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Integrative Genomic Analysis Predicts Novel Functional Enhancer-SNPs for Bone Mineral Density

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Abstract

Osteoporosis is a skeletal disorder characterized by low bone mineral density (BMD) and deterioration of bone microarchitecture. To identify novel genetic loci underlying osteoporosis, an effective strategy is to focus on scanning of variants with high potential functional impacts. Enhancers play a crucial role in regulating cell type-specific transcription. Therefore, single nucleotide polymorphisms (SNPs) located in enhancers (enhancer-SNPs) may represent strong candidate functional variants. Here, we performed a targeted analysis for potential functional enhancer-SNPs that may affect gene expression and biological processes in bone-related cells, specifically, osteoblasts and peripheral blood monocytes (PBMs), using five independent cohorts (n = 5,905) and the Genetics Factors for Osteoporosis (GEFOS) summary statistics, followed by comprehensive integrative genomic analyses of chromatin states, transcription, and metabolites. We identified 15 novel enhancer-SNPs associated with femoral neck and lumbar spine BMD, including 5 SNPs mapped to novel genes (e.g., *rs10840343* and *rs10770081* in *IGF2* gene) and 10 novel SNPs mapped to known BMD-associated genes (e.g., *rs2941742* in *ESR1* gene, *rs10249092* and *rs4342522* in *SHFM1* gene). Interestingly, enhancer-SNPs *rs10249092* and *rs4342522* in *SHFM1* were tightly linked but annotated to different enhancers in PBMs and osteoblasts respectively, suggesting even tightly linked SNPs may regulate the same target gene and contribute to the phenotype variation in cell type-specific manners. Importantly, 10 enhancer-SNPs may also regulate BMD variation by affecting the serum metabolite levels. Our findings revealed novel susceptibility loci that may regulate BMD variation and provided intriguing insights into the genetic mechanisms of osteoporosis.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no competing interests.

Keywords

Osteoporosis; BMD; Meta-analysis; Enhancer-SNPs; Integrative Genomic Analysis

Introduction

Osteoporosis is a major skeletal disorder characterized by deterioration of bone microarchitecture and low bone mineral density (BMD), resulting in increased skeletal fragility and fracture susceptibility (Kanis 2002). The prevalence of osteoporosis is over 20% in the United States (Wright et al. 2014), and it has become an increasingly serious public health issue in the aging population. Previous studies have revealed a strong genetic determination of BMD variation, with heritability ranging from 50% to 85% (Ralston and de Crombrughe 2006; Ralston and Uitterlinden 2010). Currently, over 200 loci have been reported to be associated with BMD at various skeletal sites (Chuan Qiu 2011; Estrada et al. 2012; Guo et al. 2010; Kemp et al. 2017; Koller et al. 2013; Medina-Gomez et al. 2018; Zhang et al. 2014; Zheng et al. 2015). Nevertheless, these loci only accounted for approximately 12% of BMD variation (Kemp et al. 2017) and the specific functional variants at these loci were generally unknown. To identify additional genetic loci and expand our understanding of the molecular basis of BMD variation, an effective strategy is to focus on testing of specific single nucleotide polymorphisms (SNPs) with high potential functional impacts, such as exonic/nonsynonymous SNPs (Kim et al. 2018; Zheng et al. 2015) and SNPs that could potentially affect regulatory factors (Koues et al. 2015; Lei et al. 2011; Niu et al. 2016; Qiu et al. 2018; Takata et al. 2016). Such approach can alleviate the multiple-testing problem of traditional genome-wide association study (GWAS) and therefore increase the power to identify novel susceptibility loci for the phenotype of interest. Furthermore, using information on prior functional evidence may help to reduce the false positive results and improve the interpretability of the results.

Enhancers are one of the major regulatory components of the genome and play a central role in regulating gene expression in a cell-type and cell-state specific manner. Interactions between enhancers and their targets may occur on the same chromosome or on different chromosomes (Muller and Schaffner 1990; Sasaki-Iwaoka et al. 1999). It was estimated that approximately 1 million putative enhancers exist in the human genome and distinct sets of ~30,000–40,000 enhancers were active in any given particular cell type (Buecker and Wysocka 2012; Xie and Ren 2013), which far exceed the number of protein-coding genes. Recent studies have shown that disease-/trait-associated variants identified through GWAS were significantly enriched in noncoding regulatory elements, particularly in putative cell type-specific enhancers (Corradin et al. 2014; Karczewski et al. 2013; Maurano et al. 2012). The SNPs in enhancers (enhancer-SNPs) may influence transcriptional output, thereby offering a mechanistic basis to explain their association with risk for many common diseases.

In this study, we performed a targeted genome-wide meta-analysis for BMD on enhancer-SNPs, followed by comprehensive integrative genomic analysis. As enhancer profiles are often cell type-specific (Heinz et al. 2015), we further narrowed down to the SNPs that are in

enhancers of bone-related cells, specifically, osteoblasts and peripheral blood monocytes (PBMs). Osteoblasts are bone-forming cells, which produce and secrete matrix proteins and transport mineral into the matrix. PBMs can act as osteoclast precursors (Fujikawa et al. 1996; Lari et al. 2009; Matayoshi et al. 1996). It can also secrete several cytokines essential for osteoclast differentiation and function, and represent a major target cell of sex hormones for bone metabolism (Higuchi et al. 1998; Komano et al. 2006). PBMs have been demonstrated as an excellent cellular model for investigating osteoporosis-associated gene/protein expression profiles and their regulatory mechanisms (Durand et al. 2013; Farber 2010; Guo et al. 2014; Kotani et al. 2013; Leung et al. 2011; Matsubara et al. 2012; Mori et al. 2008; Park et al. 2012; Soltanoff et al. 2009; Sung et al. 2009; Yang et al. 2013; Zhou et al. 2015). Therefore, the enhancer-SNPs in osteoblasts and PBMs represent prominent functional candidates for regulating BMD variation and osteoporosis risks.

Materials and Methods

Study Populations

The meta-analysis dataset comprised 5,905 subjects from five independent GWAS cohorts, of which three studies were “in-house” studies: Omaha Osteoporosis Study (OOS, $n = 987$) and Kansas-City Osteoporosis Study (KCOS, $n = 2,250$) with Caucasian Ancestry, China Osteoporosis Study (COS, $n = 1,547$) with Han Chinese Ancestry, and two “external” studies retrieved from the Database on Genotypes and Phenotypes (dbGaP) (<http://www.ncbi.nlm.nih.gov/gap/>): Women’s Health Initiative Observational Study with African Ancestry (WHI-AA, $n = 712$) and Hispanic Ancestry (WHI-HIS, $n = 409$). The characteristics of the five study populations are shown in Supplementary Table 1. These studies were approved by respective institutional ethics committees and all subjects provided written informed consent.

The replication dataset was obtained from the Genetic Factors for Osteoporosis Consortium (GEFOS), which included summary statistics of approximately 10 million SNPs for association with BMD (Zheng et al. 2015). GEFOS study is one of the largest meta-analysis study for BMD association, which included 2,882 subjects with whole-genome sequencing, 3,549 subjects with whole-exome sequencing, 26,534 genotyped subjects with deep imputation using a combined UK10K/1000 Genomes reference panel, and 20,271 subjects with *de novo* replication genotyping (Zheng et al. 2015).

Phenotype Measurements and Modeling

The dual-energy X-ray absorptiometry (DXA) scanners (Lunar Corp., USA or Hologic Inc., USA) were used to measure BMD at the femoral neck and the lumbar spine (L1-L4) according to the manufacturer’s protocols. In each individual GWAS, covariates (sex, age, age², height, weight, and scanner ID) were tested by a linear regression model with stepwise forward selection. Significant covariates were used to adjust the measurements of raw BMD. Residual phenotypes after adjustments were normalized by inverse quantile of the standard normal distribution and analyzed subsequently for SNP association.

Genotyping Imputation and Quality Control

Subjects from the five GWAS datasets for the meta-analysis were genotyped by high-throughput SNP genotyping arrays (Affymetrix Inc., USA; or Illumina Inc., USA) according to respective manufacturer's protocols. Quality control was implemented in PLINK (<http://pngu.mgh.harvard.edu/~purcell/plink/>) with the following criteria: individual missingness < 5%, SNP call rate > 95%, and Hardy-Weinberg equilibrium (HWE) p-value > 1.00×10^{-5} . To correct for potential population stratification and monitor population outliers, principal component analyses (PCA) were performed based on the genome-wide genotype data, and the first five PCs (i.e., PC1-PC5) were adjusted as covariates in the association analysis. To achieve higher genome coverage, we performed extensive genotype imputation. Briefly, we used Markov Chain Haplotyping algorithm (MACH) (Li et al. 2010) to construct the haplotypes in each individual GWAS. Then based on phased haplotypes, we imputed the untyped genotypes by Minimac (Howie et al. 2012) using the haplotype data from the 1000 Genomes Project Consortium as reference panel (Genomes Project et al. 2010). For each individual GWAS, genotypes for untyped SNPs were imputed based on haplotype reference panel of relevant population. SNPs with *imputation quality score* larger than 0.3 in at least two studies and minor allele frequency (MAF) > 0.05 in at least one study were included for subsequent analyses. Strand orientations were checked prior to genotype imputation. Imputation results were summarized as an 'allele dosage' defined as the expected number of copies of the coded allele at that SNP (i.e., a fractional value between 0 and 2) for each genotype. Imputation with the 1000 Genomes Project reference panel generated genotype data for more than 11.2 million SNPs.

Selecting Potential Functional Enhancer-SNPs

The enhancer-SNPs that are potentially functional in osteoblasts and PBMs were selected according to the following steps: 1) we retrieved enhancer elements (EnhG1/2, genic enhancers; EnhA1/2, active enhancers; and EnhWk, weak enhancers) in osteoblasts and PBMs based on the 18-state ChromHMM annotation of the human genome by the Roadmap Epigenomics Project (Roadmap Epigenomics et al. 2015); 2) intersected the genomic coordinates of retrieved enhancers with SNPs cataloged in the 1000 Genomes Project reference panel (Genomes Project et al. 2010) and our in-house whole-genome deep re-sequencing study (Shen et al. 2013).

Association Tests & Meta-analyses

Additive genetic model was used to test the association between directly-typed or imputed SNPs and BMD. For the unrelated subjects in each GWAS, the association was examined by a linear regression model with MACH2QTL (Li et al. 2010), in which allele dosage was used as a predictor of phenotype. We estimated the genomic inflation factor (λ_{GC}) (Bacanu et al. 2000) in each study. Meta-analysis was performed under weighted fixed-effect model using METAL (Willer et al. 2010), in which the weights were proportional to the square root of the sample size. *Cochran's* Q statistic and $\hat{I}^2$ were calculated as measures of between-study heterogeneity (Higgins et al. 2003). SNPs with high heterogeneity (Q statistic p-value of < 0.05 or $\hat{I}^2$ value > 50%) were analyzed by random-effect model under the standard

procedure in the METASOFT (Han and Eskin 2011). Genome-wide significance was defined as a p-value less than 5.00×10^{-8} .

Integrative Genomic Analysis of the Enhancer-SNPs

The enhancer-SNPs were annotated to candidate target genes using SNPnexus (Chelala et al. 2009) under NCBI RefSeq gene annotation system (Pruitt et al. 2007). We applied HaploReg (Ward and Kellis 2012) to further explore the potential functional importance of the identified enhancer-SNPs on regulatory chromatin states across diverse cell types, predicted effects on transcription factor binding, and the effects on gene expression (eQTL hits). To evaluate the sequence evolutionary conservation, we obtained PhyloP score for each enhancer-SNP from the UCSC Genome Browser (Kent et al. 2002). We also used 3DSNP (Lu et al. 2017) to explore the potential long-distance regulation of the identified enhancer-SNPs. Analysis of the linkage disequilibrium (LD) around susceptibility enhancer-SNPs was performed by SNIPI (Arnold et al. 2015) with the 1000 Genomes reference panel (Phase 3 v5 under European population). To evaluate whether the novel enhancer-SNPs can contribute to variation in serum metabolite levels, we retrieved metabolite datasets with summary statistics from three independent GWAS for serum metabolites (Draisma et al. 2015; Shin et al. 2014; Suhre et al. 2011). The program GOEAST (Zheng and Wang 2008) was employed to identify significantly enriched gene ontology (GO) terms among list of genes associated with identified enhancer-SNPs.

Results

Characterization of Enhancer Elements in Osteoblasts and PBMs

In this study, we obtained a total of 218,012 and 140,297 putative enhancers in osteoblast and PBMs, respectively, including 55,445 partially and 5,019 completely overlapped enhancers common in both cell types (Supplementary Table 2). Interestingly, we observed a significant (Yates corrected Chi-square test, p-value < 0.0001) enrichment of active enhancers and underrepresentation of genic enhancers in osteoblasts when compared to the distribution of PBM enhancers (Supplementary Figure 1). Since the active and genic enhancers were distinguished based on different histone modification profiles, our results suggested enhancers in different cells may be under distinct epigenomic controls.

Novel Susceptibility Enhancer-SNPs for BMD

In the osteoblast and PBM enhancers, we identified a total of ~1.75 million enhancer-SNPs, among which ~1.58 million SNPs had qualified genotype data (genotyped + imputed) and were subsequently tested for association with BMD in five independent GWAS cohorts (i.e., OOS, KCOS, COS, WHI-AA, and WHI-HIS) with a total of 5,905 subjects. The estimates of genomic inflation factor λ_{GC} ranged from 0.97 to 1.02 in individual GWAS.

Enhancer-SNPs for Femoral Neck BMD —In the meta-analysis combining the five GWAS datasets, no enhancer-SNPs were found to be associated with femoral neck BMD at the genome-wide significance level (p-value < 5.00×10^{-8}). However, we observed 44 enhancer-SNPs achieved suggestive genome-wide significance level (p-value < 5.00×10^{-5}) for association with femoral neck BMD (Supplementary Table 3). We then performed *in*

silico replication for these suggestive significant enhancer-SNPs and successfully replicated (p-value < 0.05) 8 enhancer-SNPs in the GEFOS cohort (Zheng et al. 2015) (Table 1) including a known enhancer-SNP *rs1430738* in gene *WLS*, 5 novel enhancer-SNPs (*rs6465531*, *rs10278858*, *rs10249092*, *rs10273072*, *rs4342522*) in gene *SHFM1*, and 2 novel enhancer-SNPs (*rs10840343*, *rs10770081*) in gene *IGF2*. Subsequently, a joint analysis in both the discovery and the replication cohorts yielded genome-wide significant association with femoral neck BMD at 6 enhancer-SNPs (Table 1), including the 5 novel enhancer-SNPs clustered in the genomic regions encompassing *SHFM1* gene (*rs6465531*, p-value_{joint} = 1.32×10^{-11} ; *rs10278858*, p-value_{joint} = 2.21×10^{-9} ; *rs10249092*, p-value_{joint} = 2.04×10^{-11} ; *rs10273072*, p-value_{joint} = 6.74×10^{-9} ; and *rs4342522*, p-value_{joint} = 3.37×10^{-12}) (Figure 1A) and the known enhancer-SNP *rs1430738* (p-value_{joint} = 1.56×10^{-9}) in gene *WLS*. Interestingly, *SHFM1* SNPs *rs10249092* and *rs4342522* were tightly linked ($r^2 = 1$ in European population) (Supplementary Table 4) but were located in two different enhancers in PBMs and osteoblasts, respectively (Figure 2, Supplementary Table 3), suggesting that even tightly linked SNPs may regulate the same target gene and contribute to the phenotype variation in cell type-specific manners.

Enhancer-SNPs for Lumbar Spine BMD — Similar to the results for the femoral neck BMD, no enhancer-SNPs showed association with lumbar spine BMD at the genome-wide significance level, but 97 enhancer-SNPs achieved suggestive genome-wide significance (Supplementary Table 3). By using the GEFOS cohort, we successfully replicated (p-value < 0.05) 25 enhancer-SNPs for lumbar spine BMD (Table 2), including 8 novel enhancer-SNPs (annotated to genes *SMOC1*, *AFF3*, *MPP7*, *ESR1*, *NGF*, and *SHFM1*, respectively). Notably, 12 enhancer-SNPs were clustered in the *TNFRSF11B-COLEC10* region (Figure 1B) and 4 enhancer-SNPs were mapped in the *CCDC170-ESR1* region (Supplementary Figure 2). Interestingly, several lumbar spine BMD-associated enhancer-SNPs were tightly linked ($r^2 > 0.8$) and mapped to the same enhancer in the same cell type (Supplementary Table 3 & 4), such as osteoblast enhancer-SNPs *rs2062377* and *rs2220189* ($r^2 = 0.92$ in European population) in the *TNFRSF11B-COLEC10* region, suggesting that multiple neighboring enhancer-SNPs may synergistically mediate the expression of the target genes by affecting the same regulatory element. Subsequent joint analysis of the discovery and the replication cohorts showed that 4 novel enhancer-SNPs were associated with lumbar spine BMD at the genome-wide significance level (Table 2), including *rs1471243* (p-value_{joint} = 5.66×10^{-9}) and *rs7151788* (p-value_{joint} = 9.52×10^{-9}) in gene *SMOC1*, *rs2941742* (p-value_{joint} = 2.34×10^{-9}) in gene *ESR1*, and *rs35494924* (p-value_{joint} = 4.26×10^{-9}) in gene *MPP7*.

Comprehensive Integrative Genomic Analysis

Functional Annotation of Enhancer-SNPs — To explore the potential functional importance of the identified susceptibility enhancer-SNPs, we first evaluated the chromatin states for regions containing the significant/suggestive enhancer-SNPs across multiple cell types. Specifically, the femoral neck BMD-associated SNPs *rs6465531* and *rs4342522* in *SHFM1* gene were in the region with weak transcription in osteoblasts, and SNPs *rs10249092*, *rs10278858* and *rs10273072* in *SHFM1* gene were in genic enhancers or the region with weak transcription in PBMs. The enhancer-SNPs in *IGF2* (*rs10840343*,

rs10770081) and *WLS* gene (*rs1430738*) were in the regions with active transcription in osteoblasts (Table 3). Among the 25 enhancer-SNPs for lumbar spine BMD, 21 SNPs with various enhancer types were identified from osteoblasts (Table 4). Notably, both enhancer-SNPs *rs1871859* and *rs4407910* were also reported in PBMs. The novel enhancer-SNPs *rs1471243* and *rs7151788* in *SMOC1* gene were in the region with active transcription in osteoblasts, and enhancer-SNPs in gene *AFF3* (*rs7609179*, *rs17023186*) and *NGF* (*rs11581489*) were annotated to regions with weak transcription in osteoblasts. We also observed three novel enhancer-SNPs in PBMs, enhancer-SNPs *rs2941742* (*ESR1*) and *rs10278858* (*SHFM1*) were in the regions with weak transcription and *rs35494924* (*MPP7*) were annotated to active enhancer (Table 4).

Next, we interrogate these enhancer-SNPs to other possible regulatory elements by using data from ENCODE, Roadmap Epigenomics, and GTEx projects through the HaploReg program (Ward and Kellis 2012). All 8 replicated enhancer-SNPs for femoral neck BMD were predicted to alter transcription factor binding motifs, and 7 of which were also reported as eQTLs in various tissue/cell types (Table 3). Four enhancer-SNPs *rs1430738*, *rs10249092*, *rs10273072*, and *rs4342522* were also related to circular RNA (circRNA) regulatory elements which might be a novel type of potential biomarkers or treatment targets for disease prognosis and therapy (Zhao et al. 2018). Among the 25 replicated enhancer-SNPs for lumbar spine BMD, 20 were predicted to alter the transcription factor binding motifs and 19 were reported as eQTLs in a variety of tissue/cell types (Table 4). These results highlighted the strong regulatory potential of these identified enhancer-SNPs.

Furthermore, we searched for genes that may be regulated by the identified enhancer-SNPs through three-dimensional (3D) chromatin looping and identified several interesting potential long-distance interactions between genes and SNP-containing enhancers. For example, the enhancer containing femoral neck BMD-associated SNP *rs10840343* may remotely interact with genes *H19*, *IGF2/IGF2-AS*, and *MRPL23* (Figure 3A). The other interesting example is enhancer encompassing SNP *rs35494924*, which may have distal interaction with gene *MKX* (Figure 3B). *MKX* encodes an IRX family-related homeobox protein and expresses in the embryonic progenitor cell populations of skeletal muscle, tendon, cartilage, and bone (Anderson et al. 2006; Anderson et al. 2009). In addition, we tested the evolutionary conservation of the identified enhancer-SNPs and found that the enhancer-SNP *rs10278858*, which was associated with both femoral neck and lumbar spine BMDs, was in a highly conserved region (phyloP score = 1, Table 3 & 4).

LD Analysis of Novel Enhancer-SNPs with Known BMD-associated SNPs —

Targeted analysis of putative functional variants may shed novel insights into the mechanisms underlying previously reported GWAS signals. Therefore, we investigated the LD relationships of the novel enhancer-SNPs identified in this study with the previously identified BMD-associated loci. Out of the 5 novel enhancer-SNPs in *SHFM1* locus associated with femoral neck BMD, none of them were in high LD (all $r^2 < 0.6$) with previously identified BMD-associated SNPs. To explore the possibility of secondary genome-wide significant association signals driven by the enhancer-SNPs, we performed GCTA-COJO conditional association analysis (Yang et al. 2012) conditioning on the 4 previously reported BMD-associated *SHFM1* SNPs, namely, *rs10429035* (Zhang et al.

2014), *rs4729260* (Rivadeneira et al. 2009), *rs4448201*, and *rs6965122* (Zheng et al. 2015). After adjusting the residual effect of previously reported BMD-associated SNPs, 3 novel *SHFM1* enhancer-SNPs achieved genome-wide significance (*rs6465531*, $p\text{-value}_{\text{conditional}} = 2.30 \times 10^{-8}$; *rs10278858*, $p\text{-value}_{\text{conditional}} = 2.03 \times 10^{-8}$; *rs10273072*, $p\text{-value}_{\text{conditional}} = 4.08 \times 10^{-8}$), implying these enhancer-SNPs may represent a largely independent signal from what was reported in previous GWAS. For the 8 novel enhancer-SNPs associated with lumbar spine BMD, SNPs *rs2941742* and *rs35494924* were in high LD ($r^2 > 0.8$) with several known BMD-associated SNPs (Table 5). Specifically, PBMs enhancer-SNP *rs2941742* was in high LD with 6 known BMD-associated SNPs, including 5 SNPs (*rs3020331*, *rs2941741*, *rs2941740*, *rs3020333*, and *rs3020335*) overlapped with quiescent/low chromatin state and one SNP *rs3020334* (also identified in this study) annotated to weak enhancer in PBMs. As quiescent/low segments are normally inactive for transcriptional regulation (Hoffman et al. 2013), therefore our findings suggested that the two enhancer-SNPs *rs2941742* and *rs3020334* were strong candidates of functional variants that may at least partially explain the previously observed associations between SNPs in the *CCDC170-ESR1* region and BMD variation. Similarly, 2 osteoblasts enhancer-SNPs *rs1471243* and *rs7151788* in *SMOC1* gene were in moderate LD ($r^2 = 0.75$) with a known BMD-associated SNP *rs227425*. Since SNP *rs227425* was not overlapped to any active transcriptional regulatory features (active chromatin states, active histone modification marks, and DNase I hypersensitive sites) in osteoblasts, we speculated the enhancer-SNPs *rs1471243* and *rs7151788* were more likely to be the functional variants in this region underlying the observed BMD association.

Enhancer-SNPs Contributing to Variation in Serum Metabolite Levels —

Metabolites are small molecules involved in cellular metabolism, which can reflect the physiological state of the body and may differ between individuals due to variation in genetic makeup and environmental exposure (Suhre and Gieger 2012). To further explore the biological function of the identified enhancer-SNPs, we assessed whether the enhancer-SNPs were also associated with variations in serum metabolite levels using data from recently published metabolite GWAS (Draisma et al. 2015; Shin et al. 2014; Suhre et al. 2011). We found that 10 BMD-associated enhancer-SNPs showed suggestive genome-wide significant association with various serum metabolites (Table 6). Notably, some of the identified metabolites, e.g. phosphatidylcholines, levulinate (4-oxovalerate), and dimethylarginine (SDMA + ADMA), have been linked to bone metabolism (Han et al. 2003; Xiao et al. 2001). For example, phosphatidylcholine is a major constituent of the lipid fraction and present in the calcification front during normal bone formation (Han et al. 2003). Interestingly, a recent metabolomics study by Liu et al (Liu et al. 2018) showed that phosphatidylcholine was significantly associated with hip BMD ($p\text{-value} = 6.00 \times 10^{-4}$, $\text{FDR} = 0.02$). Another metabolomics-based study performed in menopausal women showed that dimethylarginine change was a useful marker to predict estrogen deficiency and/or bone loss (Miyamoto et al. 2018). These results suggested that some of the identified enhancer-SNPs may contribute to the pathogenesis of osteoporosis by affecting the serum metabolite levels.

GO Annotation — We performed GO enrichment analysis for genes that were nearest to the identified enhancer-SNPs. Genes related to the enhancer-SNPs associated with femoral

neck BMD were significantly enriched in biological processes of protein binding and transmembrane transporter activity (Table 7, Supplementary Table 5), such as Wnt-protein binding and voltage-gated calcium channel activity. Likewise, we identified significant enrichment of biological processes for genes related to the enhancer-SNPs associated with lumbar spine BMD (Table 7, Supplementary Table 5), such as estrogen receptor activity and lipid binding. Notably, Wnt-protein binding, voltage-gated calcium channel activity, and estrogen receptor activity have all been associated with bone metabolism in previous studies (Krishnan et al. 2006; Manolagas et al. 2013; Miyauchi et al. 1990).

Discussion

Our study represents the first targeted meta-analysis testing enhancer-SNPs that are potentially functional in bone-related cells for association with BMD variation, followed by comprehensive integrative genomic analysis. Although human genome contains ~1 million potential enhancers, only a small subset of them is active in a given cell type (Heinz et al. 2015). Therefore, it is necessary to select out those enhancers that are potentially functional in disease/trait-related cells when performing focused enhancer-SNP association studies. One reasonable and commonly used strategy to predict cell type-specific enhancers is based on the combinatorial interactions between different chromatin marks in their spatial context (chromatin states) in the cells of interest (Ernst and Kellis 2010). Unfortunately, such data is currently not available in osteoclasts or osteocytes; therefore, we used predicted enhancer data from two other bone-related cell types, namely, osteoblast and PBM, to select out ~1.75 million candidate enhancer-SNPs for this study.

By using the data from five independent GWAS cohorts and the summary statistics from the GEFOS study, we identified significant/suggestive associations for 8 enhancer-SNPs with femoral neck BMD. Five novel enhancer-SNPs *rs6465531*, *rs10278858*, *rs10249092*, *rs10273072*, and *rs4342522* in gene *SHFM1* and 1 previous identified enhancer-SNP *rs1430738* in *WLS* reached genome-wide significance level in the joint analysis. *SHFM1* encodes a 26S proteasome complex subunit DSS1, a multiprotein complex involved in the ATP-dependent degradation of ubiquitinated proteins. Chromosomal rearrangements of the *SHFM1* have been linked to isolated or syndromic limb malformation (Crackower et al. 1996; Sowinska-Seidler et al. 2014). In addition, *SHFM1* has been shown to directly interact with *BRCA2*. *SHFM1* can bind and stabilize *BRCA2* and therefore involved in the control of R-loop-associated DNA damage and transcription-associated genomic instability (Bhatia et al. 2014). Interestingly, a recent study shows that women with *BRCA2* mutations who undergo oophorectomy have a high risk of bone loss (Powell et al. 2018). Therefore, variants in the *SHFM1* gene may regulate bone metabolism by affecting the *BRCA2* functionality. *WLS* encodes a Wntless Wnt ligand secretion mediator, which regulates Wnt protein sorting and secretion in a feedback regulatory mechanism. (Fu et al. 2009) Wnt proteins are a family of secreted proteins that regulate many aspects of cell growth, differentiation, function, and death (Krishnan et al. 2006) and Wnt signaling pathway has been closely linked to bone development and remodeling (Baron and Rawadi 2007). Interestingly, a recent principle component analysis of an integrated osteoporosis-related phenotype showed that both *WLS* and *SHFM1* were suggestively associated with the first principle component which was functionally annotated to osteopenia and osteoporosis (Karasik et al. 2012). In addition, we

identified another two novel femoral neck BMD-associated enhancer-SNPs *rs10840343* and *rs10770081* in gene *IGF2*. *IGF2* encodes a member of the insulin family of polypeptide growth factors, which are involved in both prenatal and postnatal body growth and development (Fisher et al. 2005; Schlegel et al. 2010). *IGF2* is strongly expressed in the proliferating zone of the growth plate (Reinecke et al. 2000; Shinar et al. 1993; Tsang et al. 2007). Begemann et al. (Begemann et al. 2015) showed that a nonsense *IGF2* mutation was associated with human postnatal growth restriction. Interestingly, a recent *IGF2* knockout mice study further demonstrated that *IGF2* controls bone growth by regulating glucose metabolism in chondrocytes (Uchimura et al. 2017). Moreover, *IGF2* can potentiate *BMP9*-induced osteogenic differentiation and bone formation through PI3K/AKT signaling pathway. (Chen et al. 2010)

We identified 25 enhancer-SNPs with significant/suggestive associations for lumbar spine BMD, including 17 enhancer-SNPs that have been associated with BMD in previous GWAS. These enhancer-SNPs were mapped to several well-known BMD-associated genes/regions, including *CCDC170-ESR1*, *FAM3C*, *TNFRSF11B-COLEC10*, and *LRP5*. We identified 4 novel enhancer-SNPs in gene *SMOC1* (*rs1471243*, *rs7151788*), *MPP7* (*rs35494924*) and *ESR1* (*rs2941742*) at genome-wide significance level. *SMOC1* encodes a multi-domain secreted protein that may have a critical role in ocular and limb development (Okada et al. 2011). Previous studies showed that knockdown of *SMOC1* significantly inhibited mineralization and the expression of osteoblast differentiation markers, while overexpression of *SMOC1* substantially increased the expression of osteoblast differentiation-related genes (Choi et al. 2010). Notably, a recent meta-analysis study also reported a significant association between *SMOC1* and BMD, suggesting *SMOC1* may be a promising candidate gene underlying osteoporosis susceptibility (Zhang et al. 2014). The other interesting gene *MPP7* encodes a member of the p55 stardust family of membrane-associated guanylate kinase (MAGUK) proteins. This family member forms a complex with the polarity protein DLG1 and facilitates epithelial cell polarity and tight junction formation (Stucke et al. 2007). An *in vivo* study in zebrafish revealed that vertebral bone mass was lower in an *MPP7* knock-down model compared with the wide-type (Xiao et al. 2012). Additionally, *MPP7* was found to have constitutive expression in human bone-derived cells during osteogenesis. (Xiao et al. 2012). Another interesting enhancer-SNP *rs11581489*, which showed suggestive signal for lumbar spine BMD, was mapped to a novel gene *NGF*. *NGF* encodes a secreted protein with nerve growth stimulating activity and is involved in the regulation of growth and the differentiation of sympathetic and certain sensory neurons (Patel et al. 2000). For the bone metabolism, *NGF* works as a bone resorption inhibitor that increases bone reconstruction after fracture by reducing the excretion of proline and calcium (Grills et al. 1997). Recently, Sang et al. (Sang et al. 2017) constructed a non-stabilized fracture model of tibia by leveraging *NGF* transgenic homozygotic mice, and they found that *NGF* potentially improved the healing of tibial fracture by promoting callus formation.

Comprehensive integrative genomic analysis further demonstrated the potential functional importance of the identified enhancer-SNPs across various cell types (Tables 3 & 4). These enhancer-SNPs were mapped to various types of regulatory elements, such as transcription factor binding motifs, eQTLs, and circRNA. Interestingly, we identified several novel BMD-associated enhancer-SNPs (Supplementary Table 4) that were tightly linked but were

mapped into either of the same enhancer (e.g., *rs1471243* and *rs7151788* for *SMOC1* gene), different enhancers of the same cell type (e.g., *rs7609179* and *rs17023186* for *AFF3* gene), or even different enhancers of different cell types (e.g., *rs10249092* and *rs4342522* for *SHFMI*), suggesting a complex set of regulatory mechanisms of enhancer-SNPs in mediating the expression of their target genes. Moreover, we also observed that four functional susceptibility enhancer-SNPs, namely, *rs2941742* (*ESR1*), *rs35494924* (*MPP7*), *rs1471243* and *rs7151788* (*SMOC1*) were in high LD with previously identified BMD-associated loci which were not annotated to any transcription-regulatory epigenetic features in bone-related cells. These results indicated that functional susceptibility enhancer-SNPs may represent the true causal candidates affecting BMD variation in bone-related cells. Importantly, we also observed ten enhancer-SNPs (*rs10249092*, *rs4342522*, *rs6465531* in *SHFMI*, *rs718766* in *FAM3C*, *rs4407910*, *rs10101385*, *rs4319131*, *rs13277230* in *TNFRSF11B*, and *rs2062377*, *rs2220189* in *COLEC10*), which may contribute to variation in bone-related metabolite levels.

Several potential limitations of this study should be concerned and addressed in the future. First, the selection of an appropriate cell model is crucial. Due to the limited epigenomic and transcriptomic studies in human primary bone cells, here we focused on enhancer-SNPs that are in osteoblast cell and an osteoclast-lineage cell, specifically, human PBMs. Although PBMs can act as precursors of osteoclasts and serve as the major target cells of sex hormones for bone metabolism, the direct and ideal model cell of osteoclastogenesis would be human primary osteoclasts. With the advancements in sequencing technology, particularly of single-cell sequencing, we expect to acquire the comprehensive epigenomic and transcriptomic profiles of human primary bone cells in the near future. Second, the functional annotations were exclusively dependent on computationally predicted regulation features, and further experimental validation should be conducted to confirm the biological significance of these potential functional enhancer-SNPs.

In conclusion, we performed a targeted meta-analysis for potential functional enhancer-SNPs in bone-related cells. Comprehensive integrative genomic analysis revealed 15 prominent novel functional enhancer-SNPs for BMD variation. Our results highlighted the power of targeted analysis of potential functional variants for the identification of novel disease susceptibility loci that have been missed by conventional GWAS approach. More importantly, our findings suggested enhancer-SNPs mediated transcriptome or metabolome changes may be the crucial biological mechanisms to be considered in interpretation of associations between common genetic variants and human complex diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The regional association plots of significant/suggestive enhancer-SNPs at A) *SHFM1* region for femoral neck BMD, and B) *TNFRSF11B-COLEC10* region for lumbar spine BMD. Genes and expressed sequence tag (ESTs) within the region are shown in the lower panel, and the unbroken blue line indicates the recombination rate within the region. Each filled circle represents the p-value for one SNP in the joint-analysis, with the top SNP shown in purple and SNPs in the region colored depending on their degree of LD (r^2) with top SNP. LD was estimated by LocusZoom (Pruim et al. 2010) on the basis of CEU (Utah residents of Northern and Western European ancestry) HapMap haplotypes data.

Figure 2. Chromatin-state annotation tracks generated by ChromHMM model and visualized in the UCSC genome browser.
Enhancer-SNP *rs10249092* is mapped to genic enhancer in PBMs and enhancer-SNP *rs4342522* is mapped to weak enhancer in osteoblasts. Gene expression profiling in osteoblasts and PBMs were obtained from ENCODE/CSHL.

Figure 3. The circular plots of chromosome interactions and epigenetic signatures associated with A) enhancer-SNP *rs10840343* in osteoblasts, and B) enhancer-SNP *rs35494924* in PBMs. In the circular plot, from outer to inner, the circles represent chromatin states, annotated genes, histone modification set (red), transcription factor set (blue), queried and associated SNPs, and 3D chromatin interactions, in corresponding cell types, respectively. The six circles in the histone modification set are for H3K4me1, H3K4me3, H3K9me3, H3K27ac, H3K27me3 and H3K36me3. The one circle in the transcription factor set is CTCF.

Table 1.

Significant/Suggestive Enhancer-SNPs for Femoral Neck BMD

Enhancer-SNPs	Chr	SNP Position	Alleles	MAF	Nearest Gene	Feature	Meta p-value	GEFOS p-value	Joint p-value
<i>rs1430738</i>	1	68664129	t/c	0.49	<i>WLS</i>	intronic	4.68E-05	1.36E-06	1.56E-09
rs6465531	7	96279746	a/g	0.26	<i>SHFM1</i>	intronic	1.31E-07	3.42E-06 *	1.32E-11
rs10278858	7	96315149	a/g	0.16	<i>SHFM1</i>	intronic	1.09E-06	8.40E-05	2.21E-09
rs10249092	7	96320604	g/t	0.26	<i>SHFM1</i>	intronic	1.96E-07	3.60E-06 *	2.04E-11
rs10273072	7	96322822	t/g	0.19	<i>SHFM1</i>	intronic	1.11E-05	2.64E-05	6.74E-09
rs4342522	7	96327293	g/a	0.26	<i>SHFM1</i>	intronic	2.27E-07	4.81E-07	3.37E-12
rs10840343	11	2116686	g/c	0.36	<i>IGF2</i>	intronic	6.80E-06	4.64E-02	5.04E-06
rs10770081	11	2116776	t/c	0.48	<i>IGF2</i>	intronic	3.77E-05	4.01E-02	2.17E-05

Note:

- Enhancer-SNPs reached genome-wide significance level (p-value 5.00×10^{-8}) in the joint analysis of discovery and replication studies are marked in bold.
- Genes/Enhancer-SNPs reported in previous GWAS for BMD are marked in italics.

* These results were based on the GEFOS 2012 data release because these SNPs were not available in the 2015 release.

Table 2.

Significant/Suggestive Enhancer-SNPs for Lumbar Spine BMD

Enhancer-SNPs	Chr	SNP Position	Alleles	MAF	Nearest Gene	Feature	Meta p-value	GEFOS p-value	Joint p-value
rs11581489	1	115805959	c/t	0.22	NGF	NA	4.16E-06	2.43E-03	1.96E-07
rs7609179	2	100425448	g/a	0.16	AFF3	intronic	8.00E-06	3.61E-02	4.64E-06
rs17023186	2	100457754	a/t	0.17	AFF3	intronic	4.62E-05	3.17E-02	2.11E-05
rs4870042	6	151888053	g/c	0.21	<i>CCDC170</i>	intronic	1.48E-05	9.52E-07	3.67E-10
rs1871859	6	151898506	c/t	0.24	<i>CCDC170</i>	intronic	1.76E-07	2.04E-09	1.31E-14
rs3020334	6	152012956	a/g	0.37	<i>ESR1</i>	intronic	1.82E-06	2.71E-08	1.56E-12
rs2941742	6	152012988	a/g	0.36	<i>ESR1</i>	intronic	1.81E-06	5.37E-05	2.34E-09
rs10278858	7	96315149	a/g	0.16	<i>SHFM1</i>	intronic	3.99E-05	2.21E-03	1.52E-06
rs718766	7	121025502	t/c	0.24	<i>FAM3C</i>	intronic	4.12E-05	2.10E-13	3.48E-16
rs4407910	8	119917117	a/g	0.36	<i>TNFRSF11B</i>	NA	1.25E-05	1.36E-15 *	7.88E-19
rs13277230	8	119922993	c/t	0.36	<i>TNFRSF11B</i>	NA	1.07E-05	1.49E-15 *	7.39E-19
rs10101385	8	119923850	a/g	0.36	<i>TNFRSF11B</i>	NA	8.07E-06	1.49E-15 *	5.63E-19
rs4355801	8	119923873	a/g	0.36	<i>TNFRSF11B</i>	NA	8.04E-06	7.77E-15	2.82E-18
rs4319131	8	119947651	a/g	0.36	<i>TNFRSF11B</i>	intronic	5.06E-06	1.82E-17	4.76E-21
rs1982763	8	119947828	c/t	0.37	<i>TNFRSF11B</i>	intronic	1.21E-05	4.66E-18	2.95E-21
rs11573824	8	119959925	a/t	0.36	<i>TNFRSF11B</i>	intronic	1.23E-05	2.62E-17	1.62E-20
rs1485307	8	120007395	t/c	0.38	<i>COLEC10</i>	intronic	1.27E-05	9.60E-19	6.54E-22
rs2062377	8	120007420	t/a	0.36	<i>COLEC10</i>	intronic	1.22E-06	6.61E-19	4.57E-23
rs2220189	8	120007708	c/g	0.37	<i>COLEC10</i>	intronic	1.53E-06	4.25E-20	3.84E-24
rs10090576	8	120037816	t/c	0.37	<i>COLEC10</i>	intronic	6.02E-06	2.92E-18	9.38E-22
rs2450083	8	120063542	t/c	0.47	<i>COLEC10</i>	intronic	3.50E-05	3.61E-17	6.20E-20
rs35494924	10	28466881	t/c	0.31	<i>MPP7</i>	intronic	3.11E-05	5.84E-06	4.26E-09
rs901823	11	68205578	t/c	0.32	<i>LRP5</i>	intronic	2.28E-05	1.57E-10	1.23E-13
rs1471243	14	70469120	t/c	0.50	<i>SMOC1</i>	intronic	5.39E-06	4.54E-05	5.66E-09
rs7151788	14	70469255	a/c	0.50	<i>SMOC1</i>	intronic	1.12E-05	3.75E-05	9.52E-09

Note:

- Enhancer-SNPs reached genome-wide significance level (p-value 5.00×10^{-8}) in the joint analysis of discovery and replication studies are marked in bold.
- Genes/Enhancer-SNPs reported in previous GWAS for BMD are marked in italics.

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* • These results were based on the GEPOS 2012 data release because these SNPs were not available in the 2015 release.

Table 3.
Functional Annotation of Significant/Suggestive Enhancer-SNPs for Femoral Neck BMD

Enhancer-SNPs	Nearest Gene	Cells with Predicted Enhancer (Number of Cell Types)	Enhancer Region (Types)	Motifs Changed	eQTL Hits	Other Regulatory Elements	Conservation PhyloP Score
rs1430738	<i>WLS</i>	Osteoblasts (40)	chr1:68662800–68665400 (Active)	MeF2, PLZF, Pou2f2	Testis, Colon	circRNA	–1.84
rs6465531	<i>SHFMI</i>	Osteoblasts (7)	chr7:96279600–96279800 (Weak)	GR, HNF4	Artery, Adipose, Lung	lncRNA	–1.89
rs10278858	<i>SHFMI</i>	PBMs (14)	chr7:96314400–96315400 (Weak)	GR, Pou2f2, Pou5f1	Testis		1
rs10249092	<i>SHFMI</i>	PBMs (7)	chr7:96320600–96321200 (Genic)	ATF3, Pax-4	Artery, Adipose, Lung	circRNA	–0.02
rs10273072	<i>SHFMI</i>	PBMs (4)	chr7:96322800–96323600 (Genic)	Bbx, NF-Y	Testis	circRNA	0.33
rs4342522	<i>SHFMI</i>	Osteoblasts (12)	chr7:96327000–96327400 (Weak)	DMRT4, Osr	Artery, Testis	circRNA	–0.46
rs10840343	<i>IGF2</i>	Osteoblasts (14)	chr11:2116600–2116800 (Active)	11 altered motifs			–0.4
rs10770081	<i>IGF2</i>	Osteoblasts (14)	chr11:2116600–2116800 (Active)	BDP1, Mrgl1::Hoxa9, TBX5, Znf143	Heart		–0.18

Note:

• Enhancer coordinates and types were based on the 18-state model from the Roadmap Epigenomics Project under human reference genome (hg19). (Roadmap Epigenomics et al. 2015)

Functional Annotation of Significant/Suggestive Enhancer-SNPs for Lumbar Spine BMD

Table 4.

Enhancer-SNPs	Nearest Gene	Cells with Predicted Enhancer (Number of Cell Types)	Enhancer Region (Types)	Motifs Changed	eQTL Hits	Other Regulatory Elements	Conservation PhyloP Score
rs11581489	<i>NGF</i>	Osteoblasts (8)	chr1:115804800–115806000 (Weak) *	RXXA			–1.00
rs7609179	<i>AFF3</i>	Osteoblasts (10)	chr2:100424000–100426800 (Weak)				–0.48
rs17023186	<i>AFF3</i>	Osteoblasts (4)	chr2:100457600–100457800 (Weak)				–0.34
rs4570042	<i>CCDC170</i>	Osteoblasts (2)	chr6:151857200–151888200 (Weak)	HMG1Y, NFAT, Pax-4	Thyroid, Adipose, Adrenal Gland		–0.24
rs1871859	<i>CCDC170</i>	Osteoblasts/PBMs (6)	chr6:151895200–151899000/chr1:151898400/151898600 (Weak/Weak)	PLZF, RBP1appa	Thyroid, Adipose	circRNA region; Chromatin interactive region/NA	0.42
rs3020334	<i>ESR1</i>	PBMs (15)	chr6:152012800–152013200 (Weak)	Foxj1, NF1appaB, Sp100, TATA			–1.21
rs2941742	<i>ESR1</i>	PBMs (15)	chr6:152012800–152013200 (Weak)	8 altered motifs			–0.23
rs10278858	<i>SHFM1</i>	PBMs (14)	chr7:96314400–96315400 (Weak)	GR, Pou2f2, Pou5f1	Testis		1
rs718766	<i>FAM3C</i>	Osteoblasts (46)	chr7:121025400–121026000 (Active)	HDAC2, Sox	Skin, Nerve	Topologically associated domain	–0.83
rs4407910	<i>TNFRSF11B</i>	Osteoblasts/PBMs (17)	chr8:119914200–119918400/chr8:119917000/19917800 (Active/Weak)		Heart		–0.50
rs13277230	<i>TNFRSF11B</i>	Osteoblasts (20)	chr8:119922000–119926400 (Active)	FXR, Zfp410	Heart		–1.79
rs10101385	<i>TNFRSF11B</i>	Osteoblasts (14)	chr8:119922000–119926400 (Active)	E2A, Foxd1, ZEB1	Heart		0.35
rs4355801	<i>TNFRSF11B</i>	Osteoblasts (14)	chr8:119922000–119926400 (Active)	Egr-1, PPAR	Heart		–0.555
rs4319131	<i>TNFRSF11B</i>	Osteoblasts (9)	chr8:119947400–119948600 (Genic)	Foxa, Pax-6	Heart, Artery		–1.24
rs1982763	<i>TNFRSF11B</i>	Osteoblasts (10)	chr8:119947400–119948600 (Genic)	Dlx1, Hlx1, Lhx3, Pou3f4, TATA	Heart, Artery		0.20
rs11573824	<i>TNFRSF11B</i>	Osteoblasts (12)	chr8:119959800–119960400 (Weak)	9 altered motifs	Heart, Artery		–0.78
rs1485307	<i>COLEC10</i>	Osteoblasts (10)	chr8:120006800–120007400 (Active)	Nkx3, PLZF	Heart, Artery		–1.20
rs2062377	<i>COLEC10</i>	Osteoblasts (10)	chr8:120007400–120007800 (Active)	Irf, Nanog, Sox	Heart, Artery		–0.18
rs2220189	<i>COLEC10</i>	Osteoblasts (9)	chr8:120007400–120007800 (Active)		Heart, Artery		–0.95
rs10090576	<i>COLEC10</i>	Osteoblasts (3)	chr8:120037800–120038200 (Weak)		Heart, Artery		–0.90
rs2450083	<i>COLEC10</i>	Osteoblasts (12)	chr8:120063400–120067200 (Active)	Cpfx, Foxp1	Heart	circRNA region	–1.59
rs35494924	<i>MPP7</i>	PBMs (22)	chr10:28466800–28467000 (Active)	Rad21, TP4			–1.02
rs901823	<i>LRP5</i>	Osteoblasts (29)	chr11:68205400–68205800 (Active)	Myf, Sin3Ak-20	Nerve, Thyroid	circRNA region; Chromatin interactive region	–0.23
rs1471243	<i>SMOC1</i>	Osteoblasts (4)	chr14:70468200–70470200 (Active)	GR, Smad3	Artery, Nerve, Thyroid	circRNA region	–0.34
rs7151788	<i>SMOC1</i>	Osteoblasts (6)	chr14:70468200–70470200 (Active)	CCNT2, Egr-1, Ets, GR, MZF1	Artery, Nerve, Testis	circRNA region	–0.14

Note:

• Enhancer coordinates and types were based on the 18-state model from the Roadmap Epigenomics Project under human reference genome (hg19). (Roadmap Epigenomics et al. 2015)

* • This enhancer is overlapped with a super-enhancer in osteoblast based on dbSUPER. (Khan and Zhang 2016)

Table 5.
Novel Enhancer-SNPs in strong or moderate LD with Previously Identified BMD-associated SNPs

Novel Enhancer-SNPs	Chr_1	SNP Position_1	Chromatin States_1	Known BMD-associated SNPs	Chr_2	SNP Position_2	Chromatin States_2	Distance (bp)	r ²
rs2941742	6	152012988	Weak Enhancer (PBMIs)	rs3020331	6	152008780	Quiescent/Low (PBMIs)	4208	0.93
				rs2941741	6	152008982	Quiescent/Low (PBMIs)	4006	0.98
				rs2941740	6	152009638	Quiescent/Low (PBMIs)	3350	0.98
				rs3020333	6	152010254	Quiescent/Low (PBMIs)	2734	0.88
				rs3020334 *	6	152012956	Weak Enhancer (PBMIs)	32	1
rs35494924	10	28466881	Active Enhancer (PBMIs)	rs3020335	6	152013223	Quiescent/Low (PBMIs)	235	0.99
rs1471243	14	70469120	Active Enhancer (Osteoblast)	rs3905706	10	28479942	Weak Transcription (PBMIs)	13061	0.88
rs7151788	14	70469255	Active Enhancer (Osteoblast)	rs227425	14	70456699	Quiescent/Low (Osteoblasts)	12421	0.75
				rs227425	14	70456699	Quiescent/Low (Osteoblasts)	12421	0.75

Note:

- *
• As shown in Table 2, enhancer-SNP *rs3020334* was also identified in our study.
• Chromatin states were based on the 18-state model from the Roadmap Epigenomics Project under human reference genome (hg19). (Roadmap Epigenomics et al. 2015)

Table 6.

BMD-associated Enhancer-SNPs Contributing to Variation in Serum Metabolite Levels

Enhancer-SNPs	Serum Metabolites	p-value	References
rs10249092, rs4342522, rs6465531	* dimethylarginine (SDMA + ADMA)	4.87×E-05	(Suhre et al. 2011)
rs718766	levulinate (4-oxovalerate)	1.48×E-05	(Shin et al. 2014)
rs4407910	SM (OH) C22:2	3.27×E-05	(Draisma et al. 2015)
	SM (OH) C16:1	1.74×E-05	(Draisma et al. 2015)
rs13277230	SM (OH) C22:2	4.84×E-05	(Draisma et al. 2015)
	SM (OH) C16:1	2.06×E-05	(Draisma et al. 2015)
rs10101385	SM (OH) C22:2	4.05×E-05	(Draisma et al. 2015)
	SM (OH) C16:1	1.72×E-05	(Draisma et al. 2015)
rs4319131	SM (OH) C22:2	8.50×E-06	(Draisma et al. 2015)
	SM (OH) C16:1	3.88×E-06	(Draisma et al. 2015)
rs2062377	SM (OH) C22:2	1.27×E-05	(Draisma et al. 2015)
	SM (OH) C16:1	1.51×E-05	(Draisma et al. 2015)
rs2220189	SM (OH) C22:2	1.95×E-06	(Draisma et al. 2015)
	PC ae C38:3	1.74×E-05	(Draisma et al. 2015)
	PC ae C36:1	1.97×E-05	(Draisma et al. 2015)
	SM C16:0	8.59×E-06	(Draisma et al. 2015)
	SM C16:1	2.89×E-06	(Draisma et al. 2015)
	SM C18:1	1.64×E-05	(Draisma et al. 2015)
	PC ae C36:2	1.53×E-05	(Draisma et al. 2015)
	SM (OH) C14:1	2.47×E-07	(Draisma et al. 2015)
	SM (OH) C16:1	3.35×E-06	(Draisma et al. 2015)

Note:

* These three enhancer-SNPs were in high LD ($r^2 > 0.8$) with SNP *rs10269596*, which showed suggestive genome-wide significant association with dimethylarginine (SDMA + ADMA). Names of lipids detected by the Biocrates AbsoluteIDQ p150 platform are abbreviated as follows: acylcarbitines, Cx:y; sphingomyelins, SMx:y; N-hydroxyacylsphingosylphosphocholine, SM (OH) x:y; phosphatidylcholines, PC (aa=diacyl, ae=acyl-alkyl). Lipid side chain composition is abbreviated as Cx:y, where x denotes the number of carbons in the side chain and y the number of double bonds.

Table 7.

The Top Ten Significant GO Terms Enriched for BMD-associated Enhancer-SNPs

GOID	Terms	Log_odds_ratio	p-value
Femoral Neck BMD			
GO:0017147	Wnt-protein binding	7.82	7.53E-18
GO:0005245	Voltage-gated calcium channel activity	6.00	3.14E-08
GO:0015085	Calcium ion transmembrane transporter activity	4.67	1.01E-05
GO:0022839	Ion gated channel activity	3.52	7.69E-04
GO:0004550	Nucleoside diphosphate kinase activity	6.08	2.89E-03
GO:0046873	Metal ion transmembrane transporter activity	2.97	5.07E-03
GO:0022838	Substrate-specific channel activity	2.95	5.33E-03
GO:0022803	Passive transmembrane transporter activity	2.89	5.97E-03
GO:0015318	Inorganic molecular entity transmembrane transporter activity	2.26	1.15E-02
GO:0016776	Phosphotransferase activity, phosphate group as acceptor	5.05	1.25E-02
Lumbar Spine BMD			
GO:0030284	Estrogen receptor activity	8.42	2.96E-06
GO:0003707	Steroid hormone receptor activity	4.92	2.66E-04
GO:0008289	Lipid binding	2.62	5.07E-04
GO:0003677	DNA binding	1.72	1.80E-03
GO:1901363	Heterocyclic compound binding	0.93	5.58E-03
GO:0003700	DNA binding transcription factor activity	2.09	5.58E-03
GO:0097159	Organic cyclic compound binding	0.92	5.71E-03
GO:0140110	Transcription regulator activity	1.92	6.66E-03
GO:0015299	Solute:proton antiporter activity	5.84	7.68E-03
GO:0005496	Steroid binding	4.60	7.83E-03

Note: GO enrichment analysis was performed in candidate genes annotated to potential functional enhancer-SNPs (p-value < 5.0×10⁻⁵). The p-values were calculated by hypergeometric tests and adjusted for multiple comparisons by stringent Yekutieli (FDR under dependency) adjustment.