. (A) Percent change in cortical bone parameters versus vehicle in femur. Tt.Ar = total (periosteal) area; Ma.Ar = marrow area; Ct.Ar = cortical area. (B) Percent change in trabecular bone parameters versus vehicle in femur. BVF = trabecular bone volume; Tb.Th = trabecular thickness; Tb.N = trabecular bone number. Histomorphometric analysis of osteoblast number and bone formation rate (C, D) in femoral trabecular bones of vehicle- and PTH-treated $ON^{A/A}$ and $ON^{B/B}$ mice ($*p < 0.05$ different from $ON^{A/A}$ mice, $n = 4-6$ /group). Osteocalcin (E) and bone sialoprotein (F) RNA in BMSCs from $ON^{A/A}$ and $ON^{B/B}$ mice undergoing osteoblastic differentiation for 2 weeks ($*p < 0.05$ different from $ON^{A/A}$, $n = 3$). Quantified alizarin red (G) and crystal violet (H) staining and alizarin red stain normalized to crystal violet (I) for BMSCs from $ON^{A/A}$ and $ON^{B/B}$ mice undergoing osteoblastic differentiation for up to 4 weeks ($*p < 0.05$ different from $ON^{A/A}$, $n = 4$).

defects in cell growth.⁽¹¹⁾ The present in vitro studies support the concept that, in BMSCs, osteonectin levels do not impact cell growth but have effects on osteoblastic differentiation.

Discussion

Skeletal phenotype is a complex genomic trait, and only a minute fraction of the genetic variants contributing to this phenotype have been identified. Further, the in vivo function of most osteoporosis-associated polymorphisms identified through GWAS and candidate gene studies is not known.⁽¹⁾ In this study, we developed a

novel knock-in mouse model to determine the in vivo impact of a human regulatory region polymorphism on skeletal phenotype. This model demonstrated that a SNP in the osteonectin 3' UTR can regulate osteonectin levels and bone volume, essentially validating the association between osteonectin 3' UTR SNP haplotypes and bone mass first identified in a cohort of white men with idiopathic osteoporosis.⁽¹³⁾ Moreover, we identified a potential molecular mechanism by which this SNP regulates osteonectin expression, through differential targeting of a miRNA (Fig. 5).

Bone matrix is enriched in osteonectin. This integrin-binding matricellular protein is important for regulating collagen matrix assembly and organization.⁽²⁶⁻²⁸⁾ In fact, many connective tissue

Table 3. Micro-CT Analyses of Trabecular and Cortical Bone Parameters in Femur of Vehicle-and PTH-Injected ON^{A/A} and ON^{B/B} Mice

	AA		BB	
	Vehicle	PTH	Vehicle	PTH
Trabecular parameters				
BVF (%)	12.00 ± 0.01	14.00 ± 0.01	12.00 ± 0.01	16.00 ± 0.01
Tb.Th. (μm)	47.03 ± 1.16	59.54 ± 1.94 [#]	46.40 ± 2.54	61.65 ± 3.81 [#]
Tb.N. (/mm)	4.43 ± 0.04	4.10 ± 0.16 [#]	4.72 ± 0.16	4.25 ± 0.21
Cortical parameters				
Tt.Ar. (mm ²)	2.15 ± 0.07	2.17 ± 0.08	2.00 ± 0.05	2.36 ± 0.06 [#]
Ma.Ar. (mm ²)	1.22 ± 0.06	1.15 ± 0.05	1.10 ± 0.03	1.21 ± 0.07
Ct.Ar. (mm ²)	0.93 ± 0.02	1.02 ± 0.02 [#]	0.90 ± 0.02	1.09 ± 0.02 [#]

BVF = trabecular bone volume fraction; Tb.Th. = trabecular thickness; Tb.N. = trabecular bone number; Tt.Ar. = total (periosteal) area; Ma.Ar. = marrow area; Ct.Ar. = cortical area.

Fourteen-week-old male haplotype A and B knock-in mice had been injected for 4 weeks with 40 μg/kg/d PTH or vehicle (*n* = 4–6 mice per group). Mean ± SEM.

[#]*p* < 0.05 different from corresponding vehicle-injected mice of the same genotype.

Table 4. Histomorphometric Analysis of Femoral Trabecular Bone of Vehicle- and PTH-Injected ON^{A/A} and ON^{B/B} Mice

Histomorphometry	AA		BB	
	Vehicle	PTH	Vehicle	PTH
Formation				
Osteoblast number (N.Ob/B.Pm./mm ²)	1.64 ± 0.26	6.28 ± 1.16 [#]	0.91 ± 0.23*	6.39 ± 1.52 [#]
Mineralizing surface (MS/BS, %)	4.77 ± 0.55	10.58 ± 1.63 [#]	7.34 ± 0.90*	8.86 ± 1.16
Bone formation rate (μm ³ /μm ² /d)	93.29 ± 1.17	317.27 ± 35.02 [#]	168.36 ± 21.90*	196.81 ± 21.34
Resorption				
Osteoclast number (N.Oc/B.Pm./mm ²)	0.91 ± 0.22	0.81 ± 0.25	0.72 ± 0.07	0.80 ± 0.21
Osteoclast surface (Oc.S/BS, %)	2.54 ± 0.64	2.27 ± 0.66	1.97 ± 0.21	2.26 ± 0.10
Eroded surface (ES/BS, %)	5.67 ± 1.14	5.63 ± 1.05	4.76 ± 0.53	5.29 ± 0.38

Fourteen-week-old haplotype A and B knock-in mice had been injected for 4 weeks with 40 μg/kg/d PTH or vehicle (*n* = 4–6 mice per group).

[#]*p* < 0.05 different from the corresponding vehicle-injected mice of the same genotype. Mean ± SEM.

**p* < 0.05 different from haplotype A mice in the same treatment group.

pathologies detected in osteonectin-null mice have been attributed to defective extracellular matrix composition.^(27–29) In vitro and in vivo studies demonstrate that collagen fibril organization is impaired in bone matrices of osteonectin-null mice, likely contributing to decreased bone mineralization.⁽²⁷⁾ In vitro, osteonectin promotes osteoblast survival, differentiation, and matrix mineralization.⁽¹¹⁾ It is possible that extracellular matrix

organization differs between ON^{A/A} and ON^{B/B} mice, which may contribute to differences in osteoblastic differentiation and mineralization; studies to address these questions are ongoing.

Although we did not analyze osteonectin mRNA or protein levels in ON^{A/A} and ON^{B/B} mice after PTH administration, studies by Turner and colleagues showed that intermittent PTH administration increased osteonectin mRNA in bone of rats subjected to hindlimb unloading.⁽³⁰⁾ Previously, we reported that although osteonectin-null and haploinsufficient mice gain bone in response to intermittent PTH therapy, their bone-anabolic response was less than that found in wild-type mice. For the most part, the response of these mice to PTH could be related to osteonectin gene dosage.⁽¹²⁾ Although the dose of PTH used in our previous study was higher than the one used in this study (80 versus 40 μg/kg/d), our results suggest that higher levels of osteonectin in bone are associated with greater PTH-mediated bone gain, particularly with regard to the cortical compartment (Fig. 4A). It is possible that the higher levels of osteonectin in ON^{B/B} mice could facilitate the anabolic response of bone to intermittent PTH treatment.

The expression of osteonectin is tightly regulated through mechanisms that alter transcription, mRNA stability, and translation.^(20,23,31–36) In osteoblasts, the osteonectin transcript is quite stable, with a half-life of >24 hours under conditions of

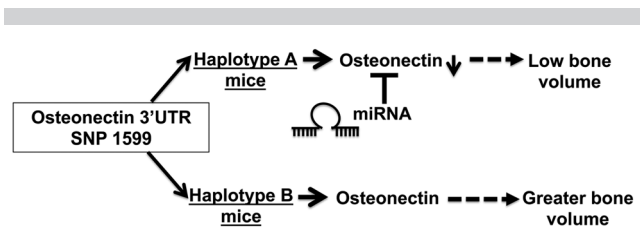


Fig. 5. Working model for osteonectin 3' UTR SNP-mediated effect on bone. In haplotype A knock-in mice, osteonectin levels in bone are lower compared with haplotype B knock-in mice. The osteonectin 3' UTR haplotype A is targeted by miRNA (miR-433), which may contribute to lower osteonectin and lower bone volume.

transcription arrest.⁽³¹⁾ Therefore, regulation of translation would likely provide the most rapid means of decreasing osteonectin synthesis. Recently, two evolutionarily conserved binding sites for the miR-29 family of miRNAs were found in the proximal region of the osteonectin 3' UTR.⁽²³⁾ Induction of miR-29 expression by canonical Wnt signaling provided potent repression of osteonectin protein synthesis, within 1 hour of treatment.⁽²⁰⁾ This example illustrates the efficiency by which miRNAs could regulate osteonectin levels.

In this study, we identified miR-433 as a miRNA that could play a role in regulating osteonectin expression. The mature miR-433 sequence is identical between mice and humans, and the organization of the miR-433 genomic locus is conserved. Previously, miR-433 was shown to decrease during BMP2-induced osteoblastic differentiation of C3H10T1/2 cells and to target the *Runx2* 3' UTR.⁽²²⁾ Our study confirms that miR-433 expression decreases during osteoblastic differentiation in human cells and that miR-433 has an inhibitory effect on differentiation (Fig. 2). We also demonstrated that SNP 1599 modulates the ability of miR-433 to regulate the human osteonectin 3' UTR (Fig. 2). Although the effect of the miR-433 inhibitor on the haplotype A construct was modest, it is consistent with effects reported in other studies and may reflect the relatively low level of miR-433 expression in the human osteoblast cell line.⁽¹⁸⁾ Moreover, osteonectin is expressed in multiple tissues, many of which likely have a complement of miRNAs that are distinct from that found in the bone cells. Other miRNAs might bind to the osteonectin 3' UTR, and differential binding of these miRNAs to the region containing SNP 1599 must be strongly considered.

Others have examined the potential association of osteonectin SNPs with disease phenotype in systemic sclerosis, hepatocellular carcinoma, glaucoma, and keratoconus.^(29,37-40) Although some studies have associated particular osteonectin 3' UTR SNPs and disease, these reports have not involved SNP 1599, nor have they described potential mechanisms. Osteonectin SNP 1599G is a common variant (Table 5).⁽⁴¹⁾ In a sample population of North Americans that are of African descent, frequency of the SNP1599G allele, present in haplotype A, is less than that for populations of European or Chinese descent (dbSNP summary for ss24686914). Studies have shown that, as a group, African Americans have higher bone mineral density compared with whites.^(42,43) Although several candidate genes have been associated with BMD within these groups, it is not clear whether decreased frequency of SNP 1599G could play a part in this effect.⁽⁴⁴⁾

In the literature, there has been argument about whether the majority of phenotypic variance is driven by rare variants with large effects or by common variants with small effects.⁽⁴⁵⁾ Most likely, rare and common variants work together, in conjunction with gene-environment interactions, to specify phenotype. The present study was performed in an inbred mouse strain, and the animals were housed in well-controlled environmental conditions. These experimental parameters allowed us to decrease the impact of genetic variance and gene-environment interactions on trabecular and cortical bone phenotype. This permitted us to assign a physiological function to a common osteonectin allele, providing support for its contribution to the complex trait of skeletal phenotype.

Presently, estimation of a patient's risk of fracture is performed using models based on clinical, demographic, and anthropomorphic information, such as BMD, previous fracture, a parent with hip fracture, smoking, and glucocorticoid and alcohol use. Although valuable, the prognostic performance of these models could be improved, especially in case of idiopathic osteoporosis. Including genetic profiling data for an individual could help

Table 5. Osteonectin SNP 1599 (rs1054204) Allele and Genotype Frequencies (From dbSNP Summary for ss24686914)⁽⁴¹⁾

Population ID	Ethnicity	Allele frequency	Genotype frequency
AFD EUR		C = 0.521	C/G = 0.542
Panel-North America	European	G = 0.479	C/C = 0.208 G/G = 0.250
AFD CHN		C = 0.604	C/G = 0.542
Panel-North America	Asian	G = 0.396	C/C = 0.333 G/G = 0.125
AFD AFR		C = 0.783	C/G = 0.348
Panel-North America	African	G = 0.217	C/C = 0.609 G/G = 0.043

improve accuracy of risk assessment and better inform treatment decisions.⁽⁴⁶⁾ Osteonectin SNP 1599 could be of importance to consider in investigations of idiopathic osteoporosis. However, the impact of a single variant on fracture risk is small, and identification of many more gene variants with an impact on the skeleton is necessary before real gains in fracture prediction can be realized.^(46,47) Data such as those reported here will contribute to the pool of SNP variants needed for individualized risk assessment and fracture prevention.

Disclosures

All authors state that they have no conflicts of interest.

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Authors' roles: NSD, KK, SPY, and AMD designed the research. NSD, KK, CBK, DJA, RCR, and AMD performed the research and analyzed the data. NSD and AMD wrote the manuscript and take responsibility for the data analysis. All authors approved the manuscript submission.

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