Human Genetics (2019) 138:167–185
https://doi.org/10.1007/s00439-019-01971-4

ORIGINAL INVESTIGATION

Integrative genomic analysis predicts novel functional enhancer-SNPs for bone mineral density

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Received: 19 September 2018 / Accepted: 3 January 2019 / Published online: 17 January 2019

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 ($n = 5905$) and the genetics factors for osteoporosis 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, and *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 that even tightly linked SNPs may regulate the same target gene and contribute to the phenotype variation in cell-type-specific manners. Importantly, ten 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.

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. The previous studies have revealed a strong genetic determination of BMD variation, with heritability ranging from 50 to 85% (Ralston and de Crombrugghe 2006; Ralston and Uitterlinden 2010). Currently, over 200 loci have been reported to be associated with BMD at various skeletal sites (Chuan Qiu et al. 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,

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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00439-019-01971-4) contains supplementary material, which is available to authorized users.

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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 the 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 data set comprised 5905 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, $n = 2250$) with Caucasian Ancestry, China Osteoporosis Study (COS, $n = 1547$) 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 data set 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 2882 subjects with whole-genome sequencing, 3549 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, age^2, 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 data sets 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; EnhWk, weak enhancers) in osteoblasts and PBMs based on the 18-state ChromHMM annotation of the human genome by the Roadmap Epigenomics Project (Roadmap 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 and 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 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 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 SNiPA (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 data sets 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 the 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 5019 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 under-representation of genic enhancers in osteoblasts when compared to the distribution of PBM enhancers (Supplementary Fig. 1). Since the active and genic enhancers were distinguished based on different histone

modification profiles, our results suggested that 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 5905 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 data sets, no enhancer-SNPs were found to be associated with femoral neck BMD at the genome-wide significance level (p value $< 5.00 \times 10^{-8}$). However, we observed 44 enhancer-SNPs achieved suggestive genome-wide significance level (p value $< 5.00 \times 10^{-5}$) 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) eight enhancer-SNPs in the GEFOS cohort (Zheng et al. 2015) (Table 1) including a known enhancer-SNP *rs1430738* in gene *WLS*, five novel enhancer-SNPs (*rs6465531, rs10278858, rs10249092, rs10273072*, and *rs4342522*) in gene *SHFM1*, and two novel enhancer-SNPs (*rs10840343* and *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 six enhancer-SNPs (Table 1), including the five novel enhancer-SNPs clustered in the genomic regions encompassing *SHFM1* gene (*rs6465531,* p value$_{joint} = 1.32 \times 10^{-11}$; *rs10278858,* p value$_{joint} = 2.21 \times 10^{-9}$; *rs10249092,* p value$_{joint} = 2.04 \times 10^{-11}$; *rs10273072,* p value$_{joint} = 6.74 \times 10^{-9}$; *rs4342522,* p value$_{joint} = 3.37 \times 10^{-12}$) (Fig. 1a) and the known enhancer-SNP *rs1430738* (p value$_{joint} = 1.56 \times 10^{-9}$) in gene *WLS*. Interestingly, *SHFM1* SNPs *rs10249092* and *rs4342522* were tightly linked ($r^2 = 1$ in European population) (Supplementary Table 4), but they were located in two different enhancers in PBMs and osteoblasts, respectively (Fig. 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). Using the GEFOS cohort, we successfully replicated (p value < 0.05) 25 enhancer-SNPs for lumbar spine BMD (Table 2), including eight 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 (Fig. 1b) and four enhancer-SNPs were mapped in the *CCDC170-ESR1* region (Supplementary Fig. 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 Tables 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 four novel enhancer-SNPs were associated with lumbar spine BMD at the genome-wide significance level

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
rs1430738	1	68664129	t/c	0.49	*WLS*	Intronic	4.68E−05	1.36E−06	1.56E−09
rs6465531	7	96279746	a/g	0.26	*SHFM1*	Intronic	1.31E−07	3.42E−06[a]	1.32E−11
rs10278858	7	96315149	a/g	0.16	*SHFM1*	Intronic	1.09E−06	8.40E−05	2.21E−09
rs10249092	7	96320604	g/t	0.26	*SHFM1*	Intronic	1.96E−07	3.60E−06[a]	2.04E−11
rs10273072	7	96322822	t/g	0.19	*SHFM1*	Intronic	1.11E−05	2.64E−05	6.74E−09
rs4342522	7	96327293	g/a	0.26	*SHFM1*	Intronic	2.27E−07	4.81E−07	3.37E−12
rs10840343	11	2116686	g/c	0.36	IGF2	Intronic	6.80E−06	4.64E−02	5.04E−06
rs10770081	11	2,116,776	t/c	0.48	IGF2	Intronic	3.77E−05	4.01E−02	2.17E−05

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

[a]These results were based on the GEFOS 2012 data release, because these SNPs were not available in the 2015 release

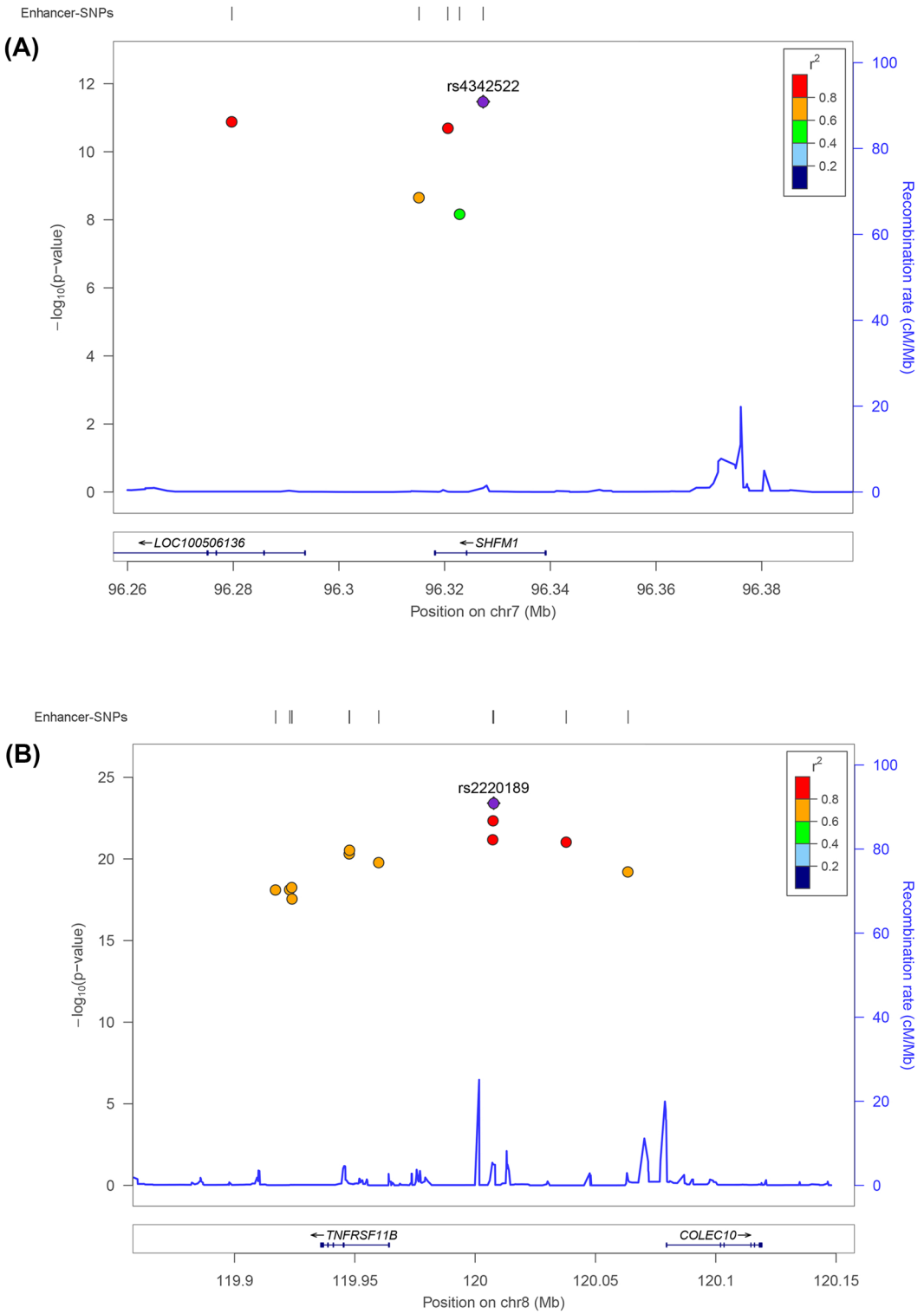

Fig. 1 The regional association plots of significant/suggestive enhancer-SNPs at **a** *SHFM1* region for femoral neck BMD and **b** *TNFRSF11B-COLEC10* region for lumbar spine BMD. Genes and expressed sequence tag (ESTs) within the region are shown in the lower panel, and the unbroken blue line indicates 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

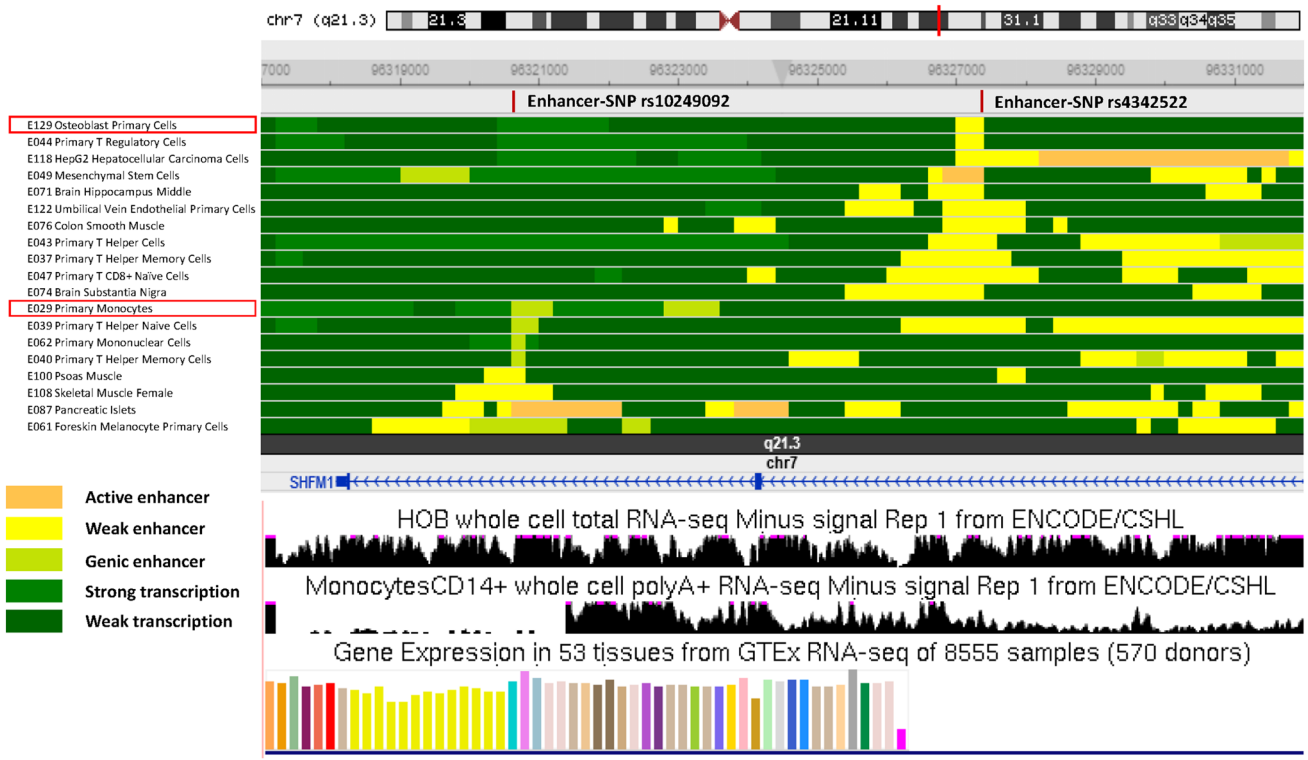

Fig. 2 Chromatin-state annotation tracks generated by ChromHMM model and visualized in the UCSC genome browser. Enhancer-SNP *rs10249092* is mapped to genic enhancer in PBMs and enhancer-SNP *rs4342522* is mapped to weak enhancer in osteoblasts. Gene expression profiling in osteoblasts and PBMs were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

(Table 2), including *rs1471243* (p value$_{\text{joint}} = 5.66 \times 10^{-9}$) and *rs7151788* ($p$ value$_{\text{joint}} = 9.52 \times 10^{-9}$) in gene *SMOC1, rs2941742* (p value$_{\text{joint}} = 2.34 \times 10^{-9}$) in gene *ESR1*, and *rs35494924* (p value$_{\text{joint}} = 4.26 \times 10^{-9}$) 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*) which 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 using data from ENCODE, Roadmap Epigenomics, and GTEx projects through the HaploReg program (Ward and Kellis 2012). All eight replicated enhancer-SNPs for femoral neck BMD were predicted to alter transcription factor binding motifs, and seven 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 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	*CCDC170*	Intronic	1.48E−05	9.52E−07	3.67E−10
rs1871859	6	151898506	c/t	0.24	*CCDC170*	Intronic	1.76E−07	2.04E−09	1.31E−14
rs3020334	6	152012956	a/g	0.37	*ESR1*	Intronic	1.82E−06	2.71E−08	1.56E−12
rs2941742	6	152012988	a/g	0.36	*ESR1*	Intronic	1.81E−06	5.37E−05	2.34E−09
rs10278858	7	96315149	a/g	0.16	*SHFM1*	Intronic	3.99E−05	2.21E−03	1.52E−06
rs718766	7	121025502	t/c	0.24	*FAM3C*	Intronic	4.12E−05	2.10E−13	3.48E−16
rs4407910	8	119917117	a/g	0.36	*TNFRSF11B*	NA	1.25E−05	1.36E−15[a]	7.88E−19
rs13277230	8	119922993	c/t	0.36	*TNFRSF11B*	NA	1.07E−05	1.49E−15[a]	7.39E−19
rs10101385	8	119923850	a/g	0.36	*TNFRSF11B*	NA	8.07E−06	1.49E−15[a]	5.63E−19
rs4355801	8	119923873	a/g	0.36	*TNFRSF11B*	NA	8.04E−06	7.77E−15	2.82E−18
rs4319131	8	119947651	a/g	0.36	*TNFRSF11B*	Intronic	5.06E−06	1.82E−17	4.76E−21
rs1982763	8	119947828	c/t	0.37	*TNFRSF11B*	Intronic	1.21E−05	4.66E−18	2.95E−21
rs11573824	8	119959925	a/t	0.36	*TNFRSF11B*	Intronic	1.23E−05	2.62E−17	1.62E−20
rs1485307	8	120007395	t/c	0.38	*COLEC10*	Intronic	1.27E−05	9.60E−19	6.54E−22
rs2062377	8	120007420	t/a	0.36	*COLEC10*	Intronic	1.22E−06	6.61E−19	4.57E−23
rs2220189	8	120007708	c/g	0.37	*COLEC10*	Intronic	1.53E−06	4.25E−20	3.84E−24
rs10090576	8	120037816	t/c	0.37	*COLEC10*	Intronic	6.02E−06	2.92E−18	9.38E−22
rs2450083	8	120063542	t/c	0.47	*COLEC10*	Intronic	3.50E−05	3.61E−17	6.20E−20
rs35494924	10	28466881	t/c	0.31	*MPP7*	Intronic	3.11E−05	5.84E−06	4.26E−09
rs901823	11	68205578	t/c	0.32	*LRP5*	Intronic	2.28E−05	1.57E−10	1.23E−13
rs1471243	14	70469120	t/c	0.50	*SMOC1*	Intronic	5.39E−06	4.54E−05	5.66E−09
rs7151788	14	70469255	a/c	0.50	*SMOC1*	Intronic	1.12E−05	3.75E−05	9.52E−09

Enhancer-SNPs reached genome-wide significance level (p value $\leq 5.00\times10^{-8}$) in the joint analysis of discovery and replication studies are marked in bold. Genes/enhancer-SNPs reported in the previous GWAS for BMD are marked in italics

[a]These results were based on the GEFOS 2012 data release, because these SNPs were not available in the 2015 release

(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* (Fig. 3a). The other interesting example is enhancer encompassing SNP *rs35494924*, which may have distal interaction with gene *MKX* (Fig. 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, 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, Tables 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 five 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 four 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,

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	*WLS*	Osteoblasts (40)	chr1:68662800–68665400 (active)	Mef2, PLZF, Pou2f2	Testis, colon	circRNA	− 1.84
rs6465531	*SHFM1*	Osteoblasts (7)	chr7:96279600–96279800 (weak)	GR, HNF4	Artery, adipose, lung	lncRNA	− 1.89
rs10278858	*SHFM1*	PBMs (14)	chr7:96314400–96315400 (weak)	GR, Pou2f2, Pou5f1	Testis		1
rs10249092	*SHFM1*	PBMs (7)	chr7:96320600–96321200 (genic)	ATF3, Pax-4	Artery, adipose, lung	circRNA	− 0.02
rs10273072	*SHFM1*	PBMs (4)	chr7:96322800–96323600 (genic)	Bbx, NF-Y	Testis	circRNA	0.33
rs4342522	*SHFM1*	Osteoblasts (12)	chr7:96327000–96327400 (weak)	DMRT4, Osr	Artery, testis	circRNA	− 0.46
rs10840343	*IGF2*	Osteoblasts (14)	chr11:2116600–2116800 (active)	11 altered motifs			− 0.4
rs10770081	*IGF2*	Osteoblasts (14)	chr11:2116600–2116800 (active)	BDP1, Mrg1::Hoxa9, TBX5, Znf143	Heart		− 0.18

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

three 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 that these enhancer-SNPs may represent a largely independent signal from what was reported in the previous GWAS. For the eight 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 six known BMD-associated SNPs, including five 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, two 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 that 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 ten 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

Table 4 Functional annotation of significant/suggestive enhancer-SNPs for lumbar spine BMD

Enhancer-SNPs	Nearest gene	Cells with predicted enhancers (number of cell types)	Enhancer region (types)	Motifs changed	eQTL hits	Other regulatory elements	Conservation PhyloP score
rs11581489	*NGF*	Osteoblasts (8)	chr1:115804800–115806000 (weak)[a]	RXRA			−1.00
rs7609179	*AFF3*	Osteoblasts (10)	chr2:100424000–100426800 (weak)				−0.48
rs17023186	*AFF3*	Osteoblasts (4)	chr2:100457600–100457800 (weak)				−0.34
rs4870042	*CCDC170*	Osteoblasts (2)	chr6:151887200–151888200 (weak)	HMG-IY, NF-AT, Pax-4	Thyroid, adipose, adrenal gland		−0.24
rs1871859	*CCDC170*	Osteoblasts/PBMs (6)	chr6:151898200–151899000/chr6:151898400–151898600 (weak/weak)	PLZF, RBP-Jkappa	Thyroid, adipose	circRNA region; chromatin interactive region/NA	0.42
rs3020334	*ESR1*	PBMs (15)	chr6:152012800–152013200 (weak)	Foxj1, NF-kappaB, Sp100, TATA			−1.21
rs2941742	*ESR1*	PBMs (15)	chr6:152012800–152013200 (weak)	8 altered motifs			−0.23
rs10278858	*SHFM1*	PBMs (14)	chr7:96314400–96315400 (weak)	GR, Pou2f2, Pou5f1	Testis		1
rs718766	*FAM3C*	Osteoblasts (46)	chr7:121025400–121026000 (active)	HDAC2, Sox	Skin, nerve	Topologically associated domain	−0.83
rs4407910	*TNFRSF11B*	Osteoblasts/PBMs (17)	chr8:119914200–119918400/chr8:119917000–119917800 (active/weak)		Heart		−0.50
rs13277230	*TNFRSF11B*	Osteoblasts (20)	chr8:119922000–119926400 (active)	FXR, Zfp410	Heart		−1.79
rs10101385	*TNFRSF11B*	Osteoblasts (14)	chr8:119922000–119926400 (active)	E2A, Foxd1, ZEB1	Heart		0.35
rs4355801	*TNFRSF11B*	Osteoblasts (14)	chr8:119922000–119926400 (active)	Egr-1, PPAR	Heart		−0.555
rs4319131	*TNFRSF11B*	Osteoblasts (9)	chr8:119947400–119948600 (genic)	Foxa, Pax-6	Heart, artery		−1.24
rs1982763	*TNFRSF11B*	Osteoblasts (10)	chr8:119947400–119948600 (genic)	Dbx1, Hlx1, Lhx3, Pou3f4, TATA	Heart, artery		0.20
rs11573824	*TNFRSF11B*	Osteoblasts (12)	chr8:119959800–119960400 (weak)	Nine altered motifs	Heart, artery		−0.78
rs1485307	*COLEC10*	Osteoblasts (10)	chr8:120006800–120007400 (active)	Nkx3, PLZF	Heart, artery		−1.20

Table 4 (continued)

Enhancer-SNPs	Nearest gene	Cells with predicted enhancers (number of cell types)	Enhancer region (types)	Motifs changed	eQTL hits	Other regulatory elements	Conservation PhyloP score
rs2062377	*COLEC10*	Osteoblasts (10)	chr8:120007400–120007800 (active)	Irf, Nanog, Sox	Heart, artery		−0.18
rs2220189	*COLEC10*	Osteoblasts (9)	chr8:120007400–120007800 (active)		Heart, artery		−0.95
rs10090576	*COLEC10*	Osteoblasts (3)	chr8:120037800–120038200 (weak)		Heart, artery		−0.90
rs2450083	*COLEC10*	Osteoblasts (12)	chr8:120063400–120067200 (active)	Cphx, Foxp1	Heart		−1.59
rs35494924	*MPP7*	PBMs (22)	chr10:28466800–28467000 (active)	Rad21, TR4		circRNA region	−1.02
rs901823	*LRP5*	Osteoblasts (29)	chr11:68205400–68205800 (active)	Myf, Sin3Ak-20	Nerve, thyroid	circRNA region; chromatin interactive region	−0.23
rs1471243	*SMOC1*	Osteoblasts (4)	chr14:70468200–70470200 (active)	GR, Smad3	Artery, nerve, thyroid	circRNA region	−0.34
rs7151788	*SMOC1*	Osteoblasts (6)	chr14:70468200–70470200 (active)	CCNT2, Egr-1, Ets, GR, MZF1	Artery, nerve, testis	circRNA region	−0.14

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

[a]This enhancer is overlapped with a super-enhancer in osteoblast based on dbSUPER (Khan and Zhang 2016)

(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. (2018) showed that phosphatidylcholine was significantly associated with hip BMD (p value $= 6.00 \times 10^{-4}$, 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 a 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 the 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

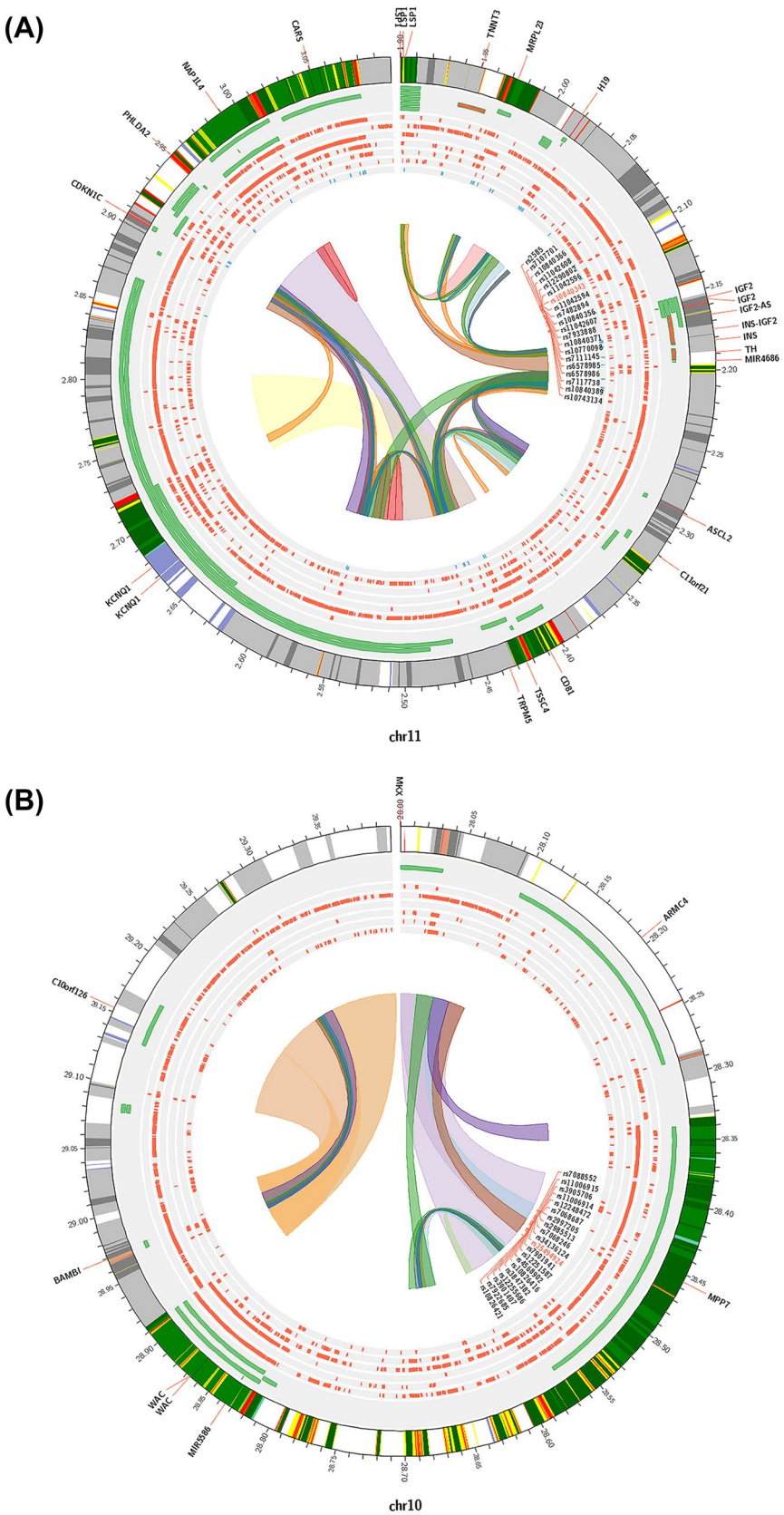

Fig. 3 The circular plots of chromosome interactions and epigenetic signatures associated with **a** enhancer-SNP *rs10840343* in osteoblasts and **b** enhancer-SNP *rs35494924* in PBMs. In the circular plot, from outer to inner, the circles represent chromatin states, annotated genes, histone modification set (red), transcription factor set (blue), queried and associated SNPs, and 3D chromatin interactions, in the 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 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 (PBMs)	rs3020331	6	152008780	Quiescent/low (PBMs)	4208	0.93
				rs2941741	6	152008982	Quiescent/low (PBMs)	4006	0.98
				rs2941740	6	152009638	Quiescent/low (PBMs)	3350	0.98
				rs3020333	6	152010254	Quiescent/low (PBMs)	2734	0.88
				rs3020334[a]	6	152012956	Weak enhancer (PBMs)	32	1
				rs3020335	6	152013223	Quiescent/low (PBMs)	235	0.99
rs35494924	10	28466881	Active enhancer (PBMs)	rs3905706	10	28479942	Weak transcription (PBMs)	13,061	0.88
rs1471243	14	70469120	Active enhancer (osteoblast)	rs227425	14	70456699	Quiescent/low (osteoblasts)	12,421	0.75
rs7151788	14	70469255	Active enhancer (osteoblast)	rs227425	14	70456699	Quiescent/low (osteoblasts)	12,421	0.75

Chromatin states were based on the 18-state model from the Roadmap Epigenomics Project under human reference genome (hg19) (Roadmap et al. 2015)

[a]As shown in Table 2, enhancer-SNP *rs3020334* was also identified in our study

their spatial context (chromatin states) in the cells of interest (Ernst and Kellis 2010). Unfortunately, such data are currently not available in osteoclasts or osteocytes; therefore, we used the 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.

Using the data from five independent GWAS cohorts and the summary statistics from the GEFOS study, we identified significant/suggestive associations for eight enhancer-SNPs with femoral neck BMD. Five novel enhancer-SNPs *rs6465531, rs10278858, rs10249092, rs10273072*, and *rs4342522* in gene *SHFM1* and one 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. (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

Table 6 BMD-associated enhancer-SNPs contributing to variation in serum metabolite levels

Enhancer-SNPs	Serum metabolites	*p* value	References
rs10249092, rs4342522, rs6465531[a]	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)

[a]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: acylcarnitines, C*x:y*; sphingomyelins, SM*x:y*; N-hydroxylacyloylsphingosylphosphocholine, SM (OH)*x:y*; phosphatidylcholines, PC (aa = diacyl, ae = acyl-alkyl). Lipid side chain composition is abbreviated as C*x:y*, where *x* denotes the number of carbons in the side chain and *y* denotes the number of double bonds

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 the 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 four novel enhancer-SNPs in gene *SMOC1* (*rs1471243* and *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). The previous studies showed that knock-down 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 that *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). In addition, *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. (2017) constructed a non-stabilized fracture model of tibia

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

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

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 *SHFM1*), 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 *SHFM1, 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 the conventional GWAS approach. More importantly, our findings suggested that enhancer-SNPs mediated transcriptome or metabolome changes may be the crucial biological mechanisms to be considered in the interpretation of associations between common genetic variants and human complex diseases.

Acknowledgements This study was partially supported or benefited by grants from the National Institutes of Health [R01AR059781, P20GM109036, R01MH107354, R01MH104680, R01GM109068, R01AR069055, and U19AG055373], the Franklin D. Dickson/Missouri Endowment, and the Edward G. Schlieder Endowment and the Drs. W. C. Tsai and P. T. Kung Professorship in Biostatistics from Tulane University. The Women's Health Initiative (WHI) program is funded by the National Heart, Lung, and Blood Institute, National Institutes of Health, U.S. Department of Health and Human Services through contracts N01WH22110, 24152, 32100-2, 32105-6, 32108-9, 32111-13, 32115, 32118-32119, 32122, 42107-26, 42129-32, and 44221. This manuscript was not prepared in collaboration with the investigators of the WHI, has not been reviewed and/or approved by, and does not necessarily reflect the opinions of the WHI investigators or the NHLBI. WHI Population Architecture Using Genomics and Epidemiology (PAGE) funded through the NHGRI Population Architecture Using Genomics and Epidemiology (PAGE) network (Grant number U01 HG004790). Assistance with phenotype harmonization, SNP selection, data cleaning, meta-analyses, data management and dissemination, and general study coordination was provided by the PAGE Coordinating Center [U01HG004801-01]. The data sets used for the analyses described in this manuscript were obtained from dbGaP at http://www.ncbi.nlm.nih.gov/sites/entrez?db=gap through dbGaP accession phs000200.v6.p2.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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