

# Identification of a Role for the *ARHGEF3* Gene in Postmenopausal Osteoporosis

Ben H. Mullin,<sup>1</sup> Richard L. Prince,<sup>1,3</sup> Ian M. Dick,<sup>1,2,3</sup> Deborah J. Hart,<sup>4</sup> Tim D. Spector,<sup>4</sup> Frank Dudbridge,<sup>5</sup> and Scott G. Wilson<sup>1,3,4,\*</sup>

Osteoporosis is a common and debilitating bone disease characterized by low bone mineral density (BMD), a highly heritable and polygenic trait. Genome-wide linkage studies have identified 3p14-p21 as a quantitative trait locus for BMD. The *ARHGEF3* gene is situated within this region and was identified as a strong positional candidate. The aim of this study was to evaluate the role of variation in *ARHGEF3* on bone density in women. Sequence variation within *ARHGEF3* was analyzed with 17 single-nucleotide polymorphisms (SNPs) in a discovery cohort of 769 female sibs. Significant associations were found with family-based association tests between five SNPs and various measures of age-adjusted BMD ( $p = 0.0007$ – $0.041$ ) with rs7646054 showing maximal association. Analysis of the data with QPDTPhase suggested that the more common G allele at rs7646054 is associated with decreased age-adjusted BMD. Significant associations were also demonstrated between 3-SNP haplotypes and age-adjusted spine and femoral-neck BMD ( $p = 0.002$  and  $0.003$ , respectively). rs7646054 was then genotyped in a replication cohort, and significant associations with hip and spine BMD were confirmed ( $p = 0.003$ – $0.038$ ), as well as an association with fracture rate ( $p = 0.02$ ). Again, the G allele was associated with a decrease in age-adjusted BMD at each site studied. In conclusion, genetic variation in *ARHGEF3* plays a role in the determination of bone density in Caucasian women. This data implicates the RhoGTPase-RhoGEF pathway in osteoporosis.

## Introduction

Postmenopausal osteoporosis is a systemic bone disease that is characterized by low bone mass and disturbed microarchitecture of bone tissue, resulting in increased fragility, and is a major risk factor for fracture.<sup>1</sup> Peak bone mass is attained in early adult life but declines in postmenopausal women as a result of a reduction in estrogen production with effects on bone as well as intestinal and renal calcium handling.<sup>2</sup> However, in addition to the effects of estrogen, calcium, and other environmental factors on bone structure and fracture, there is a strong genetic effect on peak bone mass, bone loss, and fracture rates in postmenopausal women.<sup>3–6</sup> Twin and family studies show that 50%–90% of the variance in peak bone mass is heritable.<sup>3,4,6–10</sup> The whole-genome linkage-scanning approach has identified at least 11 replicated quantitative trait loci (QTLs),<sup>11–20</sup> so that it is evident that the genetic effect for common variation in bone mineral density (BMD) is under polygenic control. The 3p14-p21 region of the human genome has been identified as one of the most replicated QTLs for BMD in multiple studies, including our own<sup>19</sup> and a meta-analysis.<sup>21</sup> The *ARHGEF3* gene, which encodes the rho guanine-nucleotide exchange factor (GEF) 3, is situated within this region.

The *ARHGEF3* gene was selected for study on the basis of a bioinformatics analysis, including physical location of the gene relative to maximum LOD score for the linkage at 3p14-p21, gene function, conserved domains found in the protein by BLASTp, and number of homologs and

gene family members identified by BLASTp. The product of this gene specifically activates two members of the RhoGTPase family: RHOA (MIM 165390),<sup>22</sup> known to play a role in bone,<sup>23,24</sup> and RHOB (MIM 165370),<sup>22</sup> with a potential role in osteoarthritis.<sup>25,26</sup> Interestingly, variation in the gene *FGD1* (MIM 300546), encoding another RhoGTPase regulatory protein, has been implicated in the Aarskog-Scott Syndrome (AAS [MIM 305400]), which is characterized by a variety of skeletal abnormalities including short stature, hypertelorism, and brachydactyly.<sup>27</sup>

This paper reports a study of the effect of polymorphisms within the *ARHGEF3* gene on bone density in a large, well-described, family-based cohort of women from Australia and the United Kingdom with replication in an independent cohort of postmenopausal women from the UK.

## Material and Methods

### Subjects

#### Discovery Cohort

A total of 769 women from 335 families were recruited in Australia and the UK. This family-based population included siblings recruited for a study of the genetics of osteoporosis in 1998.<sup>19</sup> The median BMD Z score for the proband at the spine, total-hip, and femoral-neck sites was  $Z = -1.54$  (1.03),  $-1.00$  (0.95), and  $-1.03$  (1.05), respectively (interquartile range). Sibships within the cohort included 264 families with two siblings, 49 with three, 17 with four, four with five, and one with seven siblings. The median difference in Z score between sibs with extreme BMD

<sup>1</sup>Department of Endocrinology and Diabetes, <sup>2</sup>Western Australian Institute of Medical Research, Sir Charles Gairdner Hospital, Nedlands, Western Australia, 6009, Australia; <sup>3</sup>School of Medicine and Pharmacology, University of Western Australia, Nedlands, Western Australia, 6009, Australia; <sup>4</sup>Twin & Genetic Epidemiology Research Unit, St. Thomas' Hospital Campus, King's College London, London WC2R 2LS, UK; <sup>5</sup>MRC Biostatistics Unit, Institute of Public Health, Cambridge CB2 0SR, UK

\*Correspondence: wilsonsg@cylle.uwa.edu.au

DOI 10.1016/j.ajhg.2008.04.016. ©2008 by The American Society of Human Genetics. All rights reserved.

measurements were spine  $Z = 2.2$  (1.59), total-hip  $Z = 1.5$  (1.04), and femoral-neck  $Z = 1.6$  (1.17). Exclusion criteria were applied where possible and included the presence of bone cancer, hyperparathyroidism, unstable thyroid disease, long-term steroid use (>5 mg/day for more than 6 months and presently on therapy), chronic immobility, rheumatoid arthritis, anorexia nervosa, osteomalacia, amenorrhea for > 6 months, premature cessation of regular menstruation or surgical oophorectomy  $\pm$  hormone-replacement therapy (age < 40 yr), and epilepsy with use of anticonvulsant medication for > 1 yr. All subjects from the study provided written informed consent, and the institutional ethics committees of participating institutions approved the experimental protocols.

At a clinic visit, data including age, height, weight, medical, gynecological, and lifestyle data were recorded and a blood sample was collected. Dual-energy X-ray absorptiometry (DXA) BMD was assessed (Hologic, Bedford, MA, USA) at the lumbar spine L1–L4 and the total hip, which includes an area from the femoral neck to just below the lesser trochanter. Within this area, the femoral-neck subregion is widely used in clinical practice for prediction of fracture propensity and was the phenotype chosen for use in this study. Because of the range of ages in this cohort, BMD data were adjusted for age prior to analysis by conversion to BMD Z scores. The correlations between the BMD Z scores obtained from different sites were  $r = 0.82$  (total hip and femoral neck),  $r = 0.63$  (total hip and spine), and  $r = 0.52$  (femoral neck and spine).

#### Replication Cohort

This group of subjects was recruited in 1988 to participate in a longitudinal epidemiological study of rheumatic diseases (The Chingford Study). Women between the ages of 45 and 64 were recruited from a single large general practice in Chingford, North-East London, via a population-based method. All women within this age range that were on a register of more than 11,000 patients were invited to participate in the study. No exclusion criteria were applied. This cohort has similar demographics and anthropometry to the general UK population regarding height, weight, smoking status, and hysterectomy rates.<sup>28,29</sup> Demographic and lifestyle-factor data were obtained from questionnaires completed in 1988. DNA samples were obtained from 780 individuals. Bone-density measurements were undertaken with a Hologic QDR-2000 densitometer in 1998, approximately 10 yr after the subjects were initially recruited, with hip and spine DXA BMD data obtained from 775 and 779 individuals, respectively. The correlations between the BMD measurements obtained from different sites were  $r = 0.89$  (total hip and femoral neck),  $r = 0.7$  (total hip and spine), and  $r = 0.67$  (femoral neck and spine). Subjects were categorized as fracture free or having had a previous fracture as described previously,<sup>30</sup> with fractures sustained at any skeletal site up to 2003 included in the analysis but those caused by high-impact trauma excluded. Informed consent was obtained from each individual, and the study was approved by the local ethics committee.

#### Genotyping

Genomic DNA was extracted and purified from EDTA whole blood obtained from each subject.<sup>31</sup> Genotyping in the discovery cohort was performed with the Illumina GoldenGate assay on an Illumina BeadStation 500 GX and utilized bead-array hybridization<sup>32</sup> with genomic whole-genome amplified (Repli-g) DNA. The genotype call rate with this technique was 99.8%, with an error rate of < 0.1%.

Genotyping in the replication cohort was performed with matrix-assisted laser desorption and ionization time of flight (MALDI-ToF) mass spectrometry as described previously.<sup>33</sup> For

this technique, the genotype call rate was 97.7% and the estimated error rate was < 0.1%.

#### SNP Selection

Seventeen single-nucleotide polymorphisms (SNPs) were selected in the region of the *ARHGEF3* gene for genotyping in the discovery cohort. Tagging SNPs (tSNPs) were initially selected across the region with the Perlegen Genome Browser Version 1,<sup>34</sup> which identifies tSNPs as being in linkage disequilibrium (LD) of  $r^2 \geq 0.8$  with all other SNPs in the LD bin. We attempted to tag all 13 LD bins that were identified by Perlegen within the gene and the 50 Kb region 5' of the gene and contained two or more SNPs. Because of assay design issues, we were able to tag nine of the 13 LD bins. The remaining SNPs genotyped were selected from the Perlegen Genome Browser as single SNPs not belonging to any LD bin, or they were selected from dbSNP.

#### Statistical Analysis

All SNPs were in Hardy-Weinberg equilibrium ( $\chi^2$  test,  $p < 0.05$ ). Analysis of the data from the discovery cohort was performed with the family-based association tests (FBAT) software to test for association within sib pairs.<sup>35</sup> The empirical variance estimator was used to allow for prior linkage to the region. The results were adjusted for multiple testing by permuting of the phenotypes within sibships with genotypes held constant. The minimum  $p$  values were recorded for 10,000 random reassignments of the data (with an automated script written in Perl), with an adjusted  $p$  value  $\leq 0.05$  considered significant. For individual BMD scores, with multiple SNPs adjusted for, this corresponds to an unadjusted  $p$  value of about 0.0037. For adjustment for testing BMD scores at three sites, the corresponding unadjusted  $p$  value was 0.002. To examine the effect of reducing the number of the correlated traits, we carried out principal-component analysis. The effect of menopausal status on BMD in the discovery cohort was analyzed with multiple linear regression implemented in STATISTICA version 8.0 (StatSoft, Tulsa, OK, USA). Haplotype analysis used QPDTPHASE, part of the UNPHASED suite,<sup>36</sup> which is a program for association analysis of multilocus haplotypes from unphased genotype data. Throughout, two-tailed  $p$  values are reported, with adjusted  $p \leq 0.05$  considered significant. LD between the different SNPs was evaluated with the software JLIN<sup>37</sup> and graphical overview of linkage disequilibrium (GOLD).<sup>38</sup>

Statistical analysis of the data from the replication cohort was performed with one-way ANOVA for differences between genotype groups. BMD data were adjusted for age and weight by analysis of covariance (ANCOVA). Genotype effects on the prevalent fracture rate were examined with a Chi-square test.

## Results

#### Discovery Cohort

The mean age of the discovery cohort was  $54.2 \pm 12.7$  yr; other demographic and morphometric characteristics of the populations are detailed in Table 1. The discovery cohort recorded a lower mean BMD than the replication cohort at each site studied despite a younger mean age, which was expected because of the high proportion of osteoporotic individuals in this population. For the total-hip and femoral-neck sites, menopausal status was not a significant predictor of BMD. At the spine, menopausal status was found to account for < 1% of the variance in BMD.

**Table 1. Demographics and Bone Density of the Discovery and Replication Populations**

Variable	Discovery	Replication
Age (yr)	54.2 ± 12.7 (769)	62.5 ± 5.9 (780)
Weight (kg)	62.7 ± 11.27 (699)	69.1 ± 12.6 (778)
Prevalent fractures (%)	-	34 (780)
Total-hip DXA BMD (mg/cm <sup>2</sup> )	801 ± 136 (760)	869 ± 128 (775)
Total-hip BMD Z Score	-0.420 ± 0.992 (760)	0.489 ± 0.994 (775)
Femoral-neck DXA BMD (mg/cm <sup>2</sup> )	700 ± 133 (749)	747 ± 119 (775)
Femoral-neck BMD Z score	-0.355 ± 1.050 (749)	0.276 ± 1.019 (775)
Spine L1-L4 DXA BMD (mg/cm <sup>2</sup> )	855 ± 158 (767)	955 ± 155 (779)
Spine BMD Z score	-0.669 ± 1.252 (767)	0.745 ± 1.384 (779)

Results are given as mean ± SD (number of measurements).

The location of the 17 SNPs studied relative to the splice variants of *ARHGEF3* are displayed in Figure 1. With FBAT, significant associations were seen between the SNPs rs4681928, rs1344142, rs1110866, rs7646054, and rs3772219 and various measures of BMD Z score ( $p = 0.0007$ – $0.041$ ). The chromosomal position and allele distribution of these five SNPs are detailed in Table 2. The strongest associations were observed with rs7646054, which was associated with BMD Z score at the total hip ( $p = 0.006$ ), femoral neck ( $p = 0.0007$ ), and spine ( $p = 0.006$ ). Among the other SNPs, rs4681928 was significantly associated with BMD Z score of the femoral neck ( $p = 0.01$ ) and spine ( $p = 0.03$ ), rs1344142 with spine ( $p = 0.04$ ), rs1110866 with femoral neck ( $p = 0.02$ ), and rs3772219 with spine ( $p = 0.03$ ). Significant association with rs7646054 was maintained after correction for testing multiple anatomical sites ( $p = 0.015$ ). The significant association between rs7646054 and femoral-neck BMD Z score persisted after adjustment of the data for testing multiple SNPs ( $p = 0.007$ ), as did the association corrected further for testing multiple anatomical sites ( $p = 0.024$ ).

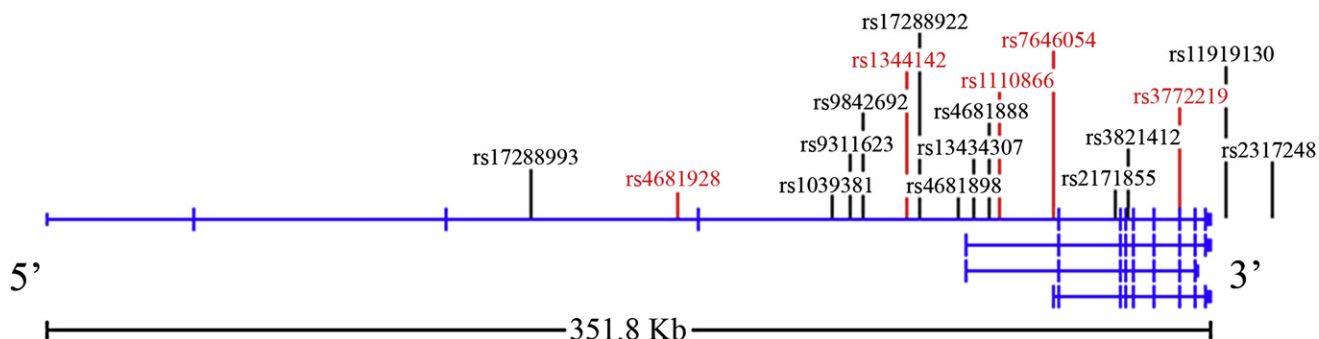
We also applied principal-component analysis to the BMD trait group. The first two principal components explained 94.5% of the trait variance. However, with the eigenvalue > 1 criterion used, only the first component, which explained 77.3% of the variance in BMD, should be retained. This component showed maximal association with rs7646054 ( $p = 0.002$ ).

The more common *G* allele at rs7646054 is associated with a lower BMD Z score at each site studied (Table 3), indicating that this allele has a negative effect on BMD. Note that the mean BMD Z scores reported by QPDTPHASE are loosely interpreted as the expected trait value for an individual carrying that particular allele and are not interpretable as additive effects on the mean.

Pair-wise linkage disequilibrium  $D'$  and  $r^2$  values for the five SNPs are shown in Figure 2. A haplotype analysis was undertaken on the femoral-neck and spine BMD Z score data with UNPHASED to determine whether any haplotypes were more strongly associated with either phenotype than individual SNPs. Each haplotype analysis incorporated only the three SNPs that were most significantly associated with the phenotype in the FBAT analysis. For femoral-neck BMD Z score, the SNPs included in the haplotype analysis were rs4681928, rs1110866, and rs7646054, whereas the SNPs rs4681928, rs7646054, and rs3772219 were included in the spine BMD Z score analysis. Significant associations were observed with both phenotypes (Table 4), including a stronger overall association with spine BMD Z score than in the individual SNP analysis, suggesting independent effects of the SNPs on BMD. In the femoral-neck BMD haplotype analysis, the significance of the overall association did not surpass that of the individual SNP analysis. However, a very strong association was observed between the AAA haplotype and femoral-neck BMD Z score in the individual haplotype analysis ( $p < 0.0004$ ).

### Replication Study

Because rs7646054 was the strongest predictor of spine and hip bone density, it was taken forward to the replication study to determine whether the effect would be detectable in a population-based cohort of postmenopausal women. Significant



**Figure 1. Diagram Showing the Localization of the 17 SNPs Analyzed in *ARHGEF3* and the Splice Variants of the Gene According to the UCSC Genome Browser, March 2006 Assembly**

The SNPs highlighted in red are those that are significantly associated with BMD parameters in the discovery cohort prior to adjustment for multiple testing.

**Table 2. Position and Allele Distribution of the Five SNPs in *ARHGEF3* that Demonstrated Significant Associations with BMD**

SNP	Chromosome Position <sup>a</sup>	Location and Function <sup>a</sup>	Genotype Distribution in the Discovery Cohort (%)
rs4681928	56901206	5' region	AA (63.6), AG (33.5), GG (2.9)
rs1344142	56832473	5' region	GG (26.4), AG (52.6), AA (21)
rs1110866	56801364	Intron 1	AA (41.2), AC (47.2), CC (11.6)
rs7646054	56784668	Intron 1	GG (29.1) (33.2) <sup>b</sup> , AG (52.1) (47.4) <sup>b</sup> , AA (18.8) (19.4) <sup>b</sup>
rs3772219	56746291	Exon 8, Change of amino acid 335 (Leu to Val)	TT (45.6), TG (42.5), GG (11.9)

<sup>a</sup> From GenBank reference sequence NM\_019555, Genome Build 36.2. <sup>b</sup> Allele distribution in the replication cohort.

associations were observed between rs7646054 and spine and total-hip BMD including the femoral-neck area, all of which persisted after adjustment of the BMD data for the covariates age and weight (Table 5). Consistent with the results for the discovery cohort, subjects homozygous for the G allele compared to individuals homozygous for the A allele had lower BMD at the total-hip, femoral-neck, and spine sites (−3.7%, −3.3%, and −3.5%, respectively). Compared to heterozygous individuals with the AG genotype, GG individuals again had lower BMD at the three sites (−1.8%, −2.4%, and −3.7%, respectively). No significant associations between genotype and the covariates age or weight were found.

An allelic association test of the replication cohort with BMD Z score as the phenotype was then carried out with QTPHASE to confirm replication with the same type of association test and the same phenotype as that used for the discovery cohort. Significant associations were observed between rs7646054 and total-hip BMD Z score ( $p = 0.007$ ), femoral-neck BMD Z score ( $p = 0.02$ ), and spine BMD Z score ( $p = 0.02$ ).

In the replication cohort, 265 subjects had suffered a fracture prior to 2004, giving a prevalent fracture rate of 34%. rs7646054 was found to be significantly associated with fracture rate, with GG individuals having an increased fracture rate (Table 5).

## Discussion

The data presented in this study provide evidence that variation within the *ARHGEF3* gene affects BMD in women

**Table 3. Genetic Data for rs7646054 Relevant to BMD Z Score in the Discovery Cohort**

BMD Z Score Phenotype	Mean BMD Z Score		p Value
	G Allele	A Allele	
Total hip	−0.437 ± 1.235 (837)	−0.399 ± 1.222 (683)	0.013
Femoral neck	−0.383 ± 1.325 (825)	−0.322 ± 1.264 (673)	0.001
Spine	−0.686 ± 1.619 (842)	−0.650 ± 1.538 (692)	0.008

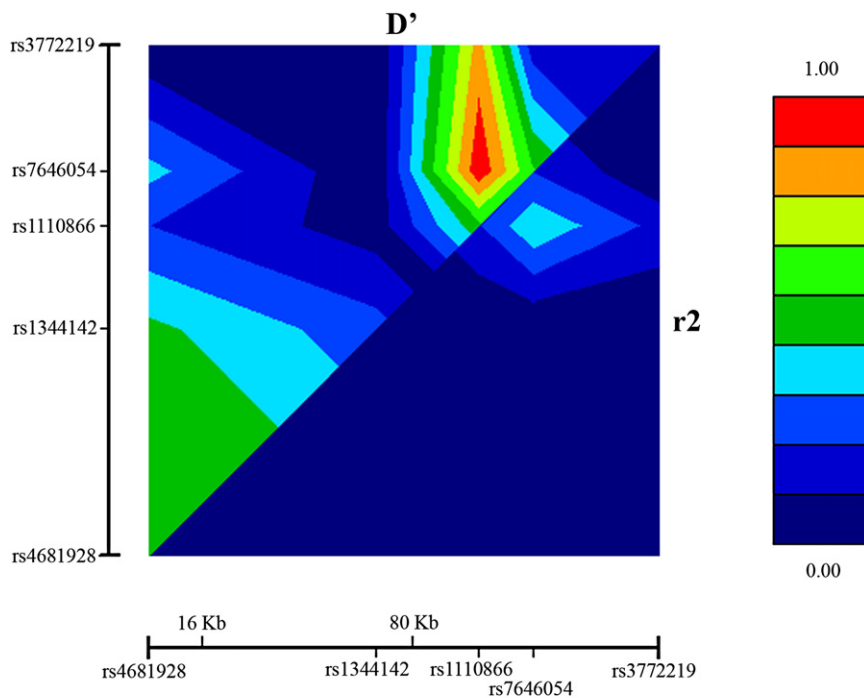
Results are given as mean ± SD (number of alleles contributing to the mean), derived from QPDTPHASE.

aged 40 to 70 in the peri- and early postmenopausal phase of life, at the time that fracture risk is rising. We found strong evidence for an influence of individual SNPs and haplotypes on spine and femoral-neck BMD Z score. rs7646054 within intron 1 of the *ARHGEF3* gene (NCBI accession number NM\_019555) was the best predictor of bone density, and the results were replicated in a second cohort including a statistically significant association with fracture rate. In general, fracture risk rises by about two times for a reduction in hip BMD of one standard deviation (SD) (15% of the mean).<sup>39</sup> We saw decreases in BMD of about 3% that equate to an increase in fracture risk for the “disease” genotype of about 20%, in keeping with the observed fracture rate in the replication cohort.

Given that rs7646054 is located within an intron, it is unlikely that polymorphism at this site would have a direct effect on the *ARHGEF3* gene product. Analysis of the SNPs in strong LD with rs7646054 provided no further evidence as to the mechanism by which the *ARHGEF3* protein could be affected by the observed polymorphism. However, rs7646054 is located within the 5' UTR of a recently described splice variant of the *ARHGEF3* gene (NCBI accession number AB209661). It is therefore possible that polymorphism at this site has an effect on the mRNA folding of this splice variant.

Using the genetic power calculator developed by Purcell and colleagues,<sup>40</sup> with QTL variance set as 0.05, QTL and marker frequency of 0.5 and 0.55, respectively,  $D' = 0.9$ , and sib correlation of 0.4, the power of the discovery cohort is 0.91 at a type I error rate of 0.05. If the QTL variance is only 0.03, then the power is 0.72. Similarly for the replication cohort, if the QTL variance is set as 0.05, then the power for this study is 0.99, whereas for a QTL variance of 0.03 the power is 0.98.

*ARHGEF3* was first described in 2002<sup>22</sup> and is a rho-family guanine-nucleotide exchange factor containing two domains: a Dbl homology domain, which is responsible for catalytic activity, and a pleckstrin homology domain, which is thought to target the GEFs to the plasma membrane and provide a site for regulation by phospholipids.<sup>41</sup> When expressed in fibroblasts, *ARHGEF3* has been shown to cause the assembly of more robust stress fibers and focal adhesions than in fibroblasts not expressing the protein through specific activation of the RHOA and RHOB GTPases.<sup>22</sup>



**Figure 2. Pairwise Linkage Disequilibrium Plot for the Five Associated SNPs in the *ARHGEF3* Gene in the Discovery Population**  
Different colors represent the strength of LD according to the scale shown on the right.

A role of the RhoGEFs such as *ARHGEF3* is to activate RhoGTPases, key actin-dynamics regulators that cycle between an inactive GDP-bound and an active GTP-bound state. RhoGEFs achieve this by catalyzing the exchange of GDP for GTP through stabilization of the nucleotide-free state.<sup>42</sup> GTP then spontaneously binds and renders the protein active. RHOA has been implicated in osteoblast differentiation. McBeath et al.<sup>43</sup> demonstrated that RHOA expression committed human mesenchymal stem cells (hMSCs) to an osteoblastic fate, whereas expression of dominant-negative RHOA caused adipogenesis. These effects even overrode the presence of differentiation factors in the media and are thought to occur through effects on cytoskeletal tension.<sup>43</sup> Meyers et al.<sup>24</sup> found that overexpression of RHOA restored actin cytoskeletal arrangement, enhanced the expression of osteoblastic genes, and suppressed the expression of adipocytic genes in hMSCs cultured in modeled microgravity (MMG). Interestingly, it was found that the quantity of activated RHOA dropped

by  $88\% \pm 2\%$  in hMSCs cultured in MMG, whereas the total expression of RHOA did not change significantly,<sup>24</sup> indicating that the RhoGEFs responsible for activating RHOA could have had a role in the effect.

Osteoclasts are highly motile cells that rely on rapid changes to their cytoskeleton to achieve the movement and attachment that is required for bone resorption.<sup>44–47</sup> Chelalaiah et al.<sup>23</sup> identified RHOA as playing a major role in this process. By transducing active and inactive RHOA into avian osteoclasts, they demonstrated that the protein is essential for podosome assembly, stress-fiber formation, osteoclast motility, and bone resorption.<sup>23</sup> In addition, farnesyl pyrophosphate synthase, the specific target of nitrogen-containing bisphosphonates widely used in the treatment of osteoporosis, is essential for the prenylation and therefore activity of RhoGTPases including RHOA.<sup>48</sup> Bisphosphonates cause loss of osteoclast activity and induction of apoptosis, possibly through the inactivation of RhoGTPases.<sup>49</sup> The known interaction of *ARHGEF3* with RHOA and potential role of *ARHGEF3* in that signal-transduction pathway strongly suggest that variation in *ARHGEF3* is associated with regulated function in bone cells—probably osteoclasts. Osteoclasts treated with dexamethasone showed prolonged longevity with decreased spreading, actin ring formation, and bone-degrading activity as the result of altered cytoskeletal organization.<sup>50</sup> This may be mediated by arrested macrophage colony-stimulating factor (M-CSF)-stimulated activation of

**Table 4. BMD Z Scores for *ARHGEF3* 3-SNP Haplotypes**

BMD Z Score Phenotype	Haplotype (rs4681928, rs1110866, rs7646054)						p Value
	AAA Allele	GAA Allele	AAG Allele	GAG Allele	ACG Allele	GCG Allele	
Femoral neck	$-0.341 \pm 1.188$ (588) <sup>c</sup>	$-0.205 \pm 1.072$ (81)	$-0.408 \pm 1.129$ (239)	$-0.359 \pm 0.781$ (65)	$-0.376 \pm 1.162$ (374)	$-0.358 \pm 0.974$ (144) <sup>a</sup>	0.003
	Haplotype (rs4681928, rs7646054, rs3772219)						
	AAT Allele	AGT Allele	GGT Allele	AAG Allele	AGG Allele	GGG Allele	p Value
Spine	$-0.573 \pm 1.299$ (375) <sup>a</sup>	$-0.677 \pm 1.378$ (450) <sup>a</sup>	$-0.725 \pm 1.161$ (151) <sup>b</sup>	$-0.720 \pm 1.187$ (228) <sup>a</sup>	$-0.665 \pm 1.171$ (177)	$-0.722 \pm 0.864$ (61)	0.002

Results are given as mean  $\pm$  SD (number of alleles contributing to the mean), derived from QPDTPHASE. <sup>a</sup>  $p < 0.05$ , <sup>b</sup>  $p < 0.01$ , and <sup>c</sup>  $p < 0.0004$  for individual haplotypes compared to all others.

**Table 5. Osteodensitometry Parameters and Fracture Rate in Relation to the Genotype Distribution of rs7646054 in the Replication Cohort**

Phenotype	AA Genotype	AG Genotype	GG genotype	p Value
Total-hip DXA BMD (mg/cm <sup>2</sup> )	889 ± 128 <sup>a</sup> (150)	872 ± 128 <sup>a</sup> (354)	857 ± 122 <sup>b</sup> (252)	0.013
Femoral-neck DXA BMD (mg/cm <sup>2</sup> )	759 ± 115 <sup>a</sup> (150)	753 ± 120 <sup>a</sup> (354)	735 ± 117 <sup>b</sup> (252)	0.038
Spine DXA BMD (mg/cm <sup>2</sup> )	967 ± 172 <sup>a</sup> (150)	969 ± 148 <sup>a</sup> (356)	934 ± 152 <sup>b</sup> (253)	0.007
Prevalent fracture (%)	36.4 (151)	29.1 (358)	39.1 (253)	0.026

Results are given as mean ± SD (number of measurements). The BMD data are adjusted for age and weight. <sup>a</sup> is significantly different from <sup>b</sup> in posthoc analysis (p < 0.05).

certain RhoGEFs and RhoGTPases including RHOA.<sup>50</sup> Finally, RHOB has a role in cartilage biology, having been linked to osteoarthritis, in which a chondrocyte disorder plays a major role.<sup>25,26</sup>

In conclusion, we have shown that genetic variation within the *ARHGEF3* gene is associated with variation in BMD in Caucasian women. There is a significant amount of evidence to suggest that the two RhoGTPases that are specifically activated by *ARHGEF3*, RHOA, and RHOB have a role in bone cell biology. Furthermore, there is evidence of a skeletal phenotype for mutations in an associated gene, *FGD1*, which regulates the RhoGTPase CDC42 (MIM 116952).

Further research needs to be conducted to determine the primary *ARHGEF3* splice variant in bone cells and the mechanism by which the identified polymorphism, rs7646054 (or a SNP in strong LD with it), affects the *ARHGEF3* protein. However, the data presented here raise the possibility that variation in the RhoGTPase pathway of cell regulation may represent new targets for pharmaceutical development in the same way that the discovery of the importance of the Wnt signaling pathway involving LRP5 in skeletal function has led to new understanding of and potential new therapy for osteoporosis.<sup>51</sup>

### Acknowledgments

The Australian study was supported by the National Health and Medical Research Council of Australia, Grants 294402 and 343603. We would also like to thank the staff and women of the Chingford study, particularly Dr. Doyle and Dr. Hakim. Research in the UK was supported in part by the Arthritis and Rheumatism Council, St. Thomas' Hospital Special Trustees, and the Wellcome Trust.

Received: February 14, 2008

Revised: April 16, 2008

Accepted: April 30, 2008

Published online: May 22, 2008

### Web Resources

The URLs for data presented herein are as follows:

BLASTp, <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>

dbSNP, <http://www.ncbi.nlm.nih.gov/SNP/>

Ensembl Genome Browser, <http://www.ensembl.org/index.html/>

Genetic Power Calculator, <http://pngu.mgh.harvard.edu/~purcell/gpc/>

International HapMap Project, <http://www.hapmap.org/>

NCBI, <http://www.ncbi.nlm.nih.gov/>

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/>

Perlegen Genome Browser Version 1, [http://genome.perlegen.com/browser/index\\_v1.html](http://genome.perlegen.com/browser/index_v1.html)

Primer3, <http://frodo.wi.mit.edu/>

UCSC Genome Bioinformatics, <http://genome.ucsc.edu/>

### References

1. Kanis, J.A., Melton, L.J. 3rd, Christiansen, C., Johnston, C.C., and Khaltav, N. (1994). The diagnosis of osteoporosis. *J. Bone Miner. Res.* 9, 1137–1141.
2. Prince, R.L., and Dick, I. (1997). Oestrogen effects on calcium membrane transport: A new view of the inter-relationship between oestrogen deficiency and age-related osteoporosis. *Osteoporos. Int.* 7 (Suppl 3), S150–S154.
3. Flicker, L., Hopper, J.L., Rodgers, L., Kaymakci, B., Green, R.M., and Wark, J.D. (1995). Bone density determinants in elderly women: A twin study. *J. Bone Miner. Res.* 10, 1607–1613.
4. Krall, E.A., and Dawson-Hughes, B. (1993). Heritable and lifestyle determinants of bone mineral density. *J. Bone Miner. Res.* 8, 1–9.
5. Michaelsson, K., Melhus, H., Ferm, H., Ahlbom, A., and Pederesen, N.L. (2005). Genetic liability to fractures in the elderly. *Arch. Intern. Med.* 165, 1825–1830.
6. Pocock, N.A., Eisman, J.A., Hopper, J.L., Yeates, M.G., Sambrook, P.N., and Eberl, S. (1987). Genetic determinants of bone mass in adults. A twin study. *J. Clin. Invest.* 80, 706–710.
7. Evans, R.A., Marel, G.M., Lancaster, E.K., Kos, S., Evans, M., and Wong, S.Y. (1988). Bone mass is low in relatives of osteoporotic patients. *Ann. Intern. Med.* 109, 870–873.
8. Smith, D.M., Nance, W.E., Kang, K.W., Christian, J.C., and Johnston, C.C. Jr. (1973). Genetic factors in determining bone mass. *J. Clin. Invest.* 52, 2800–2808.
9. Seeman, E., Hopper, J.L., Young, N.R., Formica, C., Goss, P., and Tsalamandris, C. (1996). Do genetic factors explain associations between muscle strength, lean mass, and bone density? A twin study. *Am. J. Physiol.* 270, E320–E327.
10. Young, D., Hopper, J.L., Nowson, C.A., Green, R.M., Sherwin, A.J., Kaymakci, B., Smid, M., Guest, C.S., Larkins, R.G., and Wark, J.D. (1995). Determinants of bone mass in 10- to 26-year-old females: A twin study. *J. Bone Miner. Res.* 10, 558–567.
11. Deng, H.W., Xu, F.H., Huang, Q.Y., Shen, H., Deng, H., Conway, T., Liu, Y.J., Liu, Y.Z., Li, J.L., Zhang, H.T., et al. (2002). A whole-genome linkage scan suggests several genomic regions potentially containing quantitative trait Loci for osteoporosis. *J. Clin. Endocrinol. Metab.* 87, 5151–5159.

12. Devoto, M., Shimoya, K., Caminis, J., Ott, J., Tenenhouse, A., Whyte, M.P., Sereda, L., Hall, S., Considine, E., Williams, C.J., et al. (1998). First-stage autosomal genome screen in extended pedigrees suggests genes predisposing to low bone mineral density on chromosomes 1p, 2p and 4q. *Eur. J. Hum. Genet.* *6*, 151–157.
13. Devoto, M., Specchia, C., Li, H.H., Caminis, J., Tenenhouse, A., Rodriguez, H., and Spotila, L.D. (2001). Variance component linkage analysis indicates a QTL for femoral neck bone mineral density on chromosome 1p36. *Hum. Mol. Genet.* *10*, 2447–2452.
14. Kammerer, C.M., Schneider, J.L., Cole, S.A., Hixson, J.E., Samollow, P.B., O'Connell, J.R., Perez, R., Dyer, T.D., Almasy, L., Blangero, J., et al. (2003). Quantitative trait loci on chromosomes 2p, 4p, and 13q influence bone mineral density of the forearm and hip in Mexican Americans. *J. Bone Miner. Res.* *18*, 2245–2252.
15. Karasik, D., Myers, R.H., Cupples, L.A., Hannan, M.T., Gagnon, D.R., Herbert, A., and Kiel, D.P. (2002). Genome screen for quantitative trait loci contributing to normal variation in bone mineral density: The Framingham Study. *J. Bone Miner. Res.* *17*, 1718–1727.
16. Koller, D.L., Econs, M.J., Morin, P.A., Christian, J.C., Hui, S.L., Parry, P., Curran, M.E., Rodriguez, L.A., Conneally, P.M., Joslyn, G., et al. (2000). Genome screen for QTLs contributing to normal variation in bone mineral density and osteoporosis. *J. Clin. Endocrinol. Metab.* *85*, 3116–3120.
17. Ralston, S.H., Galwey, N., MacKay, I., Albagha, O.M., Cardon, L., Compston, J.E., Cooper, C., Duncan, E., Keen, R., Langdahl, B., et al. (2005). Loci for regulation of bone mineral density in men and women identified by genome wide linkage scan: The FAMOS study. *Hum. Mol. Genet.* *14*, 943–951.
18. Shen, H., Zhang, Y.Y., Long, J.R., Xu, F.H., Liu, Y.Z., Xiao, P., Zhao, L.J., Xiong, D.H., Liu, Y.J., Dvornyk, V., et al. (2004). A genome-wide linkage scan for bone mineral density in an extended sample: Evidence for linkage on 11q23 and Xq27. *J. Med. Genet.* *41*, 743–751.
19. Wilson, S.G., Reed, P.W., Bansal, A., Chiano, M., Lindersson, M., Langdown, M., Prince, R.L., Thompson, D., Thompson, E., Bailey, M., et al. (2003). Comparison of genome screens for two independent cohorts provides replication of suggestive linkage of bone mineral density to 3p21 and 1p36. *Am. J. Hum. Genet.* *72*, 144–155.
20. Wynne, F., Drummond, F.J., Daly, M., Brown, M., Shanahan, F., Molloy, M.G., and Quane, K.A. (2003). Suggestive linkage of 2p22–25 and 11q12–13 with low bone mineral density at the lumbar spine in the Irish population. *Calcif. Tissue Int.* *72*, 651–658.
21. Ioannidis, J.P., Ng, M.Y., Sham, P.C., Zintzaras, E., Lewis, C.M., Deng, H.W., Econs, M.J., Karasik, D., Devoto, M., Kammerer, C.M., et al. (2007). Meta-analysis of genome-wide scans provides evidence for sex- and site-specific regulation of bone mass. *J. Bone Miner. Res.* *22*, 173–183.
22. Arthur, W.T., Ellerbroek, S.M., Der, C.J., BurrIDGE, K., and Wenerberg, K. (2002). XPLN, a guanine nucleotide exchange factor for RhoA and RhoB, but not RhoC. *J. Biol. Chem.* *277*, 42964–42972.
23. Chellaiah, M.A., Soga, N., Swanson, S., McAllister, S., Alvarez, U., Wang, D., Dowdy, S.F., and Hruska, K.A. (2000). Rho-A is critical for osteoclast podosome organization, motility, and bone resorption. *J. Biol. Chem.* *275*, 11993–12002.
24. Meyers, V.E., Zayzafoon, M., Douglas, J.T., and McDonald, J.M. (2005). RhoA and cytoskeletal disruption mediate reduced osteoblastogenesis and enhanced adipogenesis of human mesenchymal stem cells in modeled microgravity. *J. Bone Miner. Res.* *20*, 1858–1866.
25. Gebhard, P.M., Soder, S., Bau, B., and Aigner, T. (2004). Down-regulation of the GTPase RhoB might be involved in the pre-apoptotic phenotype of osteoarthritic chondrocytes. *Front. Biosci.* *9*, 827–833.
26. Mahr, S., Burmester, G.R., Hilke, D., Gobel, U., Grutzkau, A., Haupl, T., Hauschild, M., Koczan, D., Krenn, V., Neidel, J., et al. (2006). Cis- and trans-acting gene regulation is associated with osteoarthritis. *Am. J. Hum. Genet.* *78*, 793–803.
27. Orrico, A., Galli, L., Obregon, M.G., de Castro Perez, M.F., Falciani, M., and Sorrentino, V. (2007). Unusually severe expression of craniofacial features in Aarskog-Scott syndrome due to a novel truncating mutation of the FGD1 gene. *Am. J. Med. Genet. A.* *143*, 58–63.
28. Arden, N.K., Griffiths, G.O., Hart, D.J., Doyle, D.V., and Spector, T.D. (1996). The association between osteoarthritis and osteoporotic fracture: The Chingford Study. *Br. J. Rheumatol.* *35*, 1299–1304.
29. Hart, D.J., and Spector, T.D. (1993). The relationship of obesity, fat distribution and osteoarthritis in women in the general population: The Chingford Study. *J. Rheumatol.* *20*, 331–335.
30. Keen, R.W., Hart, D.J., Arden, N.K., Doyle, D.V., and Spector, T.D. (1999). Family history of appendicular fracture and risk of osteoporosis: A population-based study. *Osteoporos. Int.* *10*, 161–166.
31. Johns, M.B. Jr., and Paulus-Thomas, J.E. (1989). Purification of human genomic DNA from whole blood using sodium perchlorate in place of phenol. *Anal. Biochem.* *180*, 276–278.
32. Illumina (2004) Golden Gate Assay: Technical Specifications and Operation. Illumina Technical Bulletin Pub. No. 370–2004–006.
33. Mullin, B.H., Wilson, S.G., Islam, F.M., Calautti, M., Dick, I.M., Devine, A., and Prince, R.L. (2005). Klotho gene polymorphisms are associated with osteocalcin levels but not bone density of aged postmenopausal women. *Calcif. Tissue Int.* *77*, 145–151.
34. Hinds, D.A., Stuve, L.L., Nilsen, G.B., Halperin, E., Eskin, E., Ballinger, D.G., Frazer, K.A., and Cox, D.R. (2005). Whole-genome patterns of common DNA variation in three human populations. *Science* *307*, 1072–1079.
35. Laird, N.M., Horvath, S., and Xu, X. (2000). Implementing a unified approach to family-based tests of association. *Genet. Epidemiol.* *19* (Suppl 1), S36–S42.
36. Dudbridge, F. (2003). Pedigree disequilibrium tests for multilocus haplotypes. *Genet. Epidemiol.* *25*, 115–121.
37. Carter, K.W., McCaskie, P.A., and Palmer, L.J. (2006). JLIN: A java based linkage disequilibrium plotter. *BMC Bioinformatics* *7*, 60.
38. Abecasis, G.R., and Cookson, W.O. (2000). GOLD—graphical overview of linkage disequilibrium. *Bioinformatics* *16*, 182–183.
39. Marshall, D., Johnell, O., and Wedel, H. (1996). Meta-analysis of how well measures of bone mineral density predict occurrence of osteoporotic fractures. *BMJ* *312*, 1254–1259.
40. Purcell, S., Cherny, S.S., and Sham, P.C. (2003). Genetic Power Calculator: Design of linkage and association genetic mapping studies of complex traits. *Bioinformatics* *19*, 149–150.

41. Schmidt, A., and Hall, A. (2002). Guanine nucleotide exchange factors for Rho GTPases: Turning on the switch. *Genes Dev.* *16*, 1587–1609.
42. Rossman, K.L., Der, C.J., and Sondek, J. (2005). GEF means go: Turning on RHO GTPases with guanine nucleotide-exchange factors. *Nat. Rev. Mol. Cell Biol.* *6*, 167–180.
43. McBeath, R., Pirone, D.M., Nelson, C.M., Bhadriraju, K., and Chen, C.S. (2004). Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Dev. Cell* *6*, 483–495.
44. Kanehisa, J., and Heersche, J.N. (1988). Osteoclastic bone resorption: In vitro analysis of the rate of resorption and migration of individual osteoclasts. *Bone* *9*, 73–79.
45. Lakkakorpi, P.T., Helfrich, M.H., Horton, M.A., and Vaananen, H.K. (1993). Spatial organization of microfilaments and vitronectin receptor, alpha v beta 3, in osteoclasts. A study using confocal laser scanning microscopy. *J. Cell Sci.* *104*, 663–670.
46. Lakkakorpi, P.T., and Vaananen, H.K. (1991). Kinetics of the osteoclast cytoskeleton during the resorption cycle in vitro. *J. Bone Miner. Res.* *6*, 817–826.
47. Lakkakorpi, P.T., and Vaananen, H.K. (1996). Cytoskeletal changes in osteoclasts during the resorption cycle. *Microsc. Res. Tech.* *33*, 171–181.
48. Reszka, A.A., and Rodan, G.A. (2004). Nitrogen-containing bisphosphonate mechanism of action. *Mini Rev. Med. Chem.* *4*, 711–719.
49. Denoyelle, C., Hong, L., Vannier, J.P., Soria, J., and Soria, C. (2003). New insights into the actions of bisphosphonate zoledronic acid in breast cancer cells by dual RhoA-dependent and -independent effects. *Br. J. Cancer* *88*, 1631–1640.
50. Kim, H.J., Zhao, H., Kitaura, H., Bhattacharyya, S., Brewer, J.A., Muglia, L.J., Ross, F.P., and Teitelbaum, S.L. (2006). Glucocorticoids suppress bone formation via the osteoclast. *J. Clin. Invest.* *116*, 2152–2160.
51. Krishnan, V., Bryant, H.U., and Macdougald, O.A. (2006). Regulation of bone mass by Wnt signaling. *J. Clin. Invest.* *116*, 1202–1209.