Original Article

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Identification of novel susceptibility genes associated with bone density and osteoporosis in Korean women

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Purpose: Osteoporosis is a common calcium and metabolic skeletal disease which is characterized by decreased bone mass, microarchitectural deterioration of bone tissue and impaired bone strength, thereby leading to enhanced risk of bone fragility. In this study, we aimed to identify novel genes for susceptibility to osteoporosis and/or bone density.

Materials and Methods: To identify differentially expressed genes (DEGs) between control and osteoporosis-induced cells, annealing control primer-based differential display reverse-transcription polymerase chain reaction (RT-PCR) was carried out in pre-osteoblast MC3T3-E1 cells. Expression levels of the identified DEGs were evaluated by quantitative RT-PCR. Association studies for the quantitative bone density analysis and osteoporosis case-control analysis of single nucleotide polymorphism (SNPs) were performed in Korean women (3,570 subjects) from the Korean Association REsource (KARE) study cohort.

Results: Comparison analysis of expression levels of the identified DEGs by quantitative RT-PCR found seven genes, *Anxa6, Col5a1, Col6a2, Eno1, Myof, Nfib*, and *Scara5*, that showed significantly different expression between the dexamethason-treated and untreated MC3T3-E1 cells and between the ovariectomized osteoporosis-induced mice and sham mice. Association studies revealed that there was a significant association between the SNPs in the five genes, *ANXA6, COL5A1, ENO1, MYOF,* and *SCARA5*, and bone density and/or osteoporosis.

Conclusion: Using a whole-genome comparative expression analysis, gene expression evaluation analysis, and association analysis, we found five genes that were significantly associated with bone density and/or osteoporosis. Notably, the association *P*-values of the SNPs in the *ANXA6* and *COL5A1* genes were below the Bonferroni-corrected significance level.

Key words: Osteoporosis, Differentially expressed gene, Single nucleotide polymorphism, Genetic variation, Association study.

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Conflict of interest: The authors declare that they do not have any conflicts of interest.

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Introduction

Osteoporosis is a calcium and metabolic disorder characterized by decreased bone mass, enhanced risk of bone fragility and susceptibility to fracture [1,2] caused by a failure of bone homeostasis, which is due to both an increase in osteoclastic bone resorption and a decrease in osteoblastic bone formation [3,4]. Osteoporotic fractures are an important cause of morbidity and mortality, particularly in elderly women and men [5].

Osteoporosis is involved in the interactions of multiple genetic and environmental risk factors [6,7]. Recently, genetic factors have attracted much attention of many investigators due to their high importance in the pathogenesis of osteoporosis [8,9]. Severe osteoporosis may be related to mutation in a single gene, otherwise, bone mineral density (BMD) or bone mineral mass can be accounted for by the common genetic variations in multiple genes with relatively small effects or by the rare genetic variations in specific genes with relatively large effects [10,11]. Heritability studies in twins and families have demonstrated that between 50% and 85% of the variance in peak BMD is genetically determined [11,12].

The identification of genetic variants that contribute to osteoporosis and BMD phenotypes can be helpful not only for elucidation of the molecular mechanisms of osteoporosis, but also for the development of effective treatment for osteoporosis. So far, many osteoporosis susceptibility loci and genes have been identified by genome-wide linkage analysis [13,14] , candidate gene association studies [15] and genome-wide association studies (GWAS) [16-18], and recent important and representative findings through molecular genetic studies of gene identification for osteoporosis have been well summarized [19]. However, the vast majority of the monogenic and polygenic genetic factors in osteoporosis still remain to be discovered.

In this study, we aimed to identify the novel genes for susceptibility to osteoporosis that influence the pathogenesis of osteoporosis in humans in a particular way, by using the *in vitro* and *in vivo* models for osteoporosis. As an *in vitro* model for osteoporosis, we used the glucocorticoid (GC)-treated mouse osteoblastic MC3T3-E1 cell line in this study. GC causes osteoporosis-like bone loss due to decreasing bone formation and increasing bone resorption [20,21]. GC-induced apoptosis in osteoblastic cells has been reported [22]. The mouse MC3T3-E1 cell line has been demonstrated to be a suitable *in vitro* model of osteoblast development due to typical osteoblast differentiation and formation of a bone-like mineralized extracellular matrix [23,24]. Therefore, GC-treated osteoblastic MC3T3-E1 cells may provide a useful *in vitro* system for the first screening step of the differentially expressed genes (DEGs) between controls and osteoporosis models. In case of the *in vivo* models for osteoporosis, many animal models have been established for osteoporosis research [25,26]. Among the mouse models for osteoporosis, ovariectomized mice and GC-induced mice represent a reliable *in vivo* model to investigate bone loss in osteoporosis [27].

We first screened and selected the candidate genes that have a higher possibility to be linked with osteoporosis by comparative analysis of gene expression between controls and *in vitro* osteoporosis model. Subsequently, we carried out evaluation of the candidate genes using the *in vivo* osteoporosis model. Finally, we performed an association analysis between the genetic variants in the selected genes and bone density and osteoporosis in human subjects. This approach may provide an accurate identification of novel genes for susceptibility to osteoporosis.

Materials and Methods

1. Cell culture

Mouse osteoblastic MC3T3-E1 cells were purchased from the RIKEN cell bank (Tsukuba, Japan) and grown in α-MEM medium supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL). Cultured cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

2. Annealing control primer (ACP)-based differential display reverse-transcription polymerase chain reaction (RT-PCR)

ACP-based differential display RT-PCR was carried out using the predesigned arbitrary ACP (Seegene, Seoul, Korea) [28]. First-strand cDNA synthesis was performed using the primer dT-ACT1 (5′-CTGTGAATGCTGCGACTACGATIIIII(T)$_{18}$-3′: 'I' is inosine) and 1 μL of M-MLV reverse transcriptase (200 U/μL) (Fermentas, Burlington, Canada). PCR amplification was conducted using GeneFishing DEG kit (Seegene) in 20 μL reaction volumes containing 10 μL of 2× SeeAmp ACP Master Mix; 2 μL of 5 μM each arbitrary ACP; 1 μL of 10 μM dT-ACP2 (5′-CTGTGAATGCTGCGAC TACGATIIIII(T)$_{15}$-3′,); and 3 μL of diluted first-strand cDNA. Each kit comprises 120 different arbitrary annealing control primers. The amplified PCR products were separated in a 2% agarose gel and stained with ethidium bromide.

3. Quantitative reverse transcription-PCR (qRT-PCR)

Total RNAs from cells were treated with RNase-free DNase I (Invitrogen) at room temperature for 15 minutes to avoid am-

plification of genomic DNA; denatured at 70°C for 10 minutes; and subsequently reverse transcribed by Superscript II reverse transcriptase (Invitrogen) with 0.5 μg of oligo(dT)$_{15-18}$ primer in a volume of 20 μL according to manufacturer instructions.

The specific primers used for qRT-PCR were as follows: 5′-CACAGGGTGCCATGTACCG-3′ and 5′-GAGGTCCTTGCCATACAGGG-3′ for mouse *AnxA6*; 5′-GCTCTCCGCCGAAGTTAAGAA-3′ and 5′-TTCGCACAATATGATGCCGTC-3′ for mouse *Cnn3*; 5′-CAATTTGCCCTCAGGGGTAAC-3′ and 5′-TCCTCGGGAAAACCAGACTCA-3′ for mouse *Col5a1*; 5′-GCTCCTGATTGGGGGACTCT-3′ and 5′-CCAACACGAAATACACGTTGAC-3′ for mouse *Col6a2*; 5′-ACACTGGGCTTCATCATGCC-3′ and 5′-ACTGCGAAGATCATCCTCAGG-3′ for mouse *Gper*; 5′-CCCTGAAGACTCGGGCCTA-3′ and 5′-CAATTACAAGCGAAATGAGAGCC-3′ for mouse *Kitl*; 5′-TGATCGAGGGCCGTCAGTTAT-3′ and 5′-CTGTCACTCACCTTAAATTCCCC-3′ for mouse *Myof*; 5′-AGCACCATCATCCCGGAATAC-3′ and 5′-GTACCAGGACTGGCTCGTTTG-3′ for mouse *Nfib*; 5′-ACCGGACAGCTCGTTTTGG-3′ and 5′-AGGGGACAGTACAAGTCACCC-3′ for mouse *Scara5*; 5′-TGGAAACCATGATGCTTACGTT-3′ and 5′-GAAGCCCACTTTGCCATCTC-3′ for mouse *S100a10*; 5′-GCTGCCTCCGAGTTCTACAG-3′ and 5′- GCAGGGATTCGGTCACAGAG-3′ for mouse *Eno1*. To normalize the efficiency of qRT-PCR reactions, the mouse *Gadph* gene was used as an internal standard with the following primers: 5′-TGACCACAGTCCATGCCATC-3′ and 5′-GACGGACACATTGGGGGTAG-3′. qRT-PCR was performed using SYBR Green PCR premix (Takara, Shiga, Japan). All measurements were performed in triplicate.

4. Ovariectomized (OVX) mouse model

The OVX (n=10) and sham-operated (Sham, n=10) 8-weeks-old female ddY mice were purchased from Shizuoka Laboratory Center Inc. (Hamamatsu, Japan). Mice were maintained on a diet (5.0 g/day) of Formula-M07 (Feedlab Co., Ltd., Hanam, Korea) and tap water (15 mL/day). All mice were housed individually in clear plastic cages under controlled temperature (23±2°C), humidity (55±5%), and illumination (12-hour light/dark cycle). After 8 weeks of feeding, the BMD between the two groups of mice was measured. The animal research protocol was approved by the Animal Care and Use Committee of the Ajou University School of Medicine, and all experiments were conducted in accordance with the institutional guidelines established by the Committee.

5. Measurement of bone mineral density and tissue sample preparation

Whole body BMD of mice was measured using a PIXI-mus bone densitometer (GE Lunar, Madison, WI, USA). After anesthetization using tiletamine/zolazepam (Zoletil; Virbac Laboratories, Carros, France), the mice were placed on the specimen tray for measurements. All mice were placed carefully in the same position. After measurement of BMD, the mice were killed by CO_2 asphyxiation and cervical dislocation. Mice femurs were excised, and the isolated femur bones and skeletal muscles were then frozen by liquid nitrogen and deep-freeze, respectively. The frozen samples were homogenized using a porcelain mortar and pestle and then lysed using RIPA lysis buffer and used for Western blot analysis.

6. Human subjects

The subjects from the Korean Association Resource (KARE) study which were used in this study have been described in the previous report [18]. The participants were recruited from two community-based epidemiological cohorts, the rural community of Ansung and the urban community of Ansan cities. A total of 3,570 women subjects were investigated in this study. The basic characteristics of the study subjects are described in Table 1.

Bone density was estimated by T-score by dividing the difference of measured speed of sound (SOS) from mean SOS in healthy young adult population by the standard deviation of SOS in young adult population. Bone SOS was measured by quantitative ultrasound at the distal radius and mid-shaft tibia, using the Omnisense 7000P QUS (Sunlight Medical Ltd, Tel-Aviv, Israel). For the case-control analysis of osteoporosis, the subjects whose bone density T-scores at either the distal radius or mid-shaft tibia were less than –2.5 standard deviation (SD) were allocated to case and the subjects whose bone densities T-scores at both the distal radius and mid-shaft tibia were more than –1 SD were allocated to control, according to the general diagnostic categories to be established for adult women [29]. This study was approved by the institutional review board of the Korean National Institute of Health (KBN-2017-046). Written informed consent was obtained from all subjects.

7. Genotyping and selection of SNPs

The genotype data were provided by the Center for Genome Science, the Korea National Institute of Health. The detailed genotyping and quality control processes have been described in the previous report [18]. Briefly, most DNA samples were isolated from the peripheral blood of participants and genotyped using

Table 1. Basic characteristics of the women subjects in the KARE study cohort

Characteristics	Quantitative analysis for bone density (n=3,570)	Case-control analysis for osteoporosis		
		Control (n=1,711)	Case (n=651)	*P*-value[a]
Age (yr)	51.02±8.76	47.20±6.57	59.46±7.34	<0.0001
BMI (kg/m^2)	24.65±3.19	24.20±2.96	25.37±3.51	<0.0001
Distal radius T-score	0.20±1.55	0.99±1.14	–1.26±1.64	<0.0001
Midshaft tibia T-score	–0.81±1.55	0.31±0.93	–3.11±0.99	<0.0001

Values are presented as mean±standard deviation.
[a]Significant differences in characteristics between the control and case were determined by the two-tailed Student's *t*-test. Osteoporosis was defined as any bone density T-score of –2.5 standard deviation or below and control was defined as both bone densities T-score of –1 standard deviation over.
KARE, Korean Association REsource, BMI, body mass index.

the Affymetix Genome-Wide Human SNP array 5.0 (Affymetrix, Santa Clara, CA, USA). The accuracy of the genotyping was calculated by Bayesian Robust Linear Modeling using the Mahalanobis Distance genotyping algorithm [30]. The SNPs in the 7 genes that we analyzed were selected based on their locations within the gene boundary (5 kb upstream and downstream of the first and last exons, respectively) according to NCBI human genome build 36. The locations of the SNPs were validated with the Ensemble BioMart database (http://www.ensembl.org/biomart).

8. Statistical analysis

In the qRT-PCR analysis, all experiments were repeated at least 3 times unless stated otherwise and results were presented as the means±SD as indicated. Statistical significance between groups was calculated by a two-tailed Student's *t*-test. Probability values less than 0.05 (P<0.05) were considered statistically significant.

Statistical analyses for association studies were performed using the PLINK version 1.07 (http://pngu.mgh.harvard.edu/~purcell/plink) and IBM SPSS Statistics for Windows, Version 25.0 (IBM Co., Armonk, NY, USA). Linear regression was used for quantitative analysis of bone density, controlling for cohort and age as covariates. Logistic regression was used for case-control analysis of osteoporosis. All association tests were performed under the additive, dominant and recessive models, and *P*-values were adjusted for multiple tests by using the Bonferroni-corrected significance level (P<0.00185). The Haploview version 4.2 program (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) was used to examine the structure of the linkage disequilibrium (LD) block [31] using the KARE genotype data and the HapMap database (International HapMap Project, http://www.hapmap.org/). We examined the LD coefficient r^2 between all pairs of biallelic loci [32].

Results

1. Study design

The flow chart of the study design is shown in Fig. 1. We carried out a series of experiments on cell line model, mouse model and humans step-by-step for identifying novel genes for susceptibility to osteoporosis. The first experiment was the screening and identification of the DEGs in Dex-treated osteoblastic MC3T3-E1 cell line, using a RT-PCR-based gene expression differential display approach, the ACP-based PCR GeneFishing DEG screening method. Next, the identified DEGs were validated by quantitative real-time PCR with the gene-specific primers in the Dex-treated cells. In the next step, we tried to evaluate the accuracy of the identified DEGs *in vivo* and carried out quantitative real-time PCR with the gene-specific primers in the ovariectomized mice. Lastly, to determine whether the genetic variations of the selected DEGs were associated with bone density and osteoporosis, we performed association analysis in a large Korean Women's Cohort (n=3,570).

2. Screening and identification of the DEGs in Dex-treated osteoblastic cell line model

To identify the DEGs in the *in vitro* osteoporosis model, we performed a whole-genome comparative expression study using a RT-PCR based gene expression differential display, the ACP-based PCR GeneFishing DEG screening method [28]. To establish an *in vitro* osteoporosis model, mouse osteoblastic cell line, MC3T3-E1, was treated with 1 μM of synthetic GC, Dex for 2 days. Total RNAs were isolated from the cells and used for first-strand cDNA synthesis. The first-strand cDNAs were subjected to gene expression differential display.

Using 120 arbitrary ACP primers, GeneFishing DEG screening was performed and a total of 10 DEGs that showed clear differences between the two treatment groups were found. The

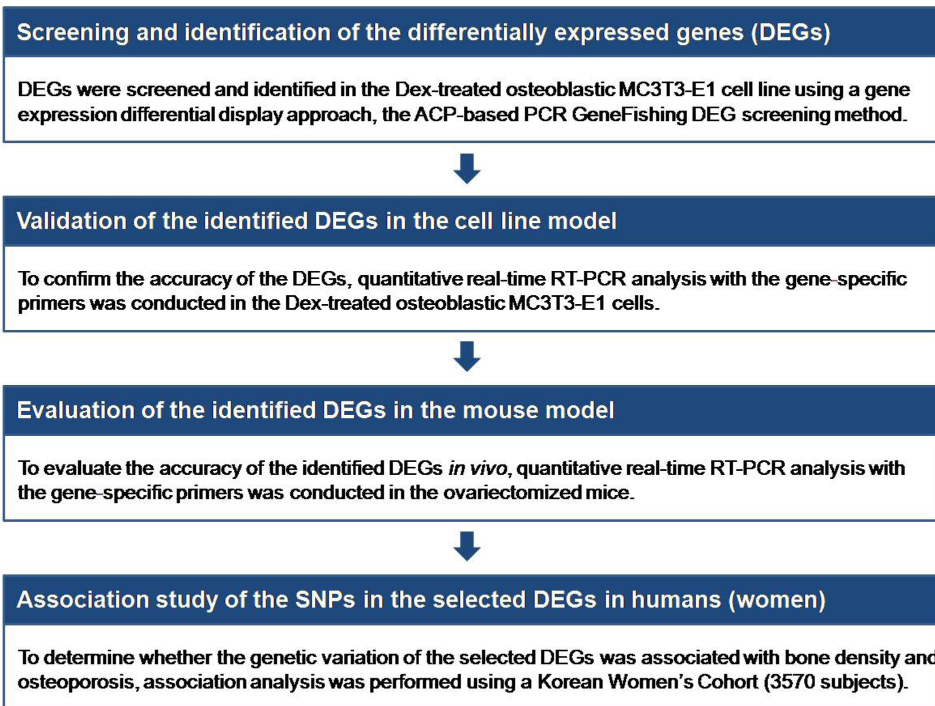

Fig. 1. The flow chart of the study. ACP, annealing control primer; RT-PCR, reverse-transcription polymerase chain reaction.

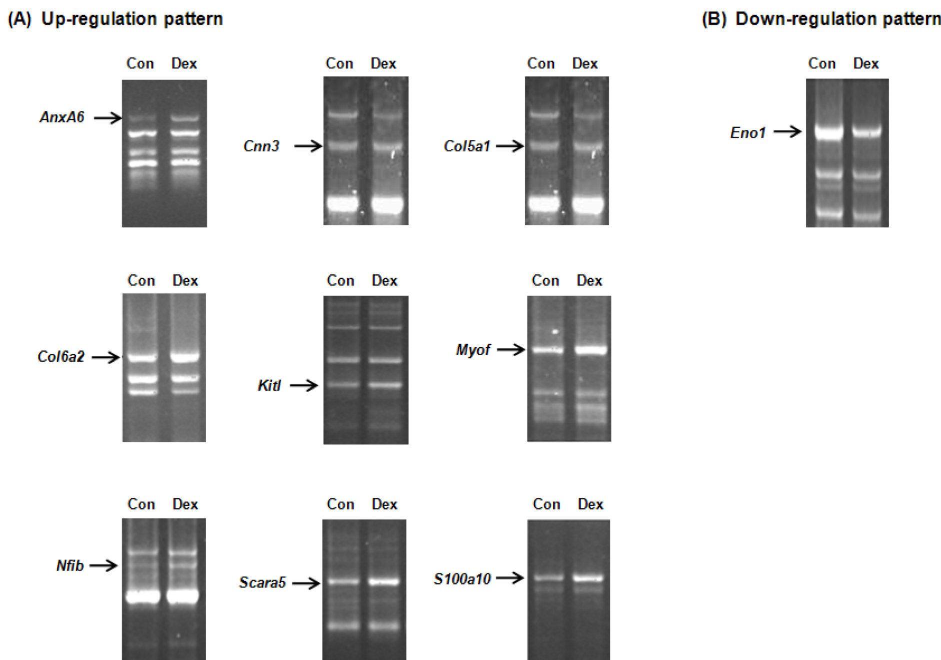

Fig. 2. Differential banding patterns of the 10 identified DEGs. The arrows with the gene name indicate the up-regulated (A) or down-regulated (B) DEGs in the Dex-treated cells compared to untreated control cells. Osteoblastic MC3T3-E1 cell line was treated with 1×PBS (Con) or 1 μM Dex, and then cultured for 2 days. Annealing control primer-based RT-PCR was performed on the total RNAs isolated from the treated cells, and RT-PCR products were resolved on 2% agarose gels and visualized by staining with ethidium bromide. DEG, differentially expressed gene; Con, control; Dex, dexamethasone; PBS, phosphate-buffered saline; RT-PCR, reverse-transcription polymerase chain reaction.

gel images for the 10 DEGs were shown in Fig. 2: 9 DEGs had increased mRNA expression levels in the Dex-treated cells compared with the controls and 1 DEG showed decreased mRNA expression level in the Dex-treated cells.

To identify the DEGs, the RT-PCR bands were extracted, re-amplified, and PCR fragments were isolated from gels, cloned, and sequenced. BLASTN and BLASTX searches in the NCBI GenBank revealed that all the 10 DEGs were known genes as listed in Table 2. The expression levels of Annexin A6 (*AnxA6*), Calponin 3 (*Cnn3*), Collagen type V alpha 1 (*Col5a1*), Collagen type VI alpha 2 (*Col6a2*), Kit ligand (*Kitl*), Myoferlin (*Myof*), Nuclear factor I/B (*Nfib*), Scavenger receptor class A member 5 (*Scara5*), and S100 calcium binding protein A10 (*S100a10*), were increased in the Dex-treated cells, however the expression level of Enolase 1

Table 2. List of the significantly DEGs in the dexamethasone-treated mouse MC3T3-E1 cells

DEG no.	Expression level		Gene symbol	GenBank Accession no.	Gene definition
	Con	Dex			
(A) Up-regulation					
1	+	++	*AnxA6*	NM_013472	Annexin A6
2	+	++	*Cnn3*	NM_028044	Calponin 3
3	+	++	*Col5a1*	NM_015734	Collagen, type V, alpha 1
4	++	+++	*Col6a2*	NM_146007	Collagen, type VI, alpha 2
5	+	++	*Kitl*	NM_013598	Kit ligand
6	+	+++	*Myof*	NM_177035	Myoferlin
7	+	++	*Nfib*	NM_008687	Nuclear factor I/B
8	+	+++	*Scara5*	NM_028903	Scavenger receptor class A, member 5
9	+	+++	*S100a10*	NM_009112	S100 calcium binding protein A10
(B) Down-regulation					
10	+++	++	*Eno1*	NM_023119	Enolase 1

DEG, differentially expressed gene; Con, control; Dex, dexamethasone.

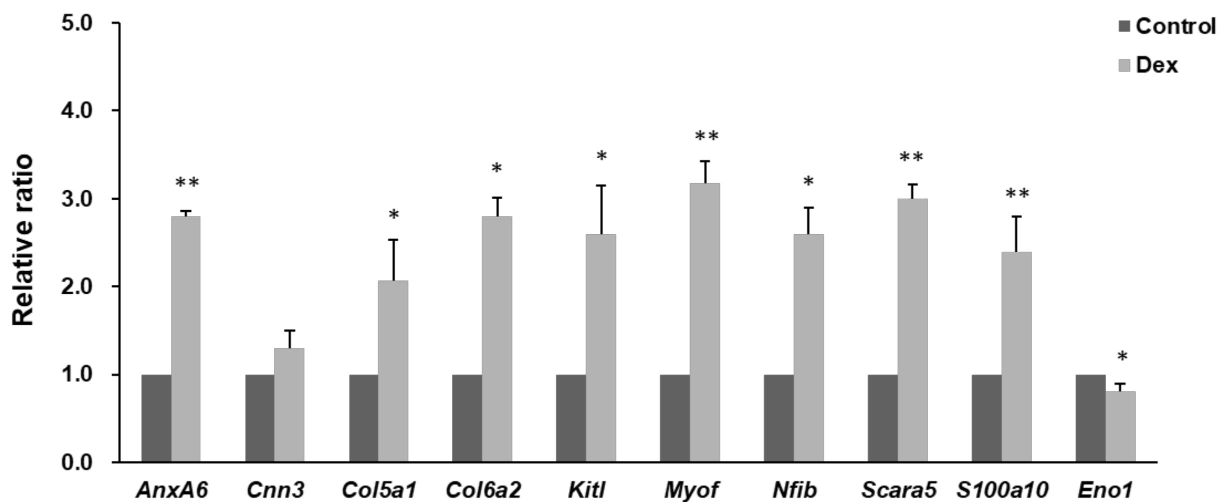

Fig. 3. Validation of the mRNA expression levels of the 10 DEGs in the Dex-treated MC3T3-E1 cells by quantitative real-time RT-PCR. Quantitative RT-PCR was performed using the total RNA samples. The mRNA expression level for each gene was quantified and the data represents the relative ratio of Dex-treated samples (Dex) compared to 1×PBS-treated samples (Con) in MC3T3-E1 cells. The Dex-insensitive housekeeping gene, *Gapdh*, was used for plotting the relative standard curve (internal control). Each experiment was repeated three times. *P<0.05, **P<0.01 vs. Control. DEG, differentially expressed gene; Dex, dexamethasone; RT-PCR, reverse-transcription polymerase chain reaction; Dex, dexamethasone; PBS, phosphate-buffered saline; Con, control.

(*Eno1*) was decreased in the Dex-treated cells.

3. Validation of the identified DEGs in the cell line model by quantitative real-time RT-PCR

To confirm the efficacy and accuracy of screening by the ACP-based differential display RT-PCR, fluorescence-monitored quantitative real-time RT-PCR analysis was employed for the 10 genes. Gene-specific primers were designed to amplify RT-PCR products ranging from 100 to 250 bp. Quantitative real-time RT-PCR results of the 10 genes are shown in Fig. 3 and are presented as relative ratios compared with the mouse *Gapdh* gene (internal control) with a value of 1.0. The mRNA expression levels of 8 genes, *AnxA6, Col5a1, Col6a2, Kitl, Myof, Nfib, Scara5* and *S100a10*, were increased significantly in the Dex-treated cells compared with the controls, and *Eno1* gene expression was decreased significantly, thereby indicating that these results are consistent with the results of ACP-based differential display shown in Fig. 2 and Table 2. The expression level of the *Cnn3* gene, however, was not different between the Dex-treated and untreated cells.

4. Evaluation of the identified DEGs in the ovariectomized mouse model by quantitative real-time RT-PCR

To evaluate the accuracy of the identified DEGs *in vivo*, we carried out comparative analysis of gene expression levels in the identified DEGs between the OVX mice group and Sham control mice group. Each of the ten 8-week-old female OVX and sham ddY mice was purchased and maintained in our laboratory for 4 weeks. Five OVX mice died during maintenance. At 16 weeks after ovariectomy, whole-body BMD of the 10 sham mice and 5 OVX mice was calculated using a PIXI-mus bone densitometer (Fig. 4A). The OVX mice group demonstrated a significant reduction in BMD (approximately 1.5-fold) compared with the sham-operated mice group. After scarifying the mice, the femur bones were excised and frozen in liquid nitrogen. Total RNAs were isolated from the frozen samples.

The mRNA expression levels of the identified DEGs were examined using the *in vivo* samples by quantitative RT-PCR analysis (Fig. 4B). The expression levels of 7 genes, *AnxA6, Col5a1, Col6a2, Eno1, Kitl, Myof*, and *Scara5*, among the 10 identified DEGs were significantly altered in the OVX group compared with the sham group, but the expression level of *Nfib* and *S100a10* genes were not significantly different between the groups. These results suggested that these 7 genes may be involved in the development of osteoporosis in the OVX mice model.

5. Association analysis of the genetic variation in the identified DEGs with bone density and osteoporosis in humans

We finally selected the 7 genes, *AnxA6, Col5a1, Col6a2, Eno1, Kitl, Myof*, and *Scara5*, as the target genes for further study in human subjects. To investigate whether the genetic variations in these 7 selected genes influenced the bone density and sus-

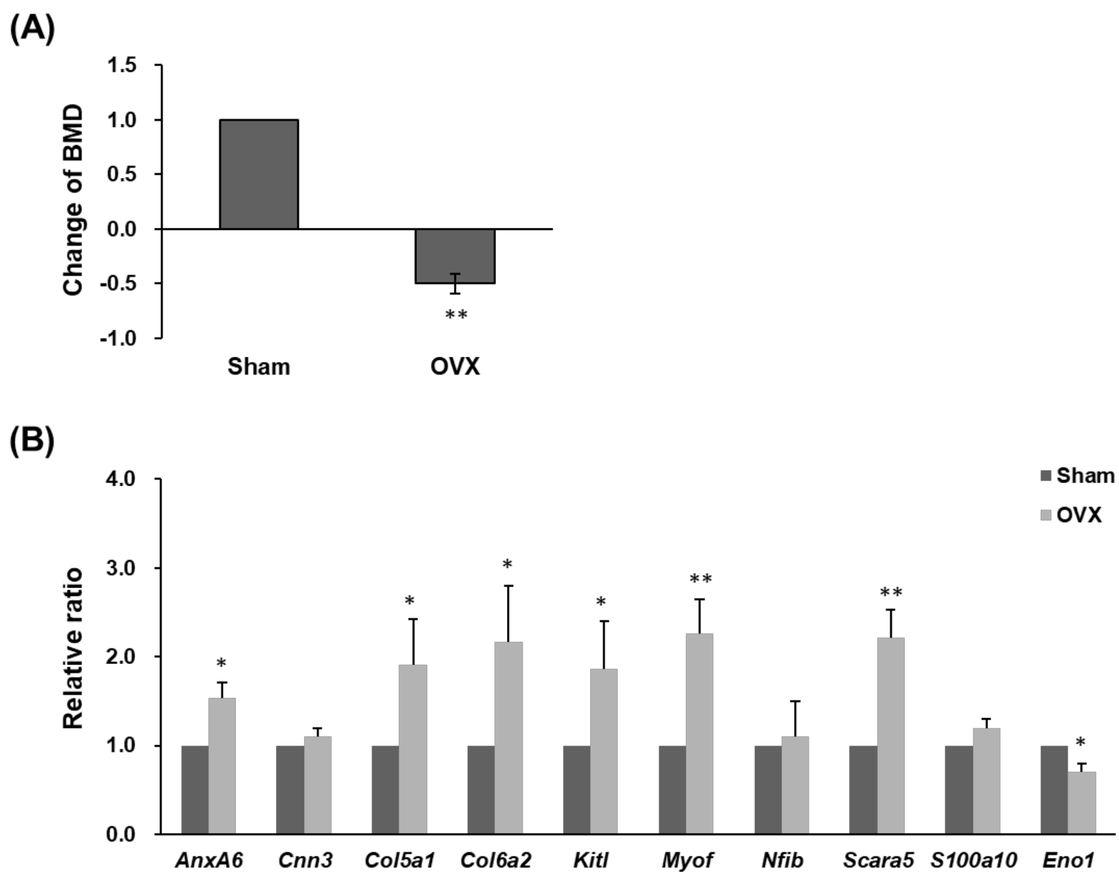

Fig. 4. Comparison of the bone mineral density and the mRNA expression levels of the 10 DEGs between the sham and OVX mice. (A) Whole-body BMD was measured in the OVX or sham-operated control (Sham) mice (10 mice per group) using on-board PIXI-mus software for small animals and adjusted for the mouse body weight. Results are expressed as a percentage change of whole-body BMD adjusted for body weight (mean±standard deviation). ***P*<0.01 vs. Sham mouse group. (B) Quantitative reverse-transcription-PCR was performed using the total RNAs from the two mouse groups. The mRNA expression level for each gene was quantified and the data represents the relative ratio of OVX mice compared to Sham mice. The housekeeping gene, *Gapdh*, was used for plotting the relative standard curve (internal control). Each experiment was repeated three times. **P*<0.05, ***P*<0.01 vs. Sham mouse group. DEG, differentially expressed gene; BMD, bone mineral density; OVX, ovariectomized.

Table 3. The results of association analysis between the SNPs in the 5 genes and bone density in the KARE women subjects

Gene	SNP	Minor allele	MAF	Women (n=3,570)					
				β±SEM	Add *P*	β±SEM	Dom *P*	β±SEM	Rec *P*
BD-RT (T-score at distal radius)									
ANXA6	rs3815725	T	0.189	0.005±0.04	0.895	0.043±0.05	0.360	–0.244±0.12	0.048[a]
	rs883887	G	0.310	0.038±0.03	0.267	0.091±0.05	0.044[a]	–0.076±0.08	0.326
COL5A1	rs6537946	A	0.082	0.120±0.06	0.041[a]	0.125±0.06	0.043[a]	0.196±0.29	0.505
	rs7874142	A	0.471	0.041±0.03	0.194	–0.039±0.05	0.439	0.162±0.05	2.5E-03[a]
	rs3811149	C	0.470	0.032±0.03	0.313	–0.042±0.05	0.406	0.140±0.05	9.4E-03[a]
MYOF	rs1614065	A	0.052	–0.007±0.07	0.925	–0.021±0.07	0.775	1.553±0.77	0.045[a]
	rs1891565	C	0.052	–0.006±0.07	0.931	–0.021±0.07	0.780	1.553±0.77	0.045[a]
	rs787695	T	0.052	–0.007±0.07	0.923	–0.021±0.07	0.772	1.553±0.77	0.045[a]
	rs787633	T	0.037	0.013±0.08	0.875	–0.005±0.09	0.951	1.553±0.77	0.045[a]
	rs17108751	A	0.254	–0.067±0.04	0.079	–0.091±0.05	0.046[a]	–0.024±0.10	0.811
	rs871427	C	0.499	0.056±0.03	0.086	0.038±0.05	0.475	0.109±0.05	0.038[a]
SCARA5	rs11778759	T	0.347	0.067±0.03	0.044[a]	0.089±0.05	0.051	0.083±0.07	0.224
BD-TT (T-score at midshaft tibia)									
ANXA6	rs17728338	T	0.095	–0.146±0.06	8.6E-03[a]	–0.128±0.06	0.032[a]	–0.707±0.25	4.6E-03[a]
	rs868641	T	0.283	0.112±0.04	1.6E-03[b]	0.141±0.05	2.0E-03[a]	0.142±0.08	0.086
	rs4958893	A	0.283	0.104±0.04	3.5E-03[a]	0.136±0.05	2.7E-03[a]	0.112±0.08	0.177
	rs4958895	T	0.331	0.073±0.03	0.033[a]	0.081±0.05	0.079	0.126±0.07	0.082
COL5A1	rs7875570	A	0.054	0.095±0.07	0.187	0.058±0.08	0.444	1.272±0.39	1.2E-03[b]
	rs10858265	C	0.055	0.096±0.07	0.178	0.063±0.08	0.405	1.113±0.38	3.2E-03[a]
	rs6537942	G	0.089	0.076±0.06	0.187	0.036±0.06	0.558	0.892±0.26	5.3E-04[b]
	rs4319175	G	0.369	0.054±0.03	0.106	0.109±0.05	0.020[a]	–0.005±0.07	0.934
	rs9308278	G	0.254	0.083±0.04	0.025[a]	0.090±0.05	0.049[a]	0.155±0.09	0.100
	rs4842173	C	0.253	0.084±0.04	0.025[a]	0.093±0.05	0.043[a]	0.145±0.09	0.128
ENO1	rs10864368	A	0.115	–0.090±0.05	0.077	-0.077±0.05	0.161	–0.450±0.22	0.043[a]
	rs11121247	T	0.067	–0.175±0.06	6.3E-03[a]	-0.184±0.07	6.5E-03[a]	–0.268±0.32	0.405
	rs6660137	G	0.067	–0.160±0.06	0.012[a]	-0.176±0.07	9.2E-03[a]	–0.108±0.31	0.730
MYOF	rs2797581	C	0.108	0.064±0.05	0.209	0.046±0.06	0.414	0.393±0.20	0.047[a]
	rs787665	G	0.109	0.063±0.05	0.221	0.042±0.06	0.457	0.461±0.21	0.027[a]
	rs787667	T	0.109	0.058±0.05	0.256	0.035±0.06	0.527	0.447±0.20	0.027[a]
	rs787668	A	0.109	0.059±0.05	0.248	0.034±0.06	0.543	0.489±0.20	0.017[a]
	rs1614065	A	0.052	–0.017±0.07	0.817	–0.032±0.07	0.666	1.626±0.79	0.039[a]
	rs1891565	C	0.052	0.002±0.07	0.980	–0.013±0.07	0.863	1.625±0.78	0.038[a]
	rs787695	T	0.052	–0.015±0.07	0.841	–0.030±0.07	0.689	1.626±0.79	0.039[a]
	rs787633	T	0.037	0.073±0.09	0.395	0.055±0.09	0.525	1.626±0.79	0.039[a]
	rs7913298	G	0.026	–0.225±0.10	0.025[a]	–0.234±0.10	0.024[a]	–0.243±0.68	0.721
SCARA5	rs7002829	A	0.122	–0.033±0.05	0.500	–0.007±0.05	0.895	–0.360±0.18	0.043[a]
	rs2859667	C	0.386	0.046±0.03	0.154	0.092±0.05	0.048[a]	0.008±0.06	0.903
	rs2726941	A	0.398	–0.079±0.03	0.015[a]	–0.082±0.05	0.085	–0.145±0.06	0.018[a]
	rs2685399	C	0.386	–0.086±0.03	8.4E-03[a]	–0.089±0.05	0.058	–0.158±0.06	0.012[a]
	rs2726934	T	0.395	–0.083±0.03	0.011[a]	–0.096±0.05	0.042[a]	–0.133±0.06	0.032[a]
	rs2727006	A	0.394	–0.089±0.03	6.4E-03[a]	–0.102±0.05	0.031[a]	–0.144±0.06	0.020[a]

[a]*P*-values below the standard significance level ($P<0.05$) are indicated. [b]*P*-values below the Bonferroni-corrected significance level ($P<0.001852$) are indicated.

SNP, single nucleotide polymorphism; KARE, Korean Association Resource; MAF, minor allele frequency; SEM, standard error; Add *P*, additive genetic model *P*-value; Dom P, dominant genetic model *P*-value; Rec P, recessive genetic model *P*-value; BD-RT, bone density estimated by T-score at distal radius; BD-TT, bone density estimated by T-score at midshaft tibia.

ceptibility to osteoporosis, we performed the quantitative trait analysis for bone density and osteoporosis case-control analysis for the SNPs of the 7 genes in the KARE Women's Study Cohort (3,570 subjects). The basic characteristics of the study subjects are shown in Table 1. The mean age of the women subjects was 51.02 years, the mean bone density estimated by T-score at the distal radius (BD-RT) was 0.20±1.55, and the mean bone density estimated by T-score at the midshaft tibia (BD-TT) was −0.81±1.55.

Linear regression analysis was used to associate the genotypes with bone density traits, controlling for age and cohort as covariates. The 116 SNPs were genotyped in the 7 genes (Supplementary Table 1). The genotyped 116 SNPs of the 7 genes were partitioned into a total 27 LD blocks, which was demonstrated by the Haplotype and PLINK program using the KARE data. Therefore, the Bonferroni-corrected significance *P*-value threshold was calculated as 0.00185 (0.05/27 LD blocks).

The results of association analysis between the 116 SNPs in the 7 genes and bone density in the 3,570 KARE women subjects are summarized in Table 3. Total 12 SNPs in the 4 genes (2 SNPs in *ANXA6*, 3 SNPs in *COL5A1*, 6 SNPs in *MYOF* and 1 SNP in *SCARA5*) were significantly associated with BD-RT trait, and total 28 SNPs in the 5 genes (4 SNPs in *ANXA6*, 6 SNPs in *COL5A1*, 3 SNPs in *ENO1*, 9 SNPs in *MYOF* and 6 SNPs in *SCARA5*) were significantly associated with BD-TT trait. Particularly, 1 SNP, rs868641 in the *ANXA6* gene and 2 SNPs, rs7875570 and rs6537942 in the *COL5A1* gene showed a highly significant association with BD-TT trait and their *P*-values satisfied the Bonferroni-corrected significance level ($P<0.001852$).

For osteoporosis case-control association analysis, the control subjects (n=1,711) and osteoporosis case subjects (n=651) were analyzed. The results of case-control association analysis between the 116 SNPs in the 7 genes and osteoporosis in the KARE women subjects are summarized in Table 4. Total 14 SNPs in 5 genes (1 SNP in *ANXA6*, 6 SNPs in *COL5A1*, 1 SNP in *ENO1*, 4 SNPs in *MYOF* and 2 SNPs in *SCARA5*) were significantly associated with osteoporosis.

Notably, 8 SNPs in the 5 genes (1 SNP in *ANXA6*, 1 SNP in *COL5A1*, 1 SNP in *ENO1*, 4 SNPs in *MYOF* and 1 SNP in *SCARA5*) were significantly associated with both the bone density and osteoporosis traits (Tables 3, 4). In all the 8 SNPs, their β-values in BD-RT and BD-TT traits were in the same direction and showed consistent trends with the odds ratios of osteoporosis. The location and basic LD of the analyzed SNPs in the *ANXA6, COL5A1, MYOF* and *SCARA5* genes are shown in Supplementary Fig. 1. The SNPs that were significantly associated with bone density and/or osteoporosis in the KARE women subjects are also indicated. Interestingly, the 4 SNPs in the *MYOF* gene showing a significant association with both bone density and osteoporosis were located in the same LD block of the gene (Supplementary Fig. 1C).

Table 4. The results of case-control association analysis between the SNPs in the 5 genes and osteoporosis in the KARE women subjects

Gene	SNP	Minor allele	MAF	Women subjects (1,711 controls, 651 cases)					
				OR (95% CI)	Add *P*	OR (95% CI)	Dom *P*	OR (95% CI)	Rec *P*
ANXA6	rs17728338	T	0.095	1.39 (1.06-1.83)	0.018[a]	1.36 (1.01-1.82)	0.041[a]	3.53 (1.07-11.62)	0.038[a]
COL5A1	rs4335205	G	0.430	1.20 (1.02-1.43)	0.031[a]	1.40 (1.08-1.81)	0.010[a]	1.13 (0.83-1.53)	0.434
	rs4319175	G	0.369	0.88 (0.74-1.05)	0.154	0.78 (0.61-0.99)	0.039[a]	1.02 (0.72-1.43)	0.931
	rs9409917	G	0.065	1.16 (0.83-1.61)	0.377	1.23 (0.87-1.74)	0.243[a]	NA	0.998
	rs12005720	G	0.127	0.85 (0.67-1.10)	0.214	0.90 (0.68-1.19)	0.448	0.39 (0.15-0.99)	0.048[a]
	rs11103535	T	0.144	1.21 (0.96-1.53)	0.106	1.16 (0.89-1.52)	0.264	2.17 (1.04-4.51)	0.039[a]
	rs10858284	A	0.148	0.73 (0.58-0.93)	0.012[a]	0.72 (0.55-0.95)	0.022[a]	0.49 (0.22-1.11)	0.087
ENO1	rs11121247	T	0.067	1.41 (1.02-1.97)	0.041[a]	1.44 (1.01-2.04)	0.042[a]	1.68 (0.28-10.07)	0.573
MYOF	rs2797581	C	0.108	0.81 (0.61-1.06)	0.121	0.86 (0.63-1.15)	0.307	0.20 (0.05-0.80)	0.022[a]
	rs787665	G	0.109	0.83 (0.63-1.09)	0.186	0.87 (0.65-1.18)	0.374	0.25 (0.06-0.99)	0.048[a]
	rs787667	T	0.109	0.84 (0.64-1.10)	0.202	0.89 (0.67-1.20)	0.461	0.21 (0.05-0.81)	0.024[a]
	rs787668	A	0.109	0.84 (0.64-1.10)	0.192	0.89 (0.66-1.20)	0.442	0.21 (0.05-0.81)	0.024[a]
SCARA5	rs2726959	T	0.298	1.19 (0.99-1.43)	0.064	1.11 (0.88-1.41)	0.390	1.79 (1.18-2.71)	6.1E-03[a]
	rs7002829	A	0.122	1.18 (0.92-1.52)	0.185	1.09 (0.82-1.45)	0.534	2.93 (1.32-6.50)	8.2E-03[a]

[a]*P*-values below the standard significance level ($P<0.05$) are indicated.
SNP, single nucleotide polymorphism; KARE, Korean Association Resource; MAF, minor allele frequency; OR, odds ratio; CI, confidence interval; Add *P*, additive genetic model *P*-value; Dom *P*, dominant genetic model *P*-value; Rec *P*, recessive genetic model *P*-value; NA, not applicable.

Discussion

Many approaches for identifying the genetic factors contributing to the pathogenesis of osteoporosis have been studied and have contributed to the detection of numerous genes for susceptibility to osteoporosis [11]. Among them, GWAS has been extensively executed for identifying the loci and genes that are significantly associated with bone density and osteoporosis. A major advantage of GWAS is that it offers the ranking for significance in multiple association signals across the genome. Since the statistical significance thresholds are very stringent due to the analysis of a large number of SNPs, many polymorphisms having a true association with osteoporosis but with a relatively small effect size can be missed [11]. This may lead to missing an opportunity to identify the novel osteoporosis susceptibility genes. In this study, to identify the novel genes more accurately as well as more effectively, we combined two methods, i.e whole genome expression profiling for screening of candidate genes and candidate gene association study.

In the series of experiments in the *in vitro* and *in vivo* osteoporosis models and eventually in human subjects, we identified 5 novel osteoporosis susceptibility genes, *ANXA6, COL5A1, ENO1, MYOF*, and *SCARA5*. The results from each step of the experiments in the cell line model, mouse model and humans are summarized in Table 5. In the screening step of the DEGs in the cell line model, 10 candidate genes were screened. During the validation and evaluation steps in the cell line and mouse models, respectively, the 5 genes showing false positive results were ruled out, and the 5 genes that passed all the steps of the experiment were finally selected.

The *ANXA6* gene encodes Annexin A6 which belongs to a family of calcium-dependent membrane and phospholipid binding proteins and is involved in matrix vesicle calcification [33,34]. Annexin A6 binds to phospholipids in cellular membranes in a dynamic and reversible fashion and is implicated in membrane-related events along the exocytosis and endocytosis pathways [35]. Previous reports have documented that Annexin A6 is involved in cell proliferation, growth, and apoptosis [36,37]. Annexin A6 participates in the regulation of EGFR/Ras signaling pathway and cholesterol homeostasis [35,38,39]. The SNPs in the *ANXA6* gene have been reported to be associated with osteonecrosis of the femoral head in the Korean population [40]. Although the phenotypes in this disease differ from those in osteoporosis, since the polymorphisms in this gene are associated with the phenotypes related with bone loss, it is very likely that the *ANXA6* gene plays an important role in the pathogenesis of osteoporosis.

The *COL5A1* gene encodes the alpha 1 chain of type V collagen, one of the low abundance fibrillar collagens. The *COL1A1* gene encoding the alpha 1 chain of type I collagen has long been implicated in the pathogenesis of osteoporosis because type I collagen is the main protein in bone. Many studies on the association between polymorphisms in the *COL1A1* gene and osteoporosis have been published [41,42], however, none of as-

Table 5. The summary of the results from each step of the experiments in the cell line, mouse model, and humans

Mouse Gene symbol	DD		qRT-PCR				Humans Gene symbol	Number of tested SNPs	Number of associated SNPs (lowest *P*-value)		
	Cell line		Cell model		Mouse model				Human (woman)		
	Con	Dex	Con	Dex	Sham	OVX			BD-RT	BD-TT	Osteoporosis
AnxA6	+	++	1.0	2.8 ± 0.1^{a}	1.0	1.5 ± 0.2^{c}	*ANXA6*	18	2 (0.044)	$4 (1.6\times10\text{-}3)^{e}$	1 (0.018)
Cnn3	+	++	1.0	1.3 ± 0.2	1.0	1.1 ± 0.1	*CNN3*	-	-	-	-
Col5a1	+	++	1.0	2.1 ± 0.5^{b}	1.0	1.9 ± 0.5^{c}	*COL5A1*	29	$3 (2.5\times10\text{-}3)$	$6 (5.3\times10\text{-}4)^{e}$	6 (0.010)
Col6a2	++	+++	1.0	2.8 ± 0.2^{b}	1.0	2.2 ± 0.6^{c}	*COL6A2*	3	0	0	0
Eno1	+++	++	1.0	0.8 ± 0.1^{b}	1.0	0.7 ± 0.1^{c}	*ENO1*	3	0	$3 (6.3\times10\text{-}3)$	1 (0.041)
Kitl	+	++	1.0	2.6 ± 0.6^{b}	1.0	1.9 ± 0.5^{c}	*KITLG*	9	0	0	0
Myof	+	+++	1.0	3.2 ± 0.2^{a}	1.0	2.3 ± 0.4^{d}	*MYOF*	29	6 (0.038)	9 (0.017)	4 (0.022)
Nfib	+	++	1.0	2.6 ± 0.3^{b}	1.0	1.1 ± 0.4	*NFIB*	-	-	-	-
Scara5	+	+++	1.0	3.0 ± 0.2^{a}	1.0	2.2 ± 0.3^{d}	*SCARA5*	25	1 (0.044)	$6 (6.4\times10\text{-}3)$	$2 (6.1\times10\text{-}3)$
S100a10	+++	++	1.0	2.4 ± 0.4^{a}	1.0	1.2 ± 0.1	*S100A10*	-	-	-	-

Values are presented as mean±standard deviation.
[a]$P<0.01$ vs. Control. [b]$P<0.05$ vs. Control. [c]$P<0.05$ vs. Sham. [d]$P<0.01$ vs. Sham. [e]The lowest *P*-value satisfying the Bonferroni-corrected significance level ($P<0.00185$).
DD, differential display; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; SNP, single nucleotide polymorphism; Con, control; Dex, dexamethasone; OVX, ovariectomy; BD-RT, bone density estimated by T-score at the distal radius; BD-TT, bone density estimated by T-score at the midshaft tibia; - , not tested.

sociation results between the *COL5A1* gene and osteoporosis have been reported. In the meantime, it has been reported that the *COL5A1* gene is associated with various diseases including chronic Achilles tendinopathy [43], Achilles tendon injuries [44,45] and anterior cruciate ligament rupture in female participants [46]. In addition, mutations within the *COL5A1* gene have been implicated in Ehlers-Danlos syndrome which is a multisystemic disorder that primarily affects the soft connective tissues [47]. These results suggested the *COL5A1* gene can be a candidate high risk factor for osteoporosis and we found a significant association between the SNPs in the *COL5A1* gene and bone density and osteoporosis phenotypes.

The *ENO1* gene encodes a key glycolytic enzyme alpha-enolase that acts as a 2-phospho-D-glycerate hydrolase. It is also involved in various processes such as growth control, hypoxia tolerance and autoimmune responses [48,49]. Previous report showed that the *ENO1* gene was significantly down-regulated in postmenopausal women compared with premenopausal women [50]. Our results also showed that the expression level of the *ENO1* gene was decreased in both the cell line and mouse models for osteoporosis (Figs. 3 and 4). Based on the fact that deficiency of estrogen level due to menopause is closely related with an increase in osteoclast life span and a concomitant decrease in osteoblast life span and further osteoporosis [51,52], these results suggest that the *ENO1* gene may be involved in bone metabolism.

The *MYOF* gene encodes Myoferlin which is a member of the ferlin family of proteins that promotes endomembrane fusion with the plasma membrane in muscle cells and endothelial cells [53]. Since Myoferlin was identified as a protein highly homologous to Dysferlin, the gene product of the limb girdle muscular dystrophy (LGMD) 2B locus, *MYOF* has been suggested as a candidate gene and potential modifier for muscular dystrophy [54]. The *SCARA5* gene encodes scavenger receptor class A member 5 which is involved in the host defense properties of populations of human epithelial cells [55]. Scara5 is also known to be a ferritin receptor mediating non-transferrin iron delivery [56]. Class A scavenger receptor promotes osteoclast differentiation [57].

By *in silico* analysis of transcription factor binding of the significant SNPs using the TRANSFAC database (http://www.cbrc.jp/research/db/TFSEARCH.html), we found the binding sites of transcription factors in several SNPs. The sequence region in the minor allele of the SNP, rs12005720 in the *COL5A1* gene contained the binding site for ETS1 (93.1 scoring point). ETS1 has been reported to be associated with systemic lupus erythematosus (SLE) [58]. SLE often accompanies osteoporosis. The sequence region in the minor allele of the SNP, rs787667 in the *MYOF* gene contained the binding site for GATA1 (93.0 scoring point). In addition, POU2F could bind to the sequence regions of the SNP, rs6660137 in the *ENO1* gene (90.0 scoring point) and the SNP, rs2726941 in the *SCARA5* gene (94.5 scoring point).

In conclusion, we identified 5 novel osteoporosis susceptibility genes through candidate gene selection in cells, evaluation of the DEGs in cells and mice, and association analysis in humans. There was a significant association between the SNPs in the 5 genes, *ANXA6, COL5A1, ENO1, MYOF*, and *SCARA5*, and bone density and/or osteoporosis. Nevertheless, further replication studies in other ethnic populations and functional studies on these 5 genes are needed. Notably, the SNPs in the *ANXA6* and *COL5A1* genes were highly significantly associated with bone density. These results indicate that these two genes may play an important role in regulation of bone metabolism and further the pathogenesis of osteoporosis.

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Authors' Contributions

Conception and design: HSJ, SYJ. Acquisition of data: BYK, EP, JK, CGL. Analysis and interpretation of data: DWK, BYK, EP. Drafting the article: BYK, DWK, EP, HSJ, SYJ. Final approval of the version to be published: HSJ, SYJ.

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