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Long Noncoding RNA Analyses for Osteoporosis Risk in Caucasian Women

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Abstract

Introduction—Osteoporosis is a prevalent bone metabolic disease characterized by bone fragility. As a key pathophysiological mechanism, the disease is caused by excessive bone resorption (by osteoclasts) over bone formation (by osteoblasts). Peripheral blood monocytes (PBMs) is a major systemic cell model for bone metabolism by serving as progenitors of osteoclasts and producing cytokines important for osteoclastogenesis. Protein-coding genes for osteoporosis have been widely studied by mRNA analyses of PBMs in high versus low hip bone mineral density (BMD) subjects. However, long noncoding RNAs (lncRNAs), which account for a large proportion of human transcriptome, have seldom been studied.

Methods—In this study, microarray analyses of monocytes were performed using Affymetrix exon 1.0 ST arrays in 73 Caucasian females (age: 47–56). LncRNA profile was generated by re-annotating exon array for lncRNAs detection, which yielded 12,007 lncRNAs mapped to the human genome.

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Compliance with ethical standards

Conflict of interest Authors Yu Zhou, Chao Xu, Wei Zhu, Hao He, Lan Zhang, Beisha Tang, Yong Zeng, Qing Tian, and Hong-Wen Deng have declared that no conflict of interest exists.

Human and Animal Rights and Informed Consent All the methods were conducted in accordance with the regulations and guidelines of the Institutional Review Boards of University of Missouri Kansas City and Tulane University. The Institutional Review Boards of University of Missouri Kansas City and Tulane University approved the study. Written informed consent was obtained from all participants before inclusion in the study.

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Results—575 lncRNAs were differentially expressed between the two groups. In the high BMD subjects, 309 lncRNAs were upregulated and 266 lncRNAs were downregulated (nominally significant, raw p -value < 0.05). To investigate the relationship between mRNAs and lncRNAs, we used two approaches to predict the target genes of lncRNAs and found that 26 candidate lncRNAs might regulate mRNA expression. The majority of these lncRNAs were further validated to be potentially correlated with BMD by GWAS analysis.

Conclusion—Overall, our findings for the first time reported the lncRNAs profiles for osteoporosis and suggested the potential regulatory mechanism of lncRNAs on protein-coding genes in bone metabolism.

Keywords

Bone mineral density; lncRNAs; Gene expression; Systems genetics; Osteoporosis

Introduction

Osteoporosis is the most common metabolic bone disease, mainly manifested as low bone mineral density (BMD). For human skeletal disorders, restrained by the difficulty in directly obtaining an adequate quantity of homogenous cells from bone tissues, finding an appropriate working cell model for clinical and (epi-)genomic studies is a challenge.

Peripheral blood monocytes (PBMs) are an appropriate cell model for studying osteoporosis [1]. First, PBMs constitute 3%–8% of the human leukocytes in the blood and can be easily harvested with high purity. Second, PBMs may differentiate into osteoclasts [2–5], the bone resorption cells. On the basis of the surface expressions of *CD14* and *CD16*, PBMs can be divided into three (classic, nonclassic, and intermediate) subsets [6, 7]. The classical PBMs, which are characterized by high level expression of *CD14* and lack expression of *CD16* (*CD14⁺⁺CD16⁻*), had been suggested to be potential osteoclast precursors [8]. Particularly PBMs provide the sole source of osteoclast precursors for adult peripheral skeleton, since the bone marrow in adult peripheral skeleton normally cannot generate mononuclear precursors [9]. Third, PBMs can secrete several cytokines important for osteoclast differentiation, activation, and apoptosis [10–13]. Therefore, our study selected PBMs as a cell model to investigate the transcriptome features of osteoporosis.

Long noncoding RNAs (lncRNAs) are defined as RNA transcripts longer than 200 nucleotides without protein-coding function [14]. Accumulating evidences suggest that lncRNAs are involved in various types of molecular mechanisms and many diseases [15]. For example, lncRNAs can act as cis-regulators via recruiting protein complexes to their transcription sites at DNA strands, like *Xist* [16] and *Kcnq1ot1* [17]. Meanwhile, lncRNAs can also recruit protein complexes to chromatin loci away from their transcription sites at DNA strands as trans-regulators, such as *HOTAIR* [18]. Recently, a pilot study showed that lncRNA *DANCR* expressed in circulating monocytes was involved in the bone-resorbing activity via inducing *IL6* and *TNF- α* [19]. The profile changes of lncRNAs were also identified during osteoclast differentiation in an in vitro study [20]. However, there are few transcriptome-wide lncRNA studies which have been conducted for osteoporosis in vivo in humans. Our study, for the first time using large sample size, by screening lncRNAs

transcriptome-wide and their potential target genes in PBMs in vivo, is useful in investigating the fundamental functional genomic basis of osteoporosis, particularly for females.

Although some exon microarrays were not designed for lncRNA detection, they contain many probes mapped to expressed sequence tags (ESTs) and prediction-based transcripts. Several studies [21–24] showed that a large portion of these probes could be re-annotated for lncRNAs. For example, Du et al. [25] successfully constructed a cancer-related lncRNA database via repurposing microarray probes. Two of the newly identified lncRNAs were confirmed to be associated with prostate cancer cell growth by experiments.

The goal of our current work was to explore the lncRNAs associated with osteoporosis. Toward this goal, we established a transcriptome-wide lncRNA expression profile by re-annotating exon array data of PBMs from 73 Caucasian females. We identified differentially expressed (DE) lncRNAs between high and low BMD groups. The correlation analysis was also performed to find potential “cis-” or “trans-”regulatory relationships among lncRNAs and protein-coding genes. In addition, we conducted validations of the candidate lncRNAs using the largest osteoporosis GWAS meta-analysis dataset from the Genetic Factors for Osteoporosis Consortium (GEFOS) and Least absolute shrinkage and selection operator (Lasso) method.

Materials and Methods Subjects

Characteristics

All the methods were conducted in accordance with the regulations and guidelines of the Institutional Review Boards of University of Missouri Kansas City and Tulane University. The Institutional Review Boards of University of Missouri Kansas City and Tulane University approved the study. Written informed consent was obtained from all participants before inclusion in the study. Subjects in this study were 73 unrelated Caucasian females with high versus low hip BMD [26]. (High BMD group: $Z_{\text{BMD}} > +0.84$ vs Low BMD group: $Z_{\text{BMD}} < -0.52$). Z_{BMD} was the Z-score of BMD. The hip BMD (g/cm^2) of each subject was measured using a Hologic 4500-W dual-energy X-ray absorptiometer (DXA) scanner (Hologic Corp., Waltham, MA). The Z-score was defined as the number of standard deviations a subject's BMD differed from the mean BMD of their age-, gender-, and ethnicity-matched reference population. It represented the location of a subject in the BMD value distribution in our study population. Here, high versus low BMD was defined as belonging to top versus bottom 30% of BMD values in our population. The individuals with diseases that might affect bone metabolism were excluded via strict exclusion criteria. These diseases included vital organs' chronic disorders, autoimmune-related diseases, metabolic diseases, skeletal diseases, hematopoietic diseases, lymphoreticular disease, and other diseases or any treatment that would potentially affect bone metabolism, such as bisphosphonates. The detailed characteristics of subjects were shown in Table 1 and Ref. [26]. Figure 1 showed the pipeline of integration analysis to identify mRNA/lncRNA co-expression networks for BMD variation.

PBM Isolation and RNA Sample Preparation

Sixty milliliter of peripheral blood was obtained from each subject and PBM isolation was performed immediately after blood collection. To minimize the potential cell-type heterogeneity, we used the Monocyte Isolation Kit II (MiltenyiBiotec, Auburn, CA, USA) to specifically isolate the classic PBM subset ($CD14^{++}CD16^{-}$) following the manufacturer's recommendation and protocols. Then, total RNA from the classic PBM subset ($CD14^{++}CD16^{-}$) was extracted using Qiagen RNeasy Mini kit (Qiagen, Inc., Valencia, CA) and the mRNA expression levels were scanned by the GeneChip Human Exon 1.0 ST Array (Affymetrix, Santa Clara, CA) based on the manufacturer's protocol. The raw microarray data for this cohort have been submitted to GEO (Gene Expression Omnibus) under the accession number GSE56814.

For mRNAs analysis, all raw CEL files were imported and processed by the Affy package in R [27]. MAS5.0 from Affy package was applied to normalize the array signals and differential expression analysis was performed using Student's *t* test through the Bioconductor's LIMMA (linear models for microarray data) package [28, 29]. The probe IDs were annotated with their corresponding official gene symbols. When multiple probe IDs were matched to the same gene symbol, the probe ID with the lowest *p*-value among these probe IDs was selected to represent that gene symbol. Because the sample size was limited (although still among the largest of such studies in the field), the adjusted *p*-values were too large after multiple testing control. We used raw *p*-value < 0.05 as threshold for nominally significant differential expression.

Re-Annotation

The microarray data were re-annotated using the software noncoder (<http://noncoder.mpi-bn.mpg.de/#>) [24]. This software was developed for annotating probes that uniquely map to lncRNAs [24]. Briefly, MAS5.0 was applied to normalize the data. Then, the probesets were filtered by the following steps: (1) lncRNAs with less than 3 probes were removed; (2) probes overlapping with protein-coding genes (defined as coding sequence regions on the same strand) were discarded; and (3) probes were only mapped to known lncRNAs in NONCODE v3.0 (which was included in the tools of noncoder) [14]. After re-annotation, the probes mapped to 12,007 lncRNAs in the human genome. The differential expression analysis was performed using the same method and threshold as the mRNA analysis described above. The power of this study was calculated by the sample size and power calculations for microarray software [30, 31].

Target Gene Prediction and lncRNA-mRNA Co-Expression Network

Using genome tool, Galaxy (<https://usegalaxy.org/>), and gene annotations at UCSC (<http://genome.ucsc.edu/>), the genes with transcripts within a 10 kb window upstream or downstream of given lncRNAs were identified as nearby genes. Ponjavic et al. [32] found that about half of the intergenic noncoding RNAs were located close (< 10 kb) to protein-coding genes. These lncRNAs were suggested to be the best candidates for investigating the cis-regulation mechanism [33]. If the nearby gene expression levels are significantly correlated with the given lncRNAs expression levels (*p*-value < 0.05), these genes will be considered as “cis-regulated target genes” [34].

The lncRNA-mRNA co-expression network was constructed among DE lncRNAs and DE mRNAs. Pearson's correlation coefficient value was calculated for each lncRNA-mRNA pair. If in these pairs, the mRNAs were not transcribed within a 10 kb window upstream or downstream of given lncRNAs and mRNA expression levels were significantly correlated (p -value < 0.05 and $r^2 > 0.64$) with the lncRNA expression levels, the mRNAs and lncRNAs were suggested to have a “trans-regulatory” relationship. This threshold was generated by reviewing literatures [35–37]. The network map was generated by Metscape software [38].

Gene Enrichment Analysis

To analyze the potential functions of lncRNAs and co-expressed genes, we uploaded all the genes in lncRNA-mRNA co-expression network and “cis-regulated target genes” into the Database for Annotation, Visualization and Integrated Discovery (DAVID). The lncRNA function was predicted by their co-expressed genes via gene ontology (GO) terms, enriched UniProtKB Keywords, and enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The threshold for significant enrichment was nominal p -values < 0.05 .

GWAS Data Validation

To further explore that the candidate lncRNAs were genetically associated with BMD in larger human populations, we used recent sequencing and imputation data from the meta-analysis for the Genetic Factors for Osteoporosis (GEFOS) Consortium (GEFOS-seq). It is the largest meta-analysis based on next-generation sequencing technologies and associated imputation to date for BMD study, including 9 GWASs and 32,965 Caucasians [39]. The candidate lncRNAs consisted of the lncRNAs involved in lncRNA-mRNA “cis-regulation” pairs or lncRNA-mRNA co-expression network described above. We first selected the single-nucleotide polymorphisms (SNPs) which located in the candidate lncRNAs regions. Then, we obtained the meta-analysis p -values of those SNPs for lumbar spine BMD (LS BMD) and femoral neck BMD (FN BMD) traits. The most significant SNP for a given lncRNA was chosen as a gene-level p -value. The threshold for significant association between lncRNAs and BMD traits was nominal p -values < 0.05 .

Least Absolute Shrinkage and Selection Operator

Glmnet R package [40] was used to verify the lncRNA signature for BMD variation. Glmnet was a package that fits a generalized linear model via penalized maximum likely-hood [40]. The basic concept of generalized linear model was to assign a coefficient (β) to each independent variable (x) to predict the dependent variable (y). In our case, x was the expression level of each differential expressed lncRNA and y was binary trait for BMD. It meant that high BMD subjects were labeled as “ $y = 1$ ” and low BMD subjects were labeled as “ $y = 0$.” We used least absolute shrinkage and selection operator (Lasso) [41] regression implemented in Glmnet package to generate the prediction signature. We used glmnet package to randomly divide the sample dataset into tenfolds and performed cross-validation to generate the optimal λ for the prediction model which gave minimum mean cross-validated error.

Results

Expression Profiles of mRNAs and lncRNAs

Through analyzing the exon array data, 591 genes were identified as nominal DE genes (raw $p < 0.05$) among the “core set genes” ($n = 22,011$, <http://www.affymetrix.com/support/technical/byproduct.affx?product=huexon-st>). In these DE genes, 482 genes were upregulated, and 109 genes were downregulated in the high BMD group. After re-annotating the probes to lncRNAs, 12,007 lncRNAs were detected in our study. Among them, 575 lncRNAs were identified to be DE between the high and low BMD subjects. In the high BMD cohorts, 266 lncRNAs were downregulated and 309 lncRNAs were upregulated (Supplementary Table 1). The statistical power was 0.85 and type II error was 0.15 (effect size as 0.5 and significance level as 0.05). Figure 2 showed the genomic classification and distribution of the DE lncRNAs. The whole dataset consisted of 57.74% intergenic lncRNAs, 17.74% intronic lncRNAs, 7.30% exonic lncRNAs, and others.

Target Gene Prediction for DE lncRNAs

Because lncRNAs can regulate the transcriptions of nearby genes via cis-regulatory effect [33, 34], we screened the 10 kb upstream and downstream of the DE lncRNAs. Four lncRNA-mRNA pairs (*n381782/RPL23AP64*, *n385273/MGC27345*, *n345180NR4A1*, *n339526/ABCG1*) were found to be co-expressed and all these associations were positive (Fig. 3).

lncRNA-mRNA Co-Expression Network

Based on the correlation analysis results, we constructed a lncRNA-mRNA co-expression network, including 164 interactions between 25 lncRNAs and 64 mRNAs (p -value < 0.05 and absolute value of correlation coefficient > 0.8 , Fig. 3 and Supplementary Table 2). The network showed the potential regulatory relationship between lncRNAs and mRNAs. For example, lncRNAs *n387169*, *n334197*, *n409366*, *n385273*, *n345283*, and *n408906* played as “hub” regulators which had numerous interactions with different mRNAs.

Gene Enrichment Analysis

Table 2 summarized the results of enrichment analysis of 64 co-expressed genes with lncRNAs. Prediction terms with raw p -value less than 0.05 were selected and ranked by raw p -values. The most significant term in UniProtKB Keywords was *osteogenesis* (raw p -value = 0.004, FDR=0.047).

Other top enriched terms included *transcription regulation*, *protein transport*, and *apoptosis*. For GO term analysis, *GO:0005515~protein binding* (molecular function aspect) and *GO:0006351transcription, DNA-templated* (biological process aspect) were found to be the most enriched terms based on the raw p -values. However, the terms directly related to bone resorption, such as metal/calcium ion binding, ossification, osteoclasts, were not identified as significant ones.

Validation by GWAS Studies

Table 3 showed the results from GEFOS-seq meta-analysis and gene-level p -values for both FN BMD and LS BMD, represented by the most significant SNP/marker in the gene. Totally, 26 lncRNAs were chosen as candidate lncRNAs for GWAS validation. Four of them were from the lncRNA-mRNA “cis-regulation” pairs and 25 lncRNAs were from “trans-regulation” lncRNA-mRNA pairs (three lncRNAs were shared by the two methods). In these candidate lncRNAs, 16 lncRNA sequences included SNPs associated with FN BMD and 13 lncRNA sequences included SNPs associated with LS BMD (11 lncRNAs overlapping between the FN and LS BMD GWAS findings).

Validation by Lasso Feature Selection

Least absolute shrinkage and selection operator (Lasso) method was performed to determine whether the subset of differential expressed lncRNAs could be used to predict BMD variation. Totally, we identified 47 lncRNAs as signatures for predicting BMD variation (Supplementary Table 3). In these signature lncRNAs, 6 lncRNAs (*n337687*, *n408906*, *n381782*, *n387169*, *n345180*, and *n342493*) were overlapped with the lncRNA-mRNA co-expression network described above (Supplementary Table 3).

Discussion

In this study, we profiled the expression of lncRNAs in PBMs by re-annotating Affymetrix Exon 1.0 ST Array and provisionally identified the DE lncRNAs and mRNAs between high and low hip BMD. These lncRNAs might affect the skeletal metabolism via regulating mRNAs expression. Thus, we applied two approaches to predict the target genes of the DE lncRNAs in DE gene list. For “cis-regulation,” we found four DE lncRNAs were associated with their nearby genes. Via constructing lncRNA-mRNA co-expression network, we found 164 interactions between DE genes and DE lncRNAs. Gene enrichment analysis was performed to further explore the biological function of DE lncRNAs in bone density regulation process. To validate the importance of DE lncRNAs for BMD, we screened the SNPs located in DE lncRNAs in the largest GWAS database (GEFOS-seq) for the bone study.

In the human genome, only about 2% are protein-coding genes, the majority of transcripts were classified to noncoding RNAs (ncRNAs) [15]. LncRNA was one kind of ncRNAs defined by its length (> 200 bp). Partially because of its length, the expression of lncRNA could be detected by mining existing microarray data, although the microarray was not designed for lncRNA analysis. The Affymetrix Exon 1.0 ST Array contained more than 5.5 million probes. Its many probes designed for expressed sequence tags (ESTs) and prediction-based transcripts could uniquely map to the lncRNA sequences [24]. Comparing with conducting a new RNA sequencing experiment, it was a relatively cost-effective approach to analyze the lncRNAs by re-proposing the microarray probes for lncRNA expression. In this study, we detected 12,007 lncRNA expression levels by re-annotating the microarray probes and 22,011 mRNA expression levels at the same time.

With the expression profiles of lncRNAs and mRNAs, we provisionally identified the DE lncRNAs and DE mRNAs between high BMD and low BMD groups. Currently, several studies were performed on the expression profiles of lncRNAs in osteoarthritis [42], osteosarcoma [43], chondrogenic differentiation [44], osteoclast differentiation [20], and osteoblast differentiation [45]. However, there was few lncRNAs profiling study in vivo in humans in the field of osteoporosis. A pioneering study [19], which only focused on one lncRNA (*DANCR*), reported that the expression of *DANCR* was upregulated in the blood mononuclear cells from postmenopausal low BMD patients. But in our study, the *DANCR* (noncode id: *n378655*) was not significantly associated with BMD variation. It ranked at 5484 with p -value = 0.45. This is not unexpected since the subjects in our study consisted of both pre- and postmenopausal women and the “ethnicity” of subjects were different (Chinese in their study and Caucasian in ours).

To infer the biological function of lncRNAs in BMD variation, we explored the potential regulatory relationship among DE lncRNAs and DE mRNAs. For “cis-regulated target genes,” 4 lncRNA-mRNA pairs, *n381782/RPL23AP64*, *n385273/MGC27345*, *n345180/NR4A1*, and *n339526/ABCG1*, were found. Xiaoxiao et al. [46] demonstrated that *NR4A1* could prevent excessive osteoclastogenesis. Their experiments showed that *NR4A1* deletion could lead to low bone mass because of increasing osteoclast differentiation and bone resorption. It was consistent with our results, *NR4A1* had lower expression level in low BMD group. Another gene, *ABCG1*, was also reported to be involved in osteoclastogenesis [47]. The expression of *ABCG1* was significantly increased during osteoclast fusion. Our results were consistent with this finding. The low BMD group had higher expression level of *ABCG1*, implying they might have higher osteoclast differentiation rates. The function of these two genes suggested that their paired lncRNAs could potentially affect osteoclastogenesis.

In addition, we constructed a lncRNA-mRNA co-expression network to predict the “trans-regulatory” relationship between lncRNAs and mRNAs. From the network, we found that lncRNA *n387169*, *n334197*, *n409366*, *n385273*, *n345283*, and *n408906* were co-expressed with multiple genes. It demonstrated that these lncRNAs may play multiple roles in bone metabolism. In the lncRNA-mRNA co-expression network, the associations between lncRNAs and mRNAs were various. Since lncRNA is a broad definition just based on their length, it contains various types of RNA transcripts with different roles in gene expression [48, 49]. For instance, *Air* lncRNA can target *G9a* to inhibit the expression of *Igf2r* gene in *cis* [50], while the *Evl* lncRNA will cis-activate the *Dlx5/6* genes [51]. At the same time, the functions of lncRNAs were still largely unknown. In our study, the co-expressed network only showed the potential regulatory mechanism between each lncRNA-mRNA pair. Further studies are required to clarify whether potential regulatory mechanisms exist or they are simply regulated similarly.

In the gene enrichment analysis, we found that “*Osteogenesis*” was the most enriched term (raw p -value < 0.005 and fold enrichment > 30.90), including *SBNO2*, *FSTL3*, and *BMP8B*. *SBNO2* was associated with lncRNAs *n345283* and *n387169* and was reported to regulate osteoclast fusion by enhancing the expression of dendritic cell-specific trans-membrane protein (*DC-STAMP*) [52]. This result further showed the potential roles of our candidate

lncRNAs in the pathogenesis of osteoporosis. To validate the genetic function of candidate lncRNAs in BMD variation, we screened the GEFOS-seq database for SNPs analysis. Greater proportion (near 60%) of candidate lncRNA sequences included SNPs associated with FN BMD or LS BMD. It not only partially supported our current findings about lncRNAs, but also provides new information for candidate lncRNAs in the human genome.

In our study, we isolated the classic PBMs subset ($CD14^{++}CD16^{-}$) as the cell model for osteoporosis research. Studies suggested this subset of PBMs contained the precursors of osteoclasts [1, 8]. One study used $RANK^{++}$ as a marker to characterize osteoclast-committed precursors in PBMs and found that this subpopulation did not significantly induce more cumulative resorption than unfractionated $CD14^{+}$ cells, indicating the mature osteoclasts with similar lifespan and functional activity to those derived from the unfractionated PBMs [53]. Therefore, the classic PBMs were “homogeneous” with respect to their ability of differentiating to mature osteoclasts.

There were several limitations of this study. First, the PBMs or RNA samples or serum samples were exhausted or not available without an additional expensive recall of subjects back to this study. So, we could not conduct some additional functional relevant assays to further illustrate our findings in the regulation of BMD or a qPCR validation for our findings. Second, to increase the statistical power, we used a mix of premenopausal and postmenopausal women and in high BMD group, the proportion of postmenopausal women was higher than the one in low BMD group. At last, replication study was lacked to validate our findings and we thus only used GWAS data and alternative statistical methods (LASSO) to partially validate the importance of lncRNAs we found in BMD variation.

In summary, we performed a study of lncRNAs for osteoporosis risk. We reported the transcriptome-wide lncRNA expression profile by re-annotating Affymetrix Human Exon 1.0 ST arrays data. Our prediction of potential regulatory relationship among DE lncRNAs and DE mRNAs may provide the novel insights of biological functions of lncRNAs in the pathophysiological mechanism of osteoporosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
The pipeline of integration analysis to identify mRNA/lncRNA co-expression networks for BMD variation

Fig. 2.

Genomic classification and distribution of the differentially expressed lncRNAs. Sense was defined as overlapping to parts of the protein-coding gene on the same strand. The lncRNAs, which were closer than 1000 bps, but nonoverlapping, to the next protein-coding gene, were divided into extension category (encoded on the same strand) and close category (opposite strand)

Fig. 3. LncRNA-mRNA co-expression network. This network includes potential trans-regulations between 25 lncRNAs and 64 mRNAs and the 4 cis-regulatory pairs of lncRNAs and mRNAs are highlighted. Red color edges and blue color edges represent up and downregulation, respectively

Table 1

Basic characteristics of subjects for monocyte microarray analyses

Category	Premenopausal women with high BMD	Postmenopausal women with high BMD	Premenopausal women with low BMD	Postmenopausal women with low BMD
Sample size	16	26	15	16
Age (years)	51.0 (1.8)	54.0 (1.8)	50.0 (2.0)	52.6 (2.5)
Height (cm)	161.60 (5.23)	162.06 (9.21)	162.12 (7.28)	161.96 (8.54)
Weight (kg)	69.62 (10.17)	75.62 (13.92)	71.44 (12.75)	68.06 (11.75)
Hip BMD (Z score)	1.54 (0.52)	1.28 (0.46)	- 0.93 (0.36)	- 1.17 (0.60)

Data are shown as mean (standard deviation)

Table 2

Gene enrichment analysis for target mRNAs

Category	Term	p-value	FDR	Genes
UP_KEYWORDS	Osteogenesis	0.004	0.047	<i>SBNO2, FSTL3, BMP8B</i>
UP_KEYWORDS	Transcription regulation	0.014	0.152	<i>MORF4L1, SBNO2, HMX1, RELB, HDAC10, FSTL3, ZNF668, NFKB2, ZNF627, HMGA1, POU3F3, HBP1, TP53INP2</i>
UP_KEYWORDS	Transcription	0.017	0.184	<i>MORF4L1, SBNO2, HMX1, RELB, HDAC10, FSTL3, ZNF668, NFKB2, ZNF627, HMGA1, POU3F3, HBP1, TP53INP2</i>
UP_KEYWORDS	Repressor	0.018	0.192	<i>SBNO2, HMX1, RELB, HDAC10, HBP1, NFKB2</i>
UP_KEYWORDS	Protein transport	0.02	0.213	<i>PEX26, AHCTF1, VPS53, CCDC22, STAM, IFT27</i>
UP_KEYWORDS	Transport	0.028	0.283	<i>CYBA, ATP6V0E2, CACNG8, PEX26, KCNK18, AHCTF1, VPS53, CCDC22, STAM, IFT27, GLTP</i>
GOTERM_MF	GO:0005515~protein binding	0.046	0.421	<i>MORF4L1, SIVA1, IER5, FSTL3, VPS53, NFKB2, MMP3, DUSP13, ILVBL, MSI2, STAM, HBP1, CCDC22, ACIN1, UBTD1, IFT27, C22ORF39, RELB, HDAC10, NTNG2, NUDT10, SOCS4, HMGA1, ZNF627, KRTAP10-9, GLTP, CYBA, PLK3, NME3, ULK1, PEX26, NCAPH2, CPNE2, TP53INP2</i>
GOTERM_BP	GO:0006351~transcription, DNA-templated	0.047	0.475	<i>MORF4L1, SBNO2, HMX1, RELB, HDAC10, FSTL3, POU3F3, HBP1, ZNF668, ZNF627, TP53INP2</i>
UP_KEYWORDS	Apoptosis	0.049	0.448	<i>SIVA1, NME3, PLK3, DADI, ACIN1</i>

Table 3

Validation in GEFOS-seq dataset

	NONCODE_id	Most significant SNP	GEFOS-seq <i>p</i> -value
FN BMD	<i>n373125</i>	<i>rs10774864</i>	< 0.001
	<i>n364391</i>	<i>rs7355020</i>	0.002
	<i>n367849</i>	<i>rs191931504</i>	0.002
	<i>n381782</i>	<i>rs192113495</i>	0.002
	<i>n385273</i>	<i>rs6963848</i>	0.004
	<i>n341694</i>	<i>rs35266980</i>	0.004
	<i>n411666</i>	<i>rs76289765</i>	0.004
	<i>n408906</i>	<i>rs1866580</i>	0.005
	<i>n342493</i>	<i>rs151231023</i>	0.006
	<i>n376323</i>	<i>rs62115593</i>	0.008
	<i>n363827</i>	<i>rs10157401</i>	0.010
	<i>n372764</i>	<i>rs1517111</i>	0.012
	<i>n345180</i>	<i>rs56399921</i>	0.017
	<i>n373215</i>	<i>rs55950506</i>	0.027
	<i>n339526</i>	<i>rs116904230</i>	0.033
	<i>n409366</i>	<i>rs151106544</i>	0.045
LS BMD	<i>n341694</i>	<i>rs6560593</i>	0.001
	<i>n407724</i>	<i>rs192719639</i>	0.002
	<i>n342493</i>	<i>rs2602433</i>	0.007
	<i>n383738</i>	<i>rs1820983</i>	0.010
	<i>n337797</i>	<i>rs12738345</i>	0.016
	<i>n411666</i>	<i>rs17137208</i>	0.016
	<i>n345180</i>	<i>rs2701113</i>	0.024
	<i>n385273</i>	<i>rs6963848</i>	0.026
	<i>n367849</i>	<i>rs76824006</i>	0.029
	<i>n372764</i>	<i>rs7110521</i>	0.029
	<i>n408906</i>	<i>rs148756298</i>	0.031
	<i>n339526</i>	<i>rs116904230</i>	0.032
	<i>n381782</i>	<i>rs10892320</i>	0.039
	<i>n364391</i>	<i>rs76156789</i>	0.045