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Cytosolic proteome profiling of monocytes for male osteoporosis

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Abstract

Summary—In male Caucasians with discordant hip bone mineral density (BMD), we applied the subcellular separation and proteome profiling to investigate the monocytic cytosol. Three BMD-associated proteins (ALDOA, MYH14, and Rap1B) were identified based on multiple omics evidence, and they may influence the pathogenic mechanisms of osteoporosis by regulating the activities of monocytes.

Introduction—Osteoporosis is a serious public health problem, leading to significant mortality not only in aging females but also in males. Peripheral blood monocytes (PBMs) play important roles in bone metabolism by acting as precursors of osteoclasts and producing cytokines important for osteoclast development. The first cytosolic sub-proteome profiling analysis was performed in male PBMs to identify differentially expressed proteins (DEPs) that are associated with BMDs and risk of osteoporosis.

Methods—Here, we conducted a comparative proteomics analysis in PBMs from Caucasian male subjects with discordant hip BMD (29 low BMD vs. 30 high BMD). To decrease the proteome complexity and expand the coverage range of the cellular proteome, we separated the PBM proteome into several subcellular compartments and focused on the cytosolic fractions, which are involved in a wide range of fundamental biochemical processes.

Results—Of the total of 3796 detected cytosolic proteins, we identified 16 significant ($P < 0.05$) and an additional 22 suggestive ($P < 0.1$) DEPs between samples with low vs. high hip BMDs. Some of the genes for DEPs, including *ALDOA*, *MYH14*, and *Rap1B*, showed an association with

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Compliance with ethical standards

Conflicts of interest None.

BMD in multiple omics studies (proteomic, transcriptomic, and genomic). Further bioinformatics analysis revealed the enrichment of DEPs in functional terms for monocyte proliferation, differentiation, and migration.

Conclusions—The combination strategy of subcellular separation and proteome profiling allows an in-depth and refined investigation into the composition and functions of cytosolic proteome, which may shed light on the monocyte-mediated pathogenic mechanisms of osteoporosis.

Keywords

Cytosolic proteome; Glucose metabolism; Osteoporosis; Peripheral blood monocytes; Regulation of the actin cytoskeleton

Introduction

Osteoporosis, one of the most common public health threats in modern society, is a chronic skeletal disorder featuring whole-body loss of bone mineral density (BMD) and micro-architectural deterioration of bone tissue [1]. Patients suffering from osteoporosis and osteoporotic fractures are vulnerable to high postoperative morbidity and mortality. Osteoporosis has been recognized as a prevalent disease for post-menopausal women, who experienced dramatic decrease in estrogen production [2]. However, males actually possess a higher risk of mortality from osteoporotic fracture than women, and male osteoporosis has been receiving increasing attention in recent years [3].

Bone tissue undergoes dynamic reconstruction in a delicate metabolism balance between bone resorption by osteoclasts and bone formation by osteoblasts to sustain calcium homeostasis and bone integrity [4]. Due to obvious difficulty in collecting fresh and sufficient osteoclasts from bone tissues in humans, peripheral blood monocytes (PBMs) are commonly used as a cell model to study osteoclastogenesis activities for the risk of osteoporosis [5, 6]. PBMs could act as precursors of osteoclasts; produce cytokines crucial for osteoclast differentiation, activation, and apoptosis; and serve as the major systemic target cells for sex hormones in bone metabolism [7]. These processes are likely to be further enhanced during fracture healing, sex hormone deficiency, or under pathological inflammatory conditions associated with various skeletal disorders, such as rheumatoid arthritis (RA) and periodontal disease [7]. Several novel osteoporosis susceptibility genes have been identified by evidence from three genetic levels (DNA, mRNA, and protein) and functional evidence from both in vivo and in vitro studies [5, 6, 8]. Recently, our group [9] and Farber [10] independently illustrated that PBM transcriptomic data can be used to extract additional biological functional information from GWAS datasets, allowing for discovery of novel osteoporosis-associated genes and prioritization of genes and SNPs for replication studies. Our group also conducted another in silico study, integrating GWASs, human protein-protein interaction (PPI) network, and monocytic gene expression from high vs. low BMD subjects, and found two modules of genes contributing to osteoporosis risk by participating in Wnt receptor signaling and osteoblast differentiation, respectively [11]. Together, these studies indicate that PBMs represent a valuable working cell model for studying mechanistic differences occurring in bone metabolism between health and disease conditions.

Quantitative proteomics via liquid chromatography (LC) in tandem with mass spectrometry (MS) has been employed as a powerful strategy to profile proteome composition and identify differentially expressed proteins associated with human bone-related disorders [8, 12]. However, the extensive complexity of proteomes undermines the analytical capacity of MS, and low abundance proteins are prone to be masked by pooled high abundance proteins [13]. Separating subcellular compartments is an efficient strategy to decrease proteome complexity and expand the coverage range of subcellular proteomes and to indicate protein transient localization and dynamic transportation in cellular functioning [14].

In osteoclast precursors, a wide range of essential biochemical processes occur in cytosol for osteoclast proliferation, migration, and fusion to form multinuclear cells. For example, glycolysis, a metabolic pathway, occurs in the monocytic cytosol, giving rise to adenosine triphosphate (ATP) as one of the main energy sources for cellular differentiation and migration [15]. Cytosolic proteins for biosynthetic substrates and metabolic enzymes for energy generation are increasingly expressed during the receptor activator of nuclear factor kappa-B ligand (RANKL)-stimulated osteoclastogenesis [16]. Importantly, proteins in monocytes may have specific functional roles depending on distinct subcellular localizations. For instance, the nuclear P21 protein inhibits cell cycle progression [17], while the cytoplasmic P21 protein induces cellular differentiation and resists apoptosis in monocytes [18]. However, comparative proteomics focused on monocytic cytosol have not yet been conducted to identify functional proteins and mechanisms for osteoporosis etiology.

In this study, we performed the first cytosolic sub-proteome profiling analysis in human PBMs from Caucasian males with extremely discordant hip BMDs and identified promising differentially expressed proteins (DEPs) that are associated with BMDs and risk of osteoporosis, and that are also supported by evidence from multiple omics analyses and by supplementary bioinformatics analyses.

Materials and methods

The discovery cohort

The study was approved by the appropriate Institutional Review Boards. Signed informed consent documents were obtained from all study participants. A total of 59 unrelated Caucasian men from Kansas City, Missouri, and its surrounding areas were enrolled in the discovery study (cohort 1). They were composed of 29 subjects with low hip BMD and 30 subjects with high hip BMD (Z-score mean $\pm$ standard deviation: -1.2 ± 0.4 vs. 1.6 ± 0.6 , respectively) (Table 1), corresponding roughly to the bottom 30% (low BMD) vs. top 19% (high BMD) of BMD distribution in age- and gender-matched Caucasian population [19]. We computed statistical power of different sample sizes for a proteomic study under the framework of ANOVA following the procedures as illustrated by Kirk [20]. According to the calculation, a sample size of 30 per comparison group will give us more than 98% power for identifying proteins with a between/within-group variance ratio of 1.5 and 90% power for detecting proteins of smaller effects (1.5 ratio 1.1). Therefore, our sample size is reasonable in that it will not only render that proteins of large effects be identified but also maintain a reasonable power for detecting those of moderate effects. Enrolling subjects with extremely discordant BMDs has been demonstrated to be an efficient approach to enhance

the statistical power for identification of genomic and functional genomic elements for human complex disorders/traits [21].

In order to empirically enhance the study power, we applied a strict set of exclusion criteria to minimize/exclude non-genetic factors that might affect bone metabolism and BMD. The exclusion criteria included chronic disorders involving vital organs (heart, lung, liver, kidney, and brain), serious metabolic diseases (such as diabetes, hypo- or hyper-parathyroidism, and hyperthyroidism), other skeletal diseases (such as Paget's disease, osteogenesis imperfecta, and rheumatoid arthritis), chronic use of drugs affecting bone metabolism (such as corticosteroid therapy, anticonvulsant drugs, estrogens, and thyroid hormones), and malnutrition conditions (such as chronic diarrhea and chronic ulcerative colitis). To further minimize effects of any known disorders or conditions that might affect gene expression of PBMs, we also adopted additional exclusion criteria, including influenza (within 1 week of recruitment), autoimmune or autoimmune-related diseases (such as systemic lupus erythematosus), immunodeficiency conditions (such as AIDS), and hematopoietic and lymphoreticular malignancies (such as leukemia).

BMD measurement

Total hip BMDs (g/cm^2) of the subjects from cohort 1 were measured with Hologic discovery dual energy X-ray absorptiometer scanners (Hologic, Waltham, MA, USA). Total hip BMD was a combined value of femoral neck, trochanter, and inter-trochanter BMD. The machines were calibrated daily with a control vertebral phantom. The coefficient of variation for repeated total hip BMD measurements was about 1.34%. For each subject, age, height, weight, and other demographic information were also measured and recorded. Basic information for the discovery cohort is shown in Table 1.

PBM isolation

PBMs can be divided into three subsets (classical, non-classical, and intermediate) with various properties in morphology and physiology, and mature osteoclasts derived from various subsets have similar functionality and lifespan [22]. However, to minimize the cell-type heterogeneity, we specifically study the classical PBM subset ($\text{CD14}^{++}\text{CD16}^{-}$), which is the predominant part of human PBMs and PBM-related osteoclast precursors [23].

Peripheral blood mononuclear cells (PBMCs) were isolated from 120 ml fresh whole blood drawn from each subject, using density gradient centrifugation with Histopaque-1077 (Sigma-Aldrich, Cat. No. 10771). PBMs were extracted from PBMCs with the Monocyte Isolation Kit II (Miltenyi Biotec, Cat. No. 130-091-153) following the manufacturer's recommendation. Flow cytometry analyses showed that the purity of isolated PBMs was generally around 90% [5].

Subcellular proteome extraction and profiling

Making use of the different solubilities of subcellular compartments in selected reagents (ProteoExtract® Subcellular Proteome Extraction Kit, Emd Millipore, Cat. NO. 539,790), four subcellular components (cytosolic fraction, membrane/ organelle protein fraction, nucleic protein fraction, and cytoskeletal fraction) were extracted from the collected PBMs.

NanoAcquity Ultra Performance Liquid Chromatography (UPLC) and Synapt High Definition Mass Spectrometry (HDMS) (Waters Corporation, Milford, MA) were used to profile PBM cytosolic proteomes. Briefly, the protein digests (~500 ng) were injected into a 300- $\mu\text{m} \times 50\text{-mm}$ XBridge PST C18 NanoEase Column (Waters) and separated by solvent A (water with 0.1% formic acid (FA)) and solvent B (acetonitrile with 0.1% FA) at a flow rate of 0.3 $\mu\text{l}/\text{min}$ using a gradient of 2 h as follows: 3% B initial, 10% B at 1.0 min, 30% B at 75 min, 40% B at 90 min, 95% B at 91 min, 95% B at 95 min, 3% B at 96 min, and equilibrate thereafter till 120 min. The eluate was analyzed by HDMS under positive ion V-mode. The following parameters were set for data acquisition: collision energy: 5 V for MS and ramp 15–40 V for MS^E; scan time: 0.6 s per scan. The HDMS machine was calibrated daily to ensure high accuracy (2.0 ppm for lock mass of m/z 785.8426).

LC-nano-ESI-MS^E original datasets were acquired from each PBM proteome digest sample. The MS^E data were processed with ProteinLynx Global Server v2.4 (Waters) using default parameters. Based on the alternating low and elevated energy nature of MS^E data, properties of each ion (mass-to-charge ratio, retention time, intensity, etc.) were determined, and a list of all precursor and product ions was produced. Specifically, the ion's intensity was derived from the areas of both the chromatographic and mass spectrometric peaks. The precursor ion intensity threshold was set at above 1000 counts. The International Protein Index v3.56, a human protein database with 153,078 protein entries, was searched using the following optimized parameters recommended by Waters Corporation: enzyme specificity: trypsin; number of missed cleavages permitted: 1; fixed modification: carbamidomethyl C; variable modifications: Acetyl N-TERM, Deamidation N, Deamidation Q, and Oxidation M; mass tolerance for precursor ions: 15 ppm; mass tolerance for product ions: 30 ppm; minimum peptide matches per protein: 1; minimum fragment ion matches per protein: 7; minimum fragment ion matches per peptide: 3; false positive rate: limited to 4% per randomized database searching. The integrated intensities of the top three matched peptides per protein were used as a measure of absolute quantification. The relative quantitative level of proteins was achieved by normalizing absolute quantification against internal reference (the standard alcohol dehydrogenase 1 (ADH1)) and exported in femtomol and nanogram [6].

Statistical analysis

To eliminate confounding effects for protein expression from physical factors including weight, height, and age, the protein expression values were adjusted by multiple linear regression, and these factors of corresponding subjects were selected as covariates. Two tailed Student's *t* test was used to identify DEPs in low vs. high BMD groups. DEPs with *P* value <0.05 or 0.1 were considered statistically nominally significant or suggestively significant, respectively. In the present study, we slightly broadened the threshold for DEPs in order to explore more candidate proteins (to minimize false negative findings at the discovery stage) and their interactive functional sets for skeletal development and BMD variation.

Gene ontology and pathway analysis

The Database for Annotation, Visualization and Integrated Discovery (DAVID) (<http://david.abcc.ncifcrf.gov/>) [24] was used to evaluate the enrichment of DEPs in specific gene

ontology (GO) terms. EASE P value is a modified Fisher exact P value that is used to measure the gene enrichment in annotation terms, the smaller P value, the higher probability of enrichment. We chose 0.05 as the EASE P value threshold and *Homo sapiens* as background. Then, the functional annotation clustering analyses of DEPs were performed in medium classification stringency. The functional annotation clustering integrates the same techniques of Kappa statistics to measure the degree of the common genes between two annotations, and fuzzy heuristic clustering to classify the groups of similar annotations according to kappa values. The cluster enrichment score, the geometric mean (in $-\log$ scale) of member's P values in a corresponding annotation cluster, is used to rank their biological significance. We chose 1.3 as the significant threshold for enrichment score, which is equal to $-\log_{10}0.05$.

The ClueGO plug-in based on Cytoscape platform [25] was used to evaluate and visualize the pathways enriched with genes of identified proteins in a functional network. The ClueGO enrolled comprehensive pathway databases including KEGG, WikiPathways, and Reactome. Here, we chose 0.05 as P value threshold to detect whether the input genes are enriched in pathway than random chance, and 10% as the lowest percentage of input genes/total genes involved in pathway was applied to minimize the false discovery rate for enrichment analysis. The functional network was created with kappa statistics and reflects the relationships between the pathways based on the similarity of their associated genes.

To further evaluate cellular mechanisms and functions of proteins of interest, the protein-protein interactions (PPI) between DEPs were visualized based on the STRING database (<http://string-db.org/>) [26], which is composed of comprehensive evidence from experiments, literature, and curated databases. Parameters for species and confidence were set as "*Homo sapiens*" and "medium confidence (0.400)," respectively.

Multiple-omics analysis

Results from several previously reported studies were used to evaluate the across gender and across population applicability of the DEP genes identified in the male discovery cohort for association with BMD at multiple omics levels. These included four in-house PBM transcriptomic profiling studies (including respectively, 73 (cohort 2), 80 (cohort 3), and 19 (cohort 4) Caucasian premenopausal and postmenopausal females, and 26 premenopausal Chinese females (cohort 5)) [27], which involved subjects with extreme discordant hip BMDs. The basic characteristics of cohorts 2–5 are summarized in Supplementary Table 1.

We also leveraged the results from the largest meta-analysis of genome-wide association studies (GWAS) for BMDs. As the latest effort of the Genetic Factors for Osteoporosis Consortium (GEFOS), GEFOS-seq is composed of 9 GWAS with a total of 32,965 male and female subjects (cohort 6) [28]. The purpose is to assess whether genes/proteins identified in PBM for risk of male osteoporosis would manifest their significance in general populations at the DNA levels for BMD variation.

In this study, we performed a gene-based integrative analysis with protein expression, mRNA expression, and SNP association data to identify the genes associated with hip BMD in Caucasians. First, gene-phenotype association scores were calculated separately for

individual uni-omics data. For gene g , we computed q_g^k ($k = 1, \dots, D$, where D is the number of different omics levels) to capture the relationship between this gene and the phenotype, at different omics (proteomics, transcriptomics, and genomics) levels. As several cohorts may be available for a specific type of omics data (e.g., 5 studies with transcriptomics data are available in this project), we first assigned the signal with the most significant P value from cohorts at k th omics level to represent overall associated levels of genes g for BMD [29], termed as p_g^k . Second, we integrated the gene-phenotype association measurements ($p_g^1, \dots, p_g^D$) across D omics levels into an overall score (S_{g_meta}) [30]:

$$S_{g_meta} = -2 \sum_{k=1}^D \ln(p_g^k) \quad (1)$$

S_{g_meta} follows a chi-squared distribution with $2D$ degrees of freedom, if there is no association between the phenotype and any omics data for gene g . Finally, P values for the overall scores S_{g_meta} were adjusted using false discovery rate (FDR) in order to correct for multiple hypothesis testing [31]. Note that if there were more than one signal (e.g., probes or SNPs) for gene g in one cohort data, the signal with the most significant association with the phenotype was chosen as the representation for the gene in this cohort. For transcriptomics data, only those probes that have consistent expression trends with that observed in cohort 1 were selected for the meta-analysis.

Results

Through cytosolic proteomic profiling analysis in cohort 1, we detected expression signals for a total of 3796 proteins in PBMs. To reduce false positive signals, we selected 400 proteins that were detected in at least 5 samples in each BMD group for subsequent DEP analysis. To assess the overall significant of genes for BMD variation, 321 proteins which have signals at each omics level were enrolled in a meta-analysis, and their P values were further adjusted by using FDR for multiple hypothesis testing.

Among the 400 tested proteins, 16 proteins showed nominally significant ($P < 0.05$) differential expression between low vs. high BMD groups, and an additional 22 proteins showed suggestively significant ($P < 0.1$) evidence for differential expressions (Table 2). For instance, CFL1 (cofilin 1, $P = 1.55 \times 10^{-3}$) and MYH14 (myosin, heavy chain 14, $P = 1.12 \times 10^{-2}$) showed significantly upregulated expression levels in low BMD subjects compared to those in the high BMD subjects, and ALDOA (aldolase, fructose-bisphosphate A, $P = 5.38 \times 10^{-2}$) and GSN (gelsolin, $P = 7.53 \times 10^{-2}$) reached the suggestive significance level.

Gene ontology analysis suggested the biological activities in which the enriched DEPs were involved (Table 3). The DEPs were significantly enriched in biological processes such as glucose catabolic process ($P = 9.1 \times 10^{-15}$), generation of precursor metabolites and energy ($P = 6.3 \times 10^{-7}$), and actin cytoskeleton ($P = 1.4 \times 10^{-7}$). Eleven DEPs including GSN, ENO1 (Enolase 1), and RAP1B (member of RAS oncogene family) were enriched in the cytosol compartment ($P = 6.1 \times 10^{-4}$). By condensing redundant terms and summarizing interrelationships, DAVID generated functional clusters composed of related annotation

terms associated with the DEP group. The top scoring cluster (enrichment score = 6.1) contained cellular activities such as glycolysis, cellular carbohydrate catabolic process, and generation of precursor metabolites and energy. Other highly ranked clusters were associated with actin cytoskeleton organization (enrichment score = 3.3) and anti-apoptosis (enrichment score = 2.1). Most of the DEPs involved in these activities were upregulated in subjects with low BMDs. Together, these functional annotation results suggest that the BMD-associated DEPs might participate in several essential biological processes occurring in cytosol to promote higher activities of osteoclastogenesis in low BMD subjects.

The 400 identified proteins were enriched in 27 pathways, which were divided into 9 functional groups (Fig. 1). For instance, glycolysis/gluconeogenesis pathway was the leading term for the energy metabolite group, and leukocyte transendothelial migration was the leading term for the actin cytoskeleton regulation group (Supplementary Table 3). Twenty identified proteins were enrolled in the glycolysis/gluconeogenesis pathway (Supplementary Table 3), and 8 DEPs and 7 other proteins (though not significant) were upregulated in subjects with low hip BMD, presenting a general enhancement of protein expression for glycolysis/gluconeogenesis activation. For the pathway of leukocyte transendothelial migration, 15 out of 21 involved proteins had higher expression in the low BMD group, including three DEPs of RAP1B, ACTN1 (actinin alpha 1) and MYL9 (myosin light chain 9) (Supplementary Table 3). In the PPI network analysis, we found that these DEPs have multiple interactions with each other, including binding, catalysis, positive activation, and posttranslational modification (Fig. 2). For example, MYH14 may influence posttranslational modification to MYL9, as does ACTB (actin, beta) to CFL1, and HSP90AA1 (heat shock protein 90 kDa alpha family class A member 1) has co-expression and interaction with HSPA5 (heat shock protein family A (Hsp70) member 5).

To further assess the significance of these DEP genes to BMD variation, we examined genes encoding the significant/ suggestive DEPs for association with BMD at transcriptomic and genomic levels using several previously reported datasets (Supplementary Table 2). Twenty-four DEPs have supportive evidence from multiple biological levels and achieved highly significant adjusted *P* values for meta-analysis (Supplementary Table 2). The significant differential expression of the *MYH14* gene was well-established in SNP association study from cohort 6 ($P = 2.15 \times 10^{-3}$) and FDR adjusted meta-analysis ($P_{\text{adj}} = 7.00 \times 10^{-3}$). The BMD association of the RAP1B gene was supported by evidence from SNP association with hip BMD ($P = 1.40 \times 10^{-3}$) and FDR adjusted meta-analysis ($P_{\text{adj}} = 1.01 \times 10^{-2}$). Additionally, differential expression of *adenylate cyclase-associated protein 1* (*CAP1*) and *thrombospondin 1* (*THBS1*) had supporting evidence from protein expression, mRNA expression, SNP association, and gene-based integrative analysis.

Discussion

Our study represents the very first cytosolic sub-proteome profiling analysis in PBMs for osteoporosis. We identified 16 significant and 22 suggestively significant DEPs between Caucasian males with low vs. high hip BMDs. DEPs were enriched in various important biological processes in the cytosol compartment, such as glycolysis, regulation of actin cytoskeleton, and leukocyte transendothelial migration, which may orchestrate PBM

proliferation, differentiation, and apoptosis and subsequently affect osteoclast activities. Some of the identified DEP genes also exhibited BMD association evidence in independent proteomic, transcriptomic, and genomic studies. With this evidence from multiple levels, genes of ALDOA, MYH14, and RAP1B are likely to be promising candidates for regulation of hip BMD variation and risk of osteoporosis (Table 4).

The serial stages of osteoclast differentiation, proliferation, migration, and final fusion into multi-nucleated cells are energy-demanding processes. The main energy source for cell activities is ATP, which is generated by glucose metabolic pathways, including glycolysis in the cytosol and tricarboxylic acid (TCA) cycle and oxidative phosphorylation in the mitochondria. Interestingly, glycolysis and mitochondrial respiration were increased at an earlier stage of the RANKL-induced osteoclastogenesis than in no-stimulated cells, which accelerated metabolic shifts for high ATP production [16]. These metabolic alternations sustained efficient osteoclast differentiation through a synergistic stimulation of RANKL-induced MAPK activation (ERK and JNK) [32]. Additionally, enhanced glycolysis can prolong survival of human PBMs when exposed to low oxygen conditions and is associated with their differentiation and maturation [33]. In this study, we found that 10 DEPs (ALDOA, ENO1, GAPDH, LDHAL6A, LDHB, LDHC, PGD, PGAM1, PGAM4, and PKM2), which account for ~30% members of functional groups, were involved in glucose metabolism. Among the 10 DEPs, the differential expression of ALDOA, lactate dehydrogenase A like 6A (LDHAL6A) and lactate dehydrogenase B (LDHB) proteins were further confirmed by individual transcriptomic and genomic studies. Notably, ALDOA was identified as a BMD-associated genetic factor in monocytes by integrating gene expression and methylation data in a trans-omics study for BMD variation [34]. The expression level of the DEPs associated with glucose metabolism were all upregulated in subjects with low BMDs, suggesting that enhanced energy production was involved to support monocyte activities for osteoclastogenesis. In addition, ALDOA exerts a biphasic effect for osteoclastogenesis by regulating β -catenin activation. Through specifically binding to GSK-3 β , aldolase isozymes (ALDOA, ALDOB, and ALDOC) detached the β -catenin degradation complex leading to the accumulation of β -catenin in cytosol [35]. In osteoclastogenesis, β -catenin induced the macrophage colony-stimulating factor (M-CSF)-mediated proliferation of osteoclast precursor, while constitutive activation of β -catenin suppressed RANKL-mediated osteoclast differentiation [36]. Therefore, enhanced expression of ALDOA promoted the proliferation of PBMs in early osteoclastogenesis, accounting for subsequent osteoclast maturation and bone resorption.

Expression of MYH14 was significantly upregulated in subjects with low BMD from the discovery study and showed validation evidence from multi-omics study. The MYH14 gene encodes the non-muscle myosin II heavy chain (NMHC IIC) proteins which determine the non-muscle myosin II (NM II) isoforms in mammalian cells. NM II is an important regulator of adhesion and polarity in cell migration involving the dynamic regulation of actin cytoskeleton. NM II determines the direction of cell migration by initiating symmetry break and forming the prospective rear of the cell, which results in the formation of a protrusion in the opposite direction [37]. In cell motility driven by actin polymerization in cellular protrusions, NM II generated a retrograde force of actin countering the advancement of the protrusion to reduce spreading rate [38]. In addition, increased NM II activation promoted

the formation of large actin bundles and mature adhesions [39], which may be prepared for cells' attachment to vessel endothelium wall, thereby launching cellular transendothelial migration [40]. Hence, the enhanced expression of MYH14 may activate and prompt transendothelial migration of monocytes from the peripheral vascular system into bone tissue, leading to subsequent bone resorption. MYH14 may also influence post-translational modification to MYL9 [41], and the phosphorylation of MYL9 mainly upregulates cellular migration and invasion by controlling actomyosin-based cell contractility and motility [42], increasing the formation of mature osteoclasts.

Significant differential expression of RAP1B was consistently detected in studies of proteomic, genomic, and gene-based integrative studies. *RAP1B* encodes a member of the RAS-like small GTP-binding protein superfamily, which regulates multiple cellular processes including cell adhesion and cell growth and differentiation [43]. It is also involved in pathways of leukocyte transendothelial migration and focal adhesion [44]. The GTP-bound form of RAP1 induces talin 1 (TLN1) to associate with cytoplasmic domain of β -integrin, thus transforming the integrin into high-affinity conformation [45]. The active integrin binds extracellular ligand with high affinity and transmits intracellular signals, including those that organize the cytoskeleton. Talin1-deficient osteoclast precursors can normally express differentiation markers when exposed to macrophage colony-stimulating factor (M-CSF) and the receptor activator of nuclear factor kappa-B (RANK) ligand, but they fail to attach to the substrate, and spread results in impaired development of mature osteoclasts [46]. Deletion of RAP1A and RAP1B in vitro produced osteoclasts showing the characteristic crenated appearance of cytoskeleton organizing dysfunction with defective bone-resorptive capacity, and the physiological mechanism was further confirmed in osteopetrotic mice with inhibited RAP1 expression [46]. This suggests that the expression levels of RAP1B may be a critical factor in physiological and pathological bone resorption.

The present study identified the significant composition of the cytosolic proteome by using integrative strategy of subcellular separation and proteome profiling, which was conducted to exploit the cytosolic-specific roles of DEPs in bone metabolism. The bioinformatic studies yielded supplementary clues for predicting the regulatory functions of proteins in bone-related gene networks and the association between proteins of interest. These potential pathophysiological mechanisms for BMD variation warrant further investigation by future molecular studies and animal studies, which may provide confirmatory evidence to validate our reasonable inferences.

Conclusion

As osteoporosis studies mainly focus on female patients, research on male osteoporosis is relatively rare. Profiling proteomes is a powerful means to infer important protein function and potential mechanisms for osteoporosis, but it has rarely been employed to specifically study cytosolic components of monocytes or any bone relevant homogeneous cells. The combination strategy of subcellular separation and proteome profiling allows an in-depth and refined investigation into the composition of the subcellular proteome and its functions, as a potent and sensitive detective tool for low abundance proteins. Comparative analyses in other subcellular proteomes of monocytes or other bone relevant homogeneous cell lines

will furnish supplemental strategies to gain insight into the distribution and dynamics of other major sub-proteome complexes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PBM s	Peripheral blood monocytes
BMD	Bone mineral density
DEP s	Differentially expressed proteins
ALDOA	Aldolase, fructose-bisphosphate A
MYH14	Myosin, heavy chain 14
RAP1B	Member of RAS oncogene family
GSN	Gelsolin
IL-1	Interleukin-1
IL-6	Interleukin-6

TNF-α	Tumor necrosis factor- α
MS	Mass spectrometer
PBMCs	Peripheral blood mononuclear cells
ATP	Adenosine triphosphate
RANKL	Receptor activator of nuclear factor kappa-B ligand
UPLC	Ultra performance liquid chromatography
HDMS	High-definition mass spectrometry
FA	Formic acid
ADH1	Alcohol dehydrogenase 1
GWAS	Genome-wide association studies
GO	Gene ontology
GEFOS	Genetic Factors for Osteoporosis Consortium
FDR	False discovery rate
DAVID	Database for Annotation, Visualization and Integrated Discovery
GO	Gene ontology
PPI	Protein-protein interactions
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
CFL1	Cofilin 1
FGG	Fibrinogen gamma chain
CBX3	Chromobox 3
CALR	Calreticulin
ACTN1	Actinin alpha 1
MYL9	Myosin light chain 9
ACTB	Actin, beta
HSP90AA1	Heat shock protein 90-kDa alpha family class A member 1
HSPA5	Heat shock protein family A (Hsp70) member 5
CAP1	Adenylate cyclase-associated protein 1
THBS1	Thrombospondin 1
TCA	Tricarboxylic acid

LDHAL6A	Lactate dehydrogenase A like 6A
LDHB	Lactate dehydrogenase B
TLN1	Talin1
M-CSF	Macrophage colony-stimulating factor
NMHC	IIC Non-muscle myosin II heavy chain
NM II	Muscle myosin II

References

1. Lin JT, Lane JM. Osteoporosis: a review. *Clin Orthop Relat Res*. 2004; 425:126–134.
2. Deng HW, et al. A whole-genome linkage scan suggests several genomic regions potentially containing quantitative trait loci for osteoporosis. *J Clin Endocrinol Metab*. 2002; 87(11):5151–5159. [PubMed: 12414886]
3. Center JR, et al. Mortality after all major types of osteoporotic fracture in men and women: an observational study. *Lancet*. 1999; 353(9156):878–882. [PubMed: 10093980]
4. Teitelbaum SL. Bone resorption by osteoclasts. *Science*. 2000; 289(5484):1504–1508. [PubMed: 10968780]
5. Liu YZ, et al. A novel pathophysiological mechanism for osteoporosis suggested by an in vivo gene expression study of circulating monocytes. *J Biol Chem*. 2005; 280(32):29011–29016. [PubMed: 15965235]
6. Deng FY, et al. Is GSN significant for hip BMD in female Caucasians? *Bone*. 2014; 63:69–75. [PubMed: 24607942]
7. Zhou Y, Deng HW, Shen H. Circulating monocytes: an appropriate model for bone-related study. *Osteoporos Int*. 2015; 26(11):2561–2572. [PubMed: 26194495]
8. Deng FY, et al. Peripheral blood monocyte-expressed ANXA2 gene is involved in pathogenesis of osteoporosis in humans. *Mol Cell Proteomics*. 2011; 10(11):M111. 011700.
9. Chen YC, et al. Integrative analysis of genomics and transcriptome data to identify potential functional genes of BMDs in females. *J Bone Miner Res*. 2016; 31(5):1041–1049. [PubMed: 26748680]
10. Farber CR. Systems-level analysis of genome-wide association data. *G3 (Bethesda)*. 2013; 3(1): 119–129. [PubMed: 23316444]
11. He H, et al. Integrative analysis of GWASs, human protein interaction, and gene expression identified gene modules associated with BMDs. *J Clin Endocrinol Metab*. 2014; 99(11):E2392–E2399. [PubMed: 25119315]
12. Lee JH, Cho JY. Proteomics approaches for the studies of bone metabolism. *BMB Rep*. 2014; 47(3):141–148. [PubMed: 24499667]
13. Chen EI, et al. Large scale protein profiling by combination of protein fractionation and multidimensional protein identification technology (MudPIT). *Mol Cell Proteomics*. 2006; 5(1): 53–56. [PubMed: 16272560]
14. Dreger M. Proteome analysis at the level of subcellular structures. *Eur J Biochem*. 2003; 270(4): 589–599. [PubMed: 12581199]
15. Morrison MS, et al. ATP is a potent stimulator of the activation and formation of rodent osteoclasts. *J Physiol*. 1998; 511(Pt 2):495–500. [PubMed: 9706025]
16. Kim JM, et al. Osteoclast precursors display dynamic metabolic shifts toward accelerated glucose metabolism at an early stage of RANKL-stimulated osteoclast differentiation. *Cell Physiol Biochem*. 2007; 20(6):935–946. [PubMed: 17982276]
17. Liu M, et al. Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U9 37. *Genes Dev*. 1996; 10(2):142–153. [PubMed: 8566748]

18. Asada M, et al. Apoptosis inhibitory activity of cytoplasmic p21(Cip1/WAF1) in monocytic differentiation. *EMBO J.* 1999; 18(5):1223–1234. [PubMed: 10064589]
19. Hoiberg M, et al. Population-based reference values for bone mineral density in young men. *Osteoporos Int.* 2007; 18(11):1507–1514. [PubMed: 17534538]
20. Kirk, RE. *Experimental design: procedures for the behavioral sciences.* 4th. Sage Publications; Thousand Oaks: 2013. p. 233–272.
21. Guey LT, et al. Power in the phenotypic extremes: a simulation study of power in discovery and replication of rare variants. *Genet Epidemiol.* 2011; 35(4):236–246. [PubMed: 21308769]
22. Ziegler-Heitbrock L, Hofer TP. Toward a refined definition of monocyte subsets. *Front Immunol.* 2013; 4:23. [PubMed: 23382732]
23. Komano Y, et al. Identification of a human peripheral blood monocyte subset that differentiates into osteoclasts. *Arthritis Res Ther.* 2006; 8(5):R152. [PubMed: 16987426]
24. Huang da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* 2009; 37(1):1–13. [PubMed: 19033363]
25. Bindea G, et al. ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics.* 2009; 25(8):1091–1093. [PubMed: 19237447]
26. Szklarczyk D, et al. The STRING database in 2011: functional interaction networks of proteins, globally integrated and scored. *Nucleic Acids Res.* 2011; 39:D561–D568. [PubMed: 21045058]
27. Liu YZ, et al. Attenuated monocyte apoptosis, a new mechanism for osteoporosis suggested by a transcriptome-wide expression study of monocytes. *PLoS One.* 2015; 10(2):e0116792. [PubMed: 25659073]
28. Zheng HF, et al. Whole-genome sequencing identifies EN1 as a determinant of bone density and fracture. *Nature.* 2015; 526(7571):112–117. [PubMed: 26367794]
29. Wang K, Li M, Bucan M. Pathway-based approaches for analysis of genomewide association studies. *Am J Hum Genet.* 2007; 81(6):1278–1283. [PubMed: 17966091]
30. Tyekucheva S, et al. Integrating diverse genomic data using gene sets. *Genome Biol.* 2011; 12(10):R105. [PubMed: 22018358]
31. Benjamini Y, Hochberg Y. Controlling the false discovery rate—a practical and powerful approach to multiple testing. *J Royal Stat Soc Series B—Methodol.* 1995; 57(1):289–300.
32. Ide R, et al. High glucose condition activates protein tyrosine phosphatases and deactivates insulin receptor function in insulin-sensitive rat 1 fibroblasts. *Biochem Biophys Res Commun.* 1994; 201(1):71–77. [PubMed: 8198614]
33. Roiniotis J, et al. Hypoxia prolongs monocyte/macrophage survival and enhanced glycolysis is associated with their maturation under aerobic conditions. *J Immunol.* 2009; 182(12):7974–7981. [PubMed: 19494322]
34. Zhang JG, et al. Integrative analysis of transcriptomic and Epigenomic data to reveal regulation patterns for BMD variation. *PLoS One.* 2015; 10(9):e0138524. [PubMed: 26390436]
35. Caspi M, et al. Aldolase positively regulates of the canonical Wnt signaling pathway. *Mol Cancer.* 2014; 13:164. [PubMed: 24993527]
36. Wei W, et al. Biphasic and dosage-dependent regulation of osteoclastogenesis by beta-catenin. *Mol Cell Biol.* 2011; 31(23):4706–4719. [PubMed: 21876000]
37. Yam PT, et al. Actin-myosin network reorganization breaks symmetry at the cell rear to spontaneously initiate polarized cell motility. *J Cell Biol.* 2007; 178(7):1207–1221. [PubMed: 17893245]
38. Cai Y, et al. Nonmuscle myosin IIA-dependent force inhibits cell spreading and drives F-actin flow. *Biophys J.* 2006; 91(10):3907–3920. [PubMed: 16920834]
39. Vicente-Manzanares M, et al. Non-muscle myosin II takes Centre stage in cell adhesion and migration. *Nat Rev Mol Cell Biol.* 2009; 10(11):778–790. [PubMed: 19851336]
40. Muller WA. Mechanisms of leukocyte transendothelial migration. *Annu Rev Pathol.* 2011; 6:323–344. [PubMed: 21073340]

41. Andersen GO, et al. Alpha(1)-AR-induced positive inotropic response in heart is dependent on myosin light chain phosphorylation. *Am J Physiol Heart Circ Physiol*. 2002; 283:H1471–H1480. [PubMed: 12234799]
42. Licht AH, et al. Junb regulates arterial contraction capacity, cellular contractility, and motility via its target Myl9 in mice. *J Clin Invest*. 2010; 120(7):2307–2318. [PubMed: 20551518]
43. Caron E. Cellular functions of the Rap1 GTP-binding protein: a pattern emerges. *J Cell Sci*. 2003; 116(Pt 3):435–440. [PubMed: 12508104]
44. Filippi MD. Leukocyte transcellular diapedesis: Rap1b is in control. *Tissue Barriers*. 2015; 3(3):e1052185. [PubMed: 26451346]
45. Tadokoro S, et al. Talin binding to integrin beta tails: a final common step in integrin activation. *Science*. 2003; 302(5642):103–106. [PubMed: 14526080]
46. Zou W, et al. Talin1 and Rap1 are critical for osteoclast function. *Mol Cell Biol*. 2013; 33(4):830–844. [PubMed: 23230271]

Fig. 2.
Protein-protein interaction network

Table 1

Basic sample information

Category	Cohort 1 (Caucasian male)	
	Low BMD	High BMD
Sample size	29	30
Age (years)	40.3(7.6)	41.1(7.5)
Height (cm)	173.7(7.3)	178.2(7.1)
Weight (kg)	75.1(15.7)	103.7(23.2)
Hip BMD (g/cm ²)	0.86(0.06)	1.22(0.08)
Hip BMD Z-score	-1.2(0.4)	1.6(0.6)
Regular exercise (numbers/week)	5(2)	5(2)
Alcohol drinking (drinks/week)	7(7)	3(3)
Milk drinking (cups/week)	10(6)	11(8)
Smoking (start age)	18(13)	16(7)

Presented as mean (standard deviation). Z-score is defined as the number of standard deviations a subject's BMD differs from the average BMD of their age-, gender-, and ethnicity-matched population

Differential expression proteins in cohort 1

Table 2

Accession no.	Gene symbol	P value	Number L-BMD	Number H-BMD	Regulation direction
IP100979518.1	<i>CFL1</i>	1.55×10^{-3}	6	7	Up
IP100220278.5	<i>MYL9</i>	7.29×10^{-3}	5	8	Up
IP100847989.3	<i>PKM2</i>	8.21×10^{-3}	8	10	Up
IP100337335.8	<i>MYH14</i>	1.12×10^{-2}	13	13	Up
IP100795257.3	<i>GAPDH</i>	1.15×10^{-2}	14	12	Up
IP100908876.1	<i>ALB</i>	1.20×10^{-2}	5	7	Up
IP100005161.3	<i>ARPC2</i>	1.29×10^{-2}	17	17	Up
IP100708398.2	<i>ALB</i>	1.40×10^{-2}	28	30	Down
IP100374975.2	<i>PGAM4</i>	1.66×10^{-2}	19	21	Up
IP100013508.5	<i>ACTN1</i>	1.91×10^{-2}	24	23	Up
IP100003362.3	<i>HSPA5</i>	2.89×10^{-2}	25	26	Up
IP100784258.3	<i>LTBP1</i>	3.13×10^{-2}	5	5	Up
IP101014435.1	<i>RAP1B</i>	3.41×10^{-2}	8	10	Up
IP101013638.1	<i>LDHC</i>	4.19×10^{-2}	9	11	Up
IP100555614.1	<i>HSP90AB3P</i>	4.19×10^{-2}	16	17	Up
IP100216134.3	<i>TPM1</i>	4.25×10^{-2}	11	7	Up
IP100218319.3	<i>TPM3</i>	5.05×10^{-2}	6	11	Up
IP100877792.1	<i>FGG</i>	5.14×10^{-2}	8	8	Down
IP100796333.1	<i>ALDOA</i>	5.38×10^{-2}	7	11	Up
IP100297579.4	<i>CBX3</i>	5.78×10^{-2}	9	7	Up
IP100236556.1	<i>MPO</i>	5.81×10^{-2}	8	11	Up
IP100979275.1	<i>LOC652797</i>	6.11×10^{-2}	7	15	Up
IP100894365.2	<i>ACTB</i>	6.25×10^{-2}	5	5	Down
IP101012981.1	<i>P4HB</i>	6.25×10^{-2}	8	5	Up
IP100939159.6	<i>CAP1</i>	6.78×10^{-2}	23	25	Up
IP100013122.1	<i>CDC37</i>	6.82×10^{-2}	14	13	Up
IP100909140.8	<i>TUBB</i>	7.26×10^{-2}	11	14	Up

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Accession no.	Gene symbol	P value	Number L-BMD	Number H-BMD	Regulation direction
IP100465248.5	<i>ENO1</i>	7.31×10^{-2}	29	29	Up
IP100549725.6	<i>PGAMI</i>	7.32×10^{-2}	24	24	Up
IP100219713.1	<i>FGG</i>	7.43×10^{-2}	20	13	Up
IP100026314.1	<i>GSN</i>	7.53×10^{-2}	8	5	Up
IP100853115.1	<i>NEFM</i>	8.06×10^{-2}	20	24	Up
IP100031523.4	<i>HSP90AA2</i>	8.39×10^{-2}	12	12	Up
IP100219217.3	<i>LDHB</i>	8.55×10^{-2}	25	27	Up
IP101012504.1	<i>PGD</i>	8.56×10^{-2}	13	14	Up
IP100296099.6	<i>THBS1</i>	9.37×10^{-2}	29	30	Up
IP100148061.3	<i>LDHAL6A</i>	9.64×10^{-2}	15	18	Up
IP100020599.1	<i>CALR</i>	9.78×10^{-2}	22	25	Up

The DEPs identified in discovery study are listed in this table, including 16 proteins that showed significant ($P < 0.05$) differential expression between low vs. high BMD groups and an additional 22 proteins showed suggestive ($P < 0.1$) evidence for differential expressions. Regulated direction shows the regulation direction of proteins in subjects with low hip BMDs

The gene ontology analysis

Table 3

Category	Number	Term	P value	Number	Percent	Genes
GOTERM_BP	GO:0006007	Glucose catabolic process	9.14×10^{-15}	10	29	LDHC, LOC652797, ALDOA, LDHB, LDHAL6A, PGAM4, PKM2, PGD, PGAM1, GAPDH, ENO1
	GO:0019320	Hexose catabolic process	4.77×10^{-14}	10	29	LDHC, LOC652797, ALDOA, LDHB, LDHAL6A, PGAM4, PKM2, PGD, PGAM1, GAPDH, ENO1
	GO:0046365	Monosaccharide catabolic process	6.23×10^{-14}	10	29	LDHC, LOC652797, ALDOA, LDHB, LDHAL6A, PGAM4, PKM2, PGD, PGAM1, GAPDH, ENO1
	GO:0006096	Glycolysis	1.48×10^{-13}	9	26	LDHC, LOC652797, ALDOA, LDHB, LDHAL6A, PGAM4, PKM2, PGAM1, GAPDH, ENO1
	GO:0044275	Cellular carbohydrate catabolic process	3.37×10^{-13}	10	29	LDHC, LOC652797, ALDOA, LDHB, LDHAL6A, PGAM4, PKM2, PGD, PGAM1, GAPDH, ENO1
	GO:0006091	Generation of precursor metabolites and energy	6.30×10^{-7}	9	26	LDHC, LOC652797, ALDOA, LDHB, LDHAL6A, PGAM4, PKM2, PGAM1, GAPDH, ENO1
GOTERM_CC	GO:0030029	Actin filament-based process	1.50×10^{-6}	8	23	ALDOA, GSN, CFL1, ACTN1, MYH14, CAPI, CALR, TPM1
	GO:0030036	Actin cytoskeleton organization	1.54×10^{-5}	7	20	ALDOA, GSN, CFL1, ACTN1, CAPI, CALR, TPM1
	GO:0006928	Cell motion	1.22×10^{-4}	8	23	ACTB, TUBB, ARPC2, CFL1, CAPI, THBS1, TPM1, TPM3
	GO:0010035	Response to inorganic substance	1.28×10^{-4}	6	17	ACTB, FGG, GSN, MPO, THBS1, TPM1
	GO:0043623	Cellular protein complex assembly	6.19×10^{-4}	5	14	HSP90AA2, FGG, TUBB, GSN, CALR
	GO:0005856	Cytoskeleton	5.28×10^{-6}	14	40	ALDOA, ACTB, ACTN1, CBX3, TPM1, TPM3, MYL9, TUBB, GSN, ARPC2, CFL1, CAPI, MYH14, NEFM
	GO:0005829	Cytosol	6.13×10^{-4}	11	31	HSP90AA2, LOC652797, ACTB, TUBB, GSN, PKM2, PGAM1, RAPIB, HSPA5, CALR, CDC37, ENO1
	GO:0003779	Actin binding	1.49×10^{-6}	9	26	ALDOA, GSN, ARPC2, CFL1, ACTN1, MYH14, CAPI, TPM1, TPM3
	GO:0008092	Cytoskeletal protein binding	3.63×10^{-5}	9	26	ALDOA, GSN, ARPC2, CFL1, ACTN1, MYH14, CAPI, TPM1, TPM3
	GO:0005200	Structural constituent of cytoskeleton	3.96×10^{-5}	5	14	ACTB, TUBB, ARPC2, TPM1, NEFM
GOTERM_MF	GO:0004457	Lactate dehydrogenase activity	9.92×10^{-5}	3	9	LDHC, LDHB, LDHAL6A
	GO:0005509	Calcium ion binding	2.12×10^{-3}	9	26	FGG, LTBP1, GSN, MPO, ACTN1, HSPA5, CALR, THBS1, MYL9

GOTERM_MF, GOTERM_BP and GOTERM_CC indicate molecular function, biological process, and cellular compartment, respectively. P value is EASE score, a modified Fisher exact P value. Number means the number of DEPs involved in terms, and % indicates the percentage

Table 4

Multiple-omics level analysis

Gene symbol	Proteomics			Transcriptomics						GWAS			Meta-analysis				
	Cohort 1		Probe ID	Cohort 2		Probe ID	Cohort 3		Probe ID	Cohort 4		Probe ID	Cohort 5		Probe ID	P value	P _{adj} value
	P value			P value			P value			P value			P value				
MYH14	1.12×10^{-2}	IP100337335.8	ND	ND	ND	ND	ND	ND	ND	ND	ND	219946_x_at	2.15×10^{-3}	rs139086374	8.70×10^{-4}	7.00×10^{-3}	
RAP1B	3.41×10^{-2}	IP01014435.1	6.07×10^{-1}	3,421,118	ND	ND	ND	ND	ND	ND	ND	ND	1.40×10^{-3}	rs73332537	1.91×10^{-3}	1.01×10^{-2}	
ALDOA	5.38×10^{-2}	IP100796333.1	3.80×10^{-1}	3,655,920	ND	ND	ND	ND	ND	ND	ND	200966_x_at	4.13×10^{-3}	rs184768500	4.59×10^{-3}	4.06×10^{-2}	

DEPs in this table have supported evidence from all three biological levels. In cohorts from mRNA expression data, genes have consistent expression trends with those in cohort 1. ND means no detected signal. Presented are the smallest *P* values and corresponding probes in studies of mRNA expression and SNPs association. Meta *P* value indicates a general score for each gene of DEPs which combine the meta-scores from protein expression, mRNA expression, and SNP association data. *P*_{adj} values means the *P* values which were adjusted using FDR in order to correct for multiple hypothesis testing. These significant *P* values and *P*_{adj} values are shown as italicized (*P* < 0.05)