Steroid and Xenobiotic Receptor SXR Mediates Vitamin K$_2$-activated Transcription of Extracellular Matrix-related Genes and Collagen Accumulation in Osteoblastic Cells*◆

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Vitamin K$_2$ is a critical nutrient required for blood coagulation. It also plays a key role in bone homeostasis and is a clinically effective therapeutic agent for osteoporosis. We previously demonstrated that vitamin K$_2$ is a transcriptional regulator of bone marker genes in osteoblastic cells and that it may potentiate bone formation by activating the steroid and xenobiotic receptor, SXR. To explore the SXR-mediated vitamin K$_2$ signaling network in bone homeostasis, we identified genes up-regulated by both vitamin K$_2$ and the prototypical SXR ligand, rifampicin, in osteoblastic cells using oligonucleotide microarray analysis and quantitative reverse transcription-PCR. Fourteen genes were up-regulated by both ligands. Among these, tsukushi, matrilin-2, and CD14 antigen were shown to be primary SXR target genes. Moreover, collagen accumulation in osteoblastic MG63 cells was enhanced by vitamin K$_2$ treatment. Gain- and loss-of-function analyses showed that the small leucine-rich proteoglycan, tsukushi, contributes to vitamin K$_2$-mediated enhancement of collagen accumulation. Our results suggest a new function for vitamin K$_2$ in bone formation as a transcriptional regulator of extracellular matrix-related genes, that are involved in the collagen assembly.

Vitamin K is an important cofactor in blood coagulation and also known as a potent stimulator of the bone building process. Vitamin K is a family of structurally similar, fat-soluble, 2-methyl-1,4-naphthoquinones, including phylloquinone (K$_1$), menaquinones (K$_2$), and menadione (K$_3$). Vitamin K$_1$ and vitamin K$_2$ are natural vitamin Ks. The former is found in plants, whereas the latter is mainly derived from animal sources and produced by intestinal bacteria. *In vitro* studies showed that vitamin K$_2$ is far more active than K$_1$ in both promoting bone formation and reducing bone loss (1–5). Human studies have demonstrated that vitamin K$_2$ is an effective treatment for osteoporosis and preventing fractures (6, 7). Menaquinone-4 (MK-4),[2] the most common form of

vitamin K$_2$ containing four isoprene units, is frequently prescribed for osteoporosis in Japan.

One of the major known functions of vitamin K is the posttranslational modification of vitamin K-dependent proteins containing γ-carboxyglutamic acid (Gla) residues, most of which are related to coagulation (as reviewed in Ref. 8). In vitamin K-dependent carboxylation reactions, the reduced form of vitamin K de-protonates glutamate via the γ-glutamyl carboxylase and the reduced vitamin K is converted to vitamin K epoxide. Two such vitamin K-dependent proteins were identified in bone: osteocalcin and matrix Gla protein (MGP). Osteocalcin is a bone protein only synthesized in osteoblasts and odontoblasts. It serves as a good biochemical marker of the metabolic turnover of bone because the osteocalcin lacking Gla residues cannot bind to hydroxyapatite, one of the major components of bone matrix (9). Levels of undercarboxylated osteocalcin increase during aging and significantly correlates with fracture risk (10). Therefore, vitamin K-modified osteocalcin plays an important role in bone homeostasis. In contrast to osteocalcin, MGP is predominantly expressed in chondrocytes and vascular smooth muscle cells. *Mgp*-deficient mice exhibited inappropriate calcification of various cartilages as well as arterial walls, indicating that MGP is a modulator of extracellular matrix mineralization (11, 12). Despite structural similarities between osteocalcin and MGP, these two Gla proteins exhibit different functions. These findings suggest that vitamin K plays a significant role in bone homeostasis, although the precise mechanisms through which bone Gla proteins regulate homeostasis are complex.

During the 60-year history of vitamin K research, most of the attention has been paid to the actions of vitamin K on γ-carboxylation. We recently identified a novel mechanism of vitamin K functions via transcriptional regulation in osteoblastic cells (13). Both vitamin K$_2$ and the known SXR ligands rifampicin (RIF) and hyperforin up-regulated expression of the prototypical SXR target gene *CYP3A4* and bone markers such as alkaline phosphatase (*ALP*) and *MGP* (13). Our findings suggested an important role for vitamin K$_2$-dependent transcriptional regulation in bone homeostasis. Until now, the contribution of distinct vitamin K$_2$ and SXR target genes to these mechanisms remained to be studied.

In the present study, we searched for SXR target genes induced by vitamin K$_2$ and RIF in osteoblastic MG63 cells using microarray analysis. Several genes were identified that are up-regulated by both agonists. We focused here on the osteoblastogenic functions of extracellular matrix-related genes as SXR targets in response to vitamin K$_2$ treatment. Furthermore, we showed that the novel SXR target, tsukushi (*TSK*), plays a role in collagen accumulation in MG63 cells. Our findings indicate that vitamin K$_2$ activates SXR to regulate the transcription of extracellular matrix-related genes that may contribute to collagen assembly.

Vitamin K$_2$ Activates SXR Target Genes in Osteoblastic Cells

EXPERIMENTAL PROCEDURES

Cell Culture—MG63 human osteosarcoma cells, 293T, and COS1 cells were grown in Dulbecco's modified Eagle's medium supplement with 10% fetal bovine serum (FBS), 50 units/ml penicillin, and 50 μg/ml streptomycin. Prior to vitamin K$_2$ treatment, cells were cultured in phenol red-free media containing 10% dextran-charcoal-stripped FBS.

Cloning and Construction of cDNAs—Human SXR (pCDG-SXR), human SXR containing the VP16 activation domain upstream to SXR (VP16-SXR), and tk-(3A4)$_3$-Luc containing three-copy SXR response elements from human cytochrome P-450 (CYP) 3A4 promoter were described previously (14–16). N-terminally FLAG-tagged pcDNA3 (Invitrogen) plasmids containing SXR (pcDNA3-FLAG-SXR) and VP16C-SXR (pcDNA3-FLAG-VP16C-SXR) were generated by PCR using pCDG-SXR and VP16-SXR as templates, respectively, and inserted in-frame to FLAG-tagged pcDNA3 at EcoRI and XhoI sites. VP16C-SXR contained 20 amino acids from the C terminus of VP16 activation domain upstream of SXR. The tsukushi (*TSK*) cDNA was isolated from first-strand cDNA derived from MG63 cells using primers 5'-CACGAATTCGCCACCATGCCGTGGCCCCTGCTG-3' and 5'-CGACTCGAGCAAGATGGTGGGGCCCCTGGC-3', inserted in-frame to C-terminally FLAG-tagged pcDNA3 at EcoRI and XhoI sites (pcDNA3-TSK-FLAG). DNA sequences of plasmids were determined by ABI PRIZM 377 Sequencer (Applied Biosystems, Foster City, CA).

Luciferase Assay—Luciferase assay was performed using MG63 cells (2 × 10^4 cells/well on 24-well plates) transfected with 115 ng of tk-(3A4)$_3$-Luc, 130 ng of pRL-CMV (Promega), and 5 ng of FLAG-pcDNA3 or FLAG-tagged SXR plasmids using the FuGENE 6 reagent (Roche Diagnostics). Twenty-four hours after transfection, cells were treated with 20 μM RIF (Nakalai Tesque, Kyoto, Japan), 20 μM MK-4 (gifted by Eisai Co., Ltd., Tokyo, Japan), or vehicle (0.2% ethanol) for 30 h in fresh media, and luciferase activities were determined by a MicroLumatPlus microplate luminometer (Berthold Technologies) using the dual-luciferase assay system (Promega). Firefly luciferase activity was normalized to *Renilla* luciferase, which was used as a transfection control. The experiments were repeated three times with similar results.

Generation of Stable Cell Lines—MG63 cells were transfected with pcDNA3-FLAG-SXR, pcDNA3-FLAG-VP16C-SXR, pcDNA3-TSK-FLAG, or empty FLAG-tagged pcDNA3 using the FuGENE 6 reagent and selected in 0.5 mg/ml G418. Expression levels of FLAG-SXR, FLAG-VP16C-SXR, and TSK-FLAG proteins were verified by Western blot analysis.

Western Blot Analysis and Immunoprecipitation—Whole cell lysates were prepared using PLC lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl$_2$, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Protein concentrations were analyzed using the BCA protein assay kit (Pierce). Proteins were resolved by SDS-PAGE and electroblotted onto Immobilon-P transfer membrane (Millipore). Membranes were incubated with primary antibodies for 90 min followed by incubation with secondary antibodies for 30 min. After extensive washing, the antibody-antigen complexes were detected using the Western blotting Chemiluminescence Luminol Reagent (Santa Cruz Biotechnology). Antibodies used included anti-PXR (pregnane X receptor)/SXR (N-16 and H-160, Santa Cruz Biotechnology), anti-α-tubulin monoclonal antibody (Zymed Laboratories), anti-FLAG M2 monoclonal antibody (Sigma), and anti-Myc polyclonal antibody (Cell Signaling Technology). For SXR detection in parental MG63 cells, 500 μg of proteins from cell lysates were incubated with anti-SXR antibody (H-160) or normal rabbit IgG (Sigma) at 4 °C overnight. The mixture of cell extracts and antibody was

incubated with Protein G-Sepharose beads (Amersham Biosciences) at 4 °C for 2 h, washed four times using PLC lysis buffer. The immunoprecipitated proteins were boiled 5 min in Laemmli sample buffer and separated by SDS-PAGE.

Preparation of cRNA—Total RNA was extracted from MG63 cells stably expressing FLAG-VP16C-SXR treated with vehicle (0.1% ethanol), MK-4 (10 μM), or RIF (10 μM) for 48 h. The methods for preparation of cRNA and subsequent steps leading to hybridization and scanning of the U133A GeneChip Arrays were provided by the manufacturer (Affymetrix). Briefly, poly(A)$^+$ RNA was isolated from 200 μg total RNA of each sample with the Oligotex^{TM}-dT30 Super mRNA purification kit (Takara Bio, Kyoto, Japan) and converted into double-stranded cDNA using the cDNA synthesis kit (SuperScript Choice, Invitrogen) with a special oligo(dT)$_{24}$ primer containing a T7 RNA polymerase promoter site added 3' of the poly(T) tract (Amersham Biosciences). After second-strand synthesis, labeled cRNA was generated from the cDNA sample by an *in vitro* transcription reaction using the bioarray high yield RNA transcript labeling kit (Enzo Life Sciences, Farmingdale, NY) supplemented with biotin-CTP and biotin-UTP (Enzo Life Sciences). The labeled cRNA was purified using RNeasy spin columns (Qiagen). Twenty μg of each cRNA sample was fragmented by mild alkaline treatment, at 94 °C for 35 min in fragmentation buffer (200 mM Tris acetate, pH 8.1, 500 mM potassium acetate, 150 mM magnesium acetate) and then used to prepare 400 μl of master hybridization mix (0.1 mg/ml herring sperm DNA (Promega), 0.5 mg/ml of acetylated bovine serum albumin in hybridization buffer containing 100 mM MES, 1 M [Na$^+$], 20 mM EDTA, 0.01% Tween 20).

Oligonucleotide Array Hybridization and Scanning—Before hybridization, the cRNA samples were heated to 99 °C for 5 min, equilibrated to 45 °C for 5 min, and clarified by centrifugation (15,000 rpm) at room temperature for 5 min. Aliquots of each sample (10 μg of cRNA in 200 μl of the master mix) were hybridized to U133A GeneChip arrays at 45 °C for 16 h in a rotisserie oven set at 60 rpm. After this, the arrays were washed with non-stringent wash buffer (6 × saline/sodium phosphate/EDTA, 0.01% Tween 20) and stringent wash buffer (100 mM MES/0.1 M [Na$^+$], 0.01% Tween 20), stained with streptavidin-phycoerythrin (Molecular Probes), washed again, and read using a microarray scanner G2500A (Affymetrix) with the 570-nm long-pass filter. Data analysis was performed by using Affymetrix Microarray Suite software. For comparing arrays, normalization was performed using data from all probe sets.

Reverse Transcription-PCR Analysis—MG63 cells were treated with 10 μM RIF, 10 μM MK-4, or vehicle for indicated times. Total RNA was isolated using the ISOGEN reagent (Nippon Gene, Tokyo, Japan). First strand cDNA was generated from RNase-free DNase I-treated total RNA by using the SuperScript II reverse transcriptase (Invitrogen) and oligo(dT)$_{20}$ primer. For PCR amplification, the primer sequences were: human *TSK*, 5'-CTGAGCGACGTGAACCTTAGC-3' and 5'-CCTGACTGTGCGTCGTGAAG-3'; human *MATN2*, 5'-ACAGATCCTTTGCCTGTCAGTGT-3' and 5'-GGTCCCCCAGAGCACAAGA-3'; human *CD14*, 5'-GACTGATGGCGGCTCTCTGT-3' and 5'-TGTGGGCGTCTCCATTCC-3'; human *CYP3A4*, 5'-TTCAGCCCATCTCCTTTCATATTT-3' and 5'-CAGTTGGGTGTTGAGGATGGA-3'; human *GAPDH*, 5'-GCCTGCCTGACCAAATGC-3' and 5'-GTGGTCGTTGAGGGCAATG-3'. mRNAs were quantified by real-time PCR using SYBR green PCR master mix (Applied Biosystems) and the ABI Prism 7000 system (Applied Biosystems) based on SYBR Green I fluorescence. The evaluation of relative differences of PCR product amounts among the treatment groups was carried out by the comparative cycle threshold (C$_T$) method, using GAPDH as an external control (17).

The experiments were independently repeated at least three times, each performed in triplicate. For cycloheximide treatment, cells were preincubated with the compound (10 μg/ml) 2 h prior to the stimulation by SXR ligands.

RNA Interference—Small interfering RNA (siRNA) duplexes to target human *SXR* and *TSK* were synthesized by Qiagen (Qiagen, Tokyo, Japan). The siRNA target sequences were: *SXR*, 5′-GGCCACTGGC-TATCACTTC-3′ (18) and *TSK*, 5′-CCTGCTCACCAGCATCTCA-3′. The siRNA specific to the luciferase gene (Luciferase GL2 Duplex, Dharmacon, Lafayette, CO) and nonspecific control VII (Dharmacon) was used as control. Cells were transfected with siRNA (70 nM) using GeneSilencer reagent (Genlantis, San Diego, CA) for 48 h, and further maintained in the culture medium containing 10% dextran-charcoal-stripped FBS with or without ligand stimulation for indicated times.

Collagen Accumulation Assay by Sirius Red Staining—Cells were cultured until confluence (day 0), and the medium was replaced by the osteoblast differentiation medium (α-minimal essential medium containing 10% FBS, 2 mM glutamine, 50 μg/ml ascorbic acid, and 5 mM β-glycerophosphate) with or without MK-4 (1 μM). Cells were fixed with Bouin's fluid (8.3% formaldehyde and 4.8% acetic acid in saturated aqueous picric acid) for 1 h at room temperature, rinsed with water, and stained with 1 mg/ml of sirius red dye (Direct Red 80) (Sigma) in saturated aqueous picric acid for 1 h. Cells were treated with 0.01 N HCl, and then the stain was extracted by 0.1 N NaOH. The absorbance of the dye solution was measured at 550 nm (19). In experiments with warfarin [3-(α-acetonylbenzyl)-4-hydroxycoumarin, Sigma) treatment, cells at confluence were pretreated with vehicle or warfarin at 5 μM or 25 μM for 1 day, then treated with vehicle or vitamin K$_2$ (1 μM) for another 3 days in the presence of warfarin (final concentration; 2.5 μM or 12.5 μM). In siRNA treatment experiments, cells were treated with the siRNA twice, 2 days before day 0 and on day 0.

Statistical Analysis—Differences between two groups were analyzed using two-sample, two-tailed Student's *t* test. A *p* value less than 0.05 was considered to be significant. All data are presented in the text and figures as the mean ± S.D.

RESULTS

Construction of SXR Expression Vectors and Generation of Osteoblastic Cells Stably Expressing SXR—Our previous studies showed the direct effect of vitamin K$_2$ on bone marker expression in osteoblastic cells. Although SXR is endogenously expressed in osteoblastic cells, it has been shown that the expression level is lower than that in cells derived from the intestine. Therefore, to identify vitamin K$_2$ and SXR target genes in osteoblastic cells, we generated MG63 cells stably expressing SXR. These cells respond more robustly to SXR ligands than do wild type MG63 cells. We constructed two FLAG-tagged expression vectors containing full-length SXR (FLAG-SXR) and SXR fused to the C-terminal portion of the VP16 activation domain (FLAG-VP16C-SXR). Both vitamin K$_2$ and RIF increased the transcriptional activity of the trimerized SXR response elements derived from the *CYP3A4* promoter in MG63 cells transiently transfected with FLAG-SXR or FLAG-VP16C-SXR (Fig. 1*A*). We also constructed an expression vector fusing SXR to the full-length VP16 activation domain. This construct was constitutively active in the absence of ligand stimulation (data not shown). In contrast, FLAG-VP16C-SXR retained the ligand-dependent activation while it had substantial higher basal transcription level than FLAG-SXR.

For further quantitative analysis of SXR actions in osteoblastic cells, we generated the stable cell lines expressing *SXR* constructs in MG63 cells. For either FLAG-SXR or FLAG-VP16C-SXR, we obtained two MG63 clones each with different expression levels (Fig. 1*B*).
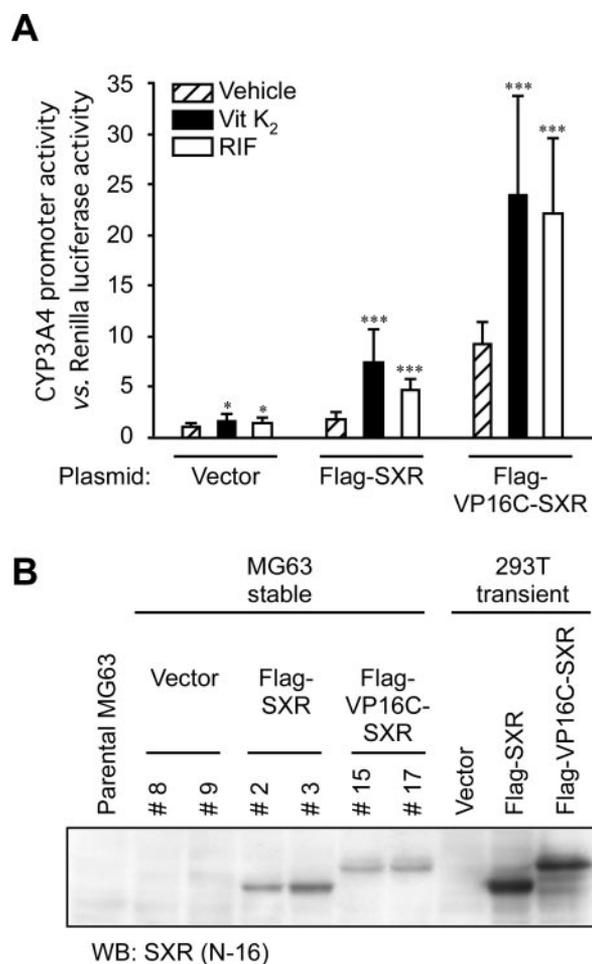

FIGURE 1. **Construction of SXR expression vectors and generation of osteoblastic cells stably expressing SXR.** *A*, activation of *CYP3A4* promoter activity by vitamin K$_2$ (*Vit K$_2$*) or RIF treatment. MG63 cells were cotransfected with tk-(CYP3A4)$_3$-Luc, pRL-CMV, and SXR expression vectors (FLAG-SXR and FLAG-VP16C-SXR) or empty vector. Twenty-four hours after transfection, cells were treated with RIF (20 μM), vitamin K$_2$ (20 μM), or vehicle (0.2% ethanol) for 30 h in fresh media. Firefly luciferase reporter activity was determined and normalized to the *Renilla* luciferase transfection control. Data are expressed as fold induction of luciferase activity by ligands *versus* vehicle in vector-transfected cells. *, $p < 0.05$; ***, $p < 0.001$ (by Student's *t* test). *B*, generation of MG63 cells stably expressing SXR constructs. SXR protein expression in MG63/FLAG-SXR clones #2 and #3 and MG63/FLAG-VP16C-SXR clones #15 and #17 was confirmed by Western blotting (*WB*) using anti-SXR antibody.

Identification of Genes Up-regulated by SXR Ligand in Osteoblastic Cells by GeneChip Analysis—To identify dual up-regulated genes by vitamin K$_2$ and RIF treatment in osteoblastic cells, we prepared biotin-labeled cRNA samples from MG63 cells expressing FLAG-VP16C-SXR treated with vitamin K$_2$, RIF, or vehicle. The Affymetrix U133A Gene-Chip array represents more than 18,000 human transcripts from ~14,000 genes. Analysis of the MG63 samples was performed by hybridizing aliquots of cRNA to the GeneChip arrays. Seventy-seven transcripts were induced 2-fold or greater by vitamin K$_2$, whereas 100 transcripts were induced by RIF. Eighteen transcripts were up-regulated by both SXR ligands. Therefore, we infer that these are potential SXR target genes.

Table 1 shows the list of 18 transcripts from 14 distinct genes up-regulated by vitamin K$_2$ and RIF. It is notable that a prototypical SXR-responsive gene ATP-binding cassette subfamily B or multidrug resistance 1 (*MDR1*) (20) was most significantly up-regulated by either vitamin K$_2$ or RIF. Among these SXR target molecules, we were particularly interested in three genes due to their putative bone-related functions. There were a small leucine-rich proteoglycan named tsukushi

TABLE 1

Dual up-regulated genes by 48-h treatment with vitamin K$_2$ (10 μM) or RIF (10 μM) in MG63/FLAG-VP16C-SXR cells identified by GeneChip analysis

Gene annotation was determined based on the probe set ID by the Array Finder on the Affymetrix web site.

Probe set ID	Ensemble ID	Gene symbol	Description	-Fold increase over control	
				Vitamin K$_2$	RIF
209994_s_at	ENSG00000085563	*ABCB1*	ATP-binding cassette, subfamily B (MDR/TAP), member 1	6.06	4.29
209993_at	ENSG00000085563	*ABCB1*	ATP-binding cassette, subfamily B (MDR/TAP), member 1	4.29	4.00
205357_s_at	ENSG00000144891	*AGTR1*	Angiotensin II receptor, type 1	2.83	2.46
212938_at	ENSG00000142156	*COL6A1*	Collagen, type VI, α1	2.64	2.14
201743_at	ENSG00000170458	*CD14*	CD14 antigen	2.46	4.92
209771_x_at	ENSG00000185275	*CD24*	CD24 antigen (small cell lung carcinoma cluster 4 antigen)	2.46	2.30
211839_s_at	ENSG00000184371	*CSF1*	Colony-stimulating factor 1 (macrophage)	2.46	2.30
212937_s_at	ENSG00000142156	*COL6A1*	Collagen, type VI, α1	2.46	2.14
216379_at	ENSG00000185275	*CD24*	CD24 antigen (small cell lung carcinoma cluster 4 antigen)	2.46	2.30
202350_s_at	ENSG00000132561	*MATN2*	Matrilin-2	2.30	6.96
203632_s_at	ENSG00000167191	*GPRC5B*	G protein-coupled receptor, family C, group 5, member B	2.30	5.28
211653_x_at	ENSG00000151632	*AKR1C2*	Aldo-keto reductase family 1, member C2	2.30	3.73
218245_at	ENSG00000182704	*TSK*	Likely ortholog of chicken tsukushi	2.30	2.64
218854_at	ENSG00000111817	*SART2*	Squamous cell carcinoma antigen recognized by T cells 2	2.30	5.28
210002_at	ENSG00000141448	*GATA6*	GATA-binding protein 6	2.14	2.14
216594_x_at	ENSG00000187134	*AKR1C1*	Aldo-keto reductase family 1, member C1	2.14	3.73
204151_x_at	ENSG00000187134	*AKR1C1*	Aldo-keto reductase family 1, member C1	2.00	3.48
212268_at	ENSG00000021355	*SERPINB1*	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 1	2.00	2.83

(*TSK*), an extracellular matrix protein matrilin-2 (*MATN2*), and CD14 antigen.

Ligand-dependent Induction of SXR Target Genes in Osteoblastic Cells—We next validated whether mRNA expression levels for these three genes could be modulated by vitamin K$_2$ and RIF in MG63 cells ectopically expressing either FLAG-VP16C-SXR or FLAG-SXR using quantitative real-time RT-PCR analysis (Fig. 2). All of the three SXR target genes, *TSK*, *MATN2*, and *CD14*, were up-regulated by SXR ligands. The time-dependent expression profiles of those genes in FLAG-VP16C-SXR and FLAG-SXR-expressing cells were quite similar, although the amplitude of induction was different in these cells. In both cell types, RIF generated stronger induction of mRNA expression than vitamin K$_2$. Nevertheless, the maximal induction by vitamin K$_2$ was greater than 2-fold for all three genes in both cell types.

Transcriptional Regulation of SXR Target Genes in Osteoblastic Cells—We next asked whether the induction of SXR target genes was dependent on direct activation of transcription or required ongoing protein synthesis. MG63 cells overexpressing FLAG-SXR were treated with vitamin K$_2$ or RIF in the presence or absence of cycloheximide. The ligand-dependent up-regulation of the three SXR target genes, including *TSK*, *MATN2*, and *CD14*, was not affected by cycloheximide treatment, indicating that the transcriptional regulation of those genes was independent of protein synthesis (Fig. 3*A*).

To further demonstrate the requirement for SXR in the regulation of *TSK*, *MATN2*, and *CD14*, we investigated the effects of siRNA on the ligand-dependent induction of gene expression. Forty-eight hour treatment with a specific siRNA duplex against SXR (siRNA-SXR), but not with a control siRNA directed against luciferase (siRNA-Luc), reduced the SXR protein level by more than 60% in MG63/SXR clone #3 (Fig. 3*B*). The effectiveness of the SXR-specific siRNA was confirmed as the vitamin K$_2$-induced up-regulation of CYP3A4 mRNA expression was diminished by the SXR siRNA in MG63/SXR clone #3 (Fig. 3*C*). In that cell system, the SXR siRNA significantly reduced either vitamin K$_2$- or RIF-activated mRNA expression for *TSK*, *MATN2*, and *CD14* (Fig. 3*D*).

We next examined whether the SXR siRNA duplex reduced the endogenous expression of SXR protein (Fig. 4). The endogenous level of SXR protein in parental MG63 cells was barely detected in Western blot analysis (Fig. 4*A*). Thus, we immunoprecipitated MG63 cell lysates with a polyclonal antibody against the hinge and a part of ligand-binding domain of SXR (H-160) and immunodetected SXR protein by another

polyclonal antibody against the SXR N terminus (N-16). The enrichment of SXR protein in immunoprecipitated fraction was also confirmed in COS1 cells transiently transfected with FLAG-SXR (Fig. 4*A*). Based on this evaluation system, we could show that the SXR siRNA reduced the level of endogenous SXR protein in MG63 (Fig. 4*B*).

Since we confirmed that the SXR siRNA duplex was effective to inhibit the endogenous expression of SXR protein, we next analyzed whether the SXR siRNA reduced the expression of the SXR target genes in parental MG63 cells. The SXR siRNA at 14 or 70 nM could significantly reduce endogenous SXR mRNA levels in natural MG63 cells (Fig. 4*C*). The expression of *TSK*, *MATN2*, and *CD14* was all up-regulated by either vitamin K$_2$ or RIF, indicating that the three genes were *bona fide* SXR targets in parental MG63 cells (Fig. 4*D*). This ligand-dependent induction of all three genes was significantly reduced by the SXR siRNA transfection in parental MG63 cells (Fig. 4*D*).

Vitamin K$_2$ and TSK Stimulate Collagen Accumulation in Osteoblastic Cells—TSK was recently identified as a bone morphogenic protein-binding protein that belongs to the small leucin-rich proteoglycan family (21), which is implicated as an extracellular matrix component. Because small leucine-rich proteoglycans such as biglycan and decorin are known to interact with matrilin-1 in the cartilage extracellular matrix (22), TSK and matrilin-1-related MATN2 are likely to be involved in the assembly of extracellular matrix, including collagens, in osteoblastic cells.

We next asked whether vitamin K$_2$ promoted collagen production or stabilized collagen levels. We evaluated collagen amounts by staining cells with a strong anionic dye Sirius red, which reacted with basic groups present in collagens via its sulfonic acid groups. It has been reported that type I and III collagens are well stained by Sirius red (19). Four-day treatment with vitamin K$_2$ exhibited significantly more intense staining by Sirius red compared with vehicle in MG63 cells under conditions favoring osteoblast differentiation (Fig. 5*A*). We also examined collagen accumulation in murine MC3T3-E1 cells, one of the cell lines with a close-to-normal osteoblast phenotype. Four-day treatment with vitamin K$_2$ increased collagen accumulation by 15.0% in this cell line. Note that RIF (1 μM) also increased collagen accumulation by 13.6% in MG63 cells after 4-day treatment. Moreover, the vitamin K$_2$-stimulated collagen accumulation in MG63 cells was not affected by warfarin treatment, suggesting that the γ-carboxylase-dependent vitamin K$_2$ action may not be involved (Fig. 5*B*).
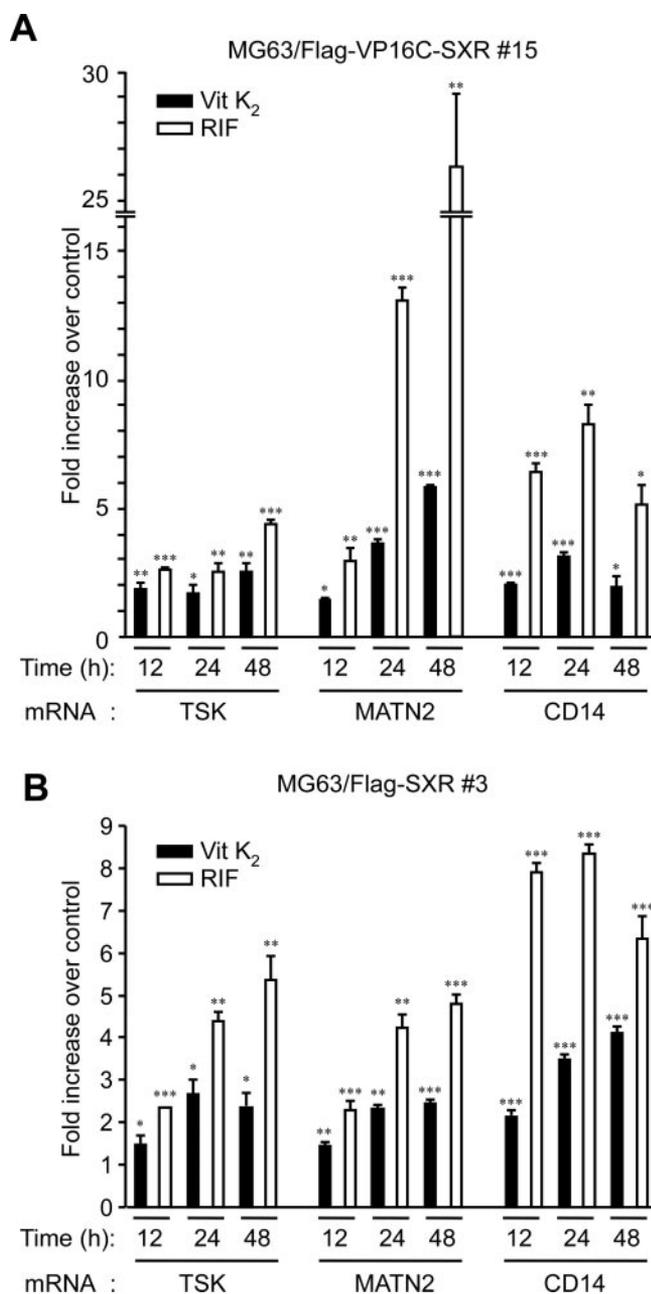
FIGURE 2. Validation of ligand-induced up-regulation of SXR target genes in osteoblastic cells stably expressing SXR. MG63/FLAG-VP16C-SXR clone #15 (*A*) and MG63/FLAG-SXR #3 (*B*) cells were treated with vitamin K$_2$ (*Vit K$_2$*) (10 μM) or RIF (10 μM) for indicated times. mRNA levels for tsukushi (*TSK*), *MATN2*, and *CD14* were determined by quantitative RT-PCR using *GAPDH* as an external control. Data represent fold induction of mRNA levels by ligands *versus* vehicle. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (by Student's *t* test).

FIGURE 3. Ligand-induced up-regulation of TSK, MATN2, and CD14 are directly regulated by SXR. *A*, effects of cyclohexamide (*CHX*) on transcription of SXR target genes. MG63/FLAG-SXR #3 cells were pretreated with CHX (10 μg/ml) or vehicle for 2 h, then stimulated by vitamin K$_2$ (*Vit K$_2$*) (10 μM) or RIF (10 μM) for 24 h. *B*, reduction of SXR expression by siRNA directed against SXR. MG63/FLAG-SXR cells #3 were transfected with siRNA against luciferase (*Luc*) or SXR (70 nM each) for 48 h. The SXR protein level was analyzed by Western blotting (*WB*). Data in the *upper graph* represent quantified levels of SXR normalized to α-tubulin as determined by NIH image software. *C*, effects of SXR siRNA transfection on vitamin K$_2$-induced up-regulation of *CYP3A4* mRNA in MG63/FLAG-SXR #3 cells. *D*, effects of SXR siRNA transfection on ligand-induced up-regulation of *TSK*, *MATN2*, and *CD14* mRNA in MG63/FLAG-SXR #3 cells. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (by Student's *t* test).

To determine whether TSK plays a role in the vitamin K$_2$-stimulated collagen accumulation, MG63 cells stably expressing FLAG-tagged TSK were generated. Two TSK-overexpressing clones were isolated, as confirmed by Western blot analysis (Fig. 5*C*). MG63 clones overexpressing TSK showed significantly enhanced collagen accumulation in 7-day culture under differentiation conditions compared with vector-transfected cells (Fig. 5*D*). During the 7-day culture, the growth of MG63 clones expressing TSK and vector was almost stationary, and there was no significant difference in proliferation between the two groups as determined by the proliferation assay using WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-
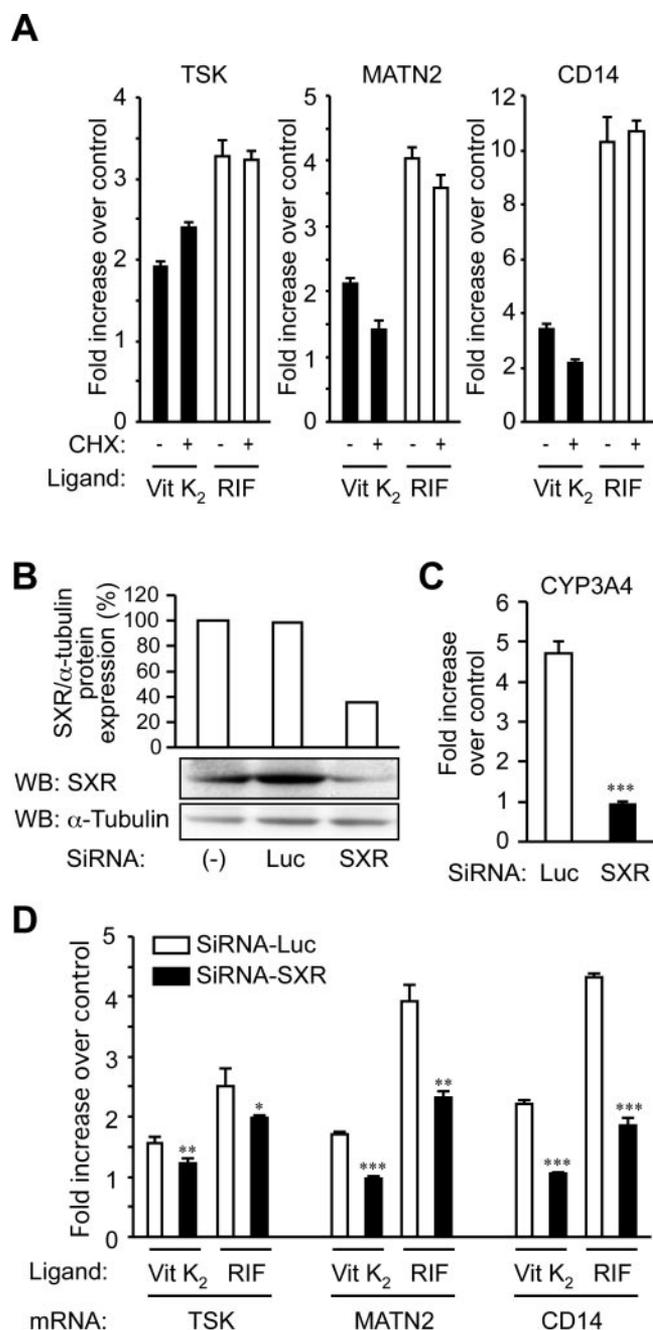
tetrazolium monosodium salt) reagent (Nacalai Tesque, Kyoto, Japan; Ref. 23) (data not shown).

We further investigated whether SXR or TSK loss-of-function affected collagen accumulation in MG63 cells. A siRNA duplex against *TSK* (70 nM) reduced the target mRNA levels by more than 40% in parental MG63 cells, verifying its efficiency (Fig. 6*A*). The *SXR*- and *TSK*-specific siRNA significantly reduced the vitamin K$_2$-stimulated
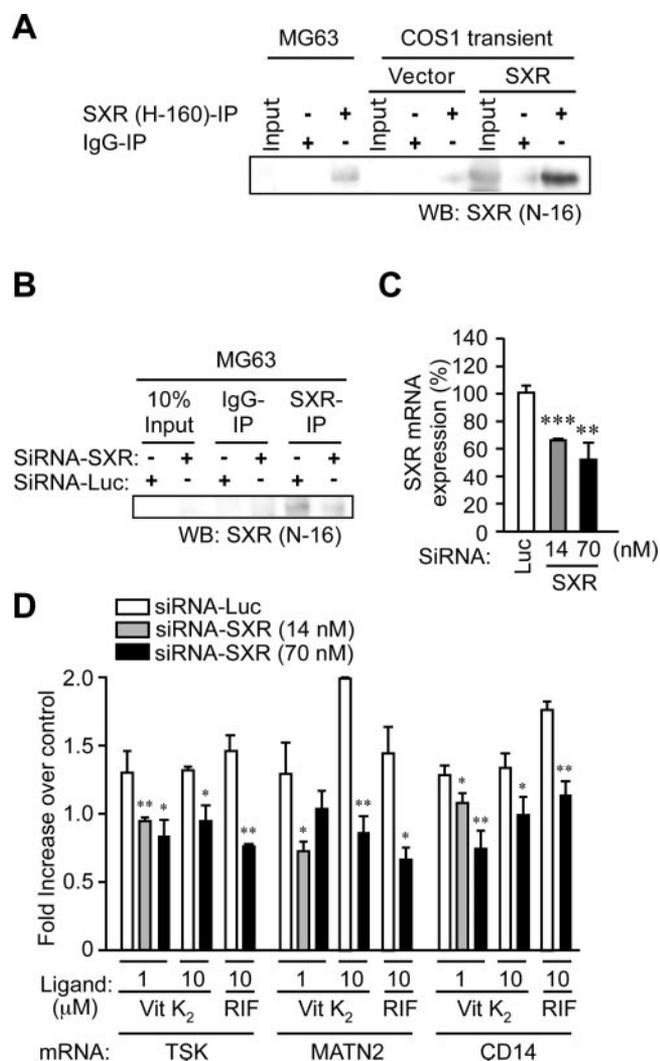
A

B

C

D

FIGURE 4. **SXR siRNA represses ligand-induced up-regulation of SXR target genes in osteoblastic cells.** *A*, detection of endogenous SXR protein enriched by immunoprecipitation (*IP*) in parental MG63 cells. Lysates from MG63 cells and COS1 cells transiently transfected with SXR or empty vector were immunoprecipitated by anti-SXR antibody (H-160), and SXR protein was immunodetected by anti-SXR (N-16) antibody. *B*, reduction of SXR protein level by *SXR* siRNA in MG63 cells. Cells were transfected with *SXR* or luciferase (*Luc*) siRNA for 48 h, and SXR protein level was analyzed as described for *A*. *C*, concentration-dependent effects of *SXR* siRNA on endogenous *SXR* mRNA levels in MG63 cells. Cells were transfected with *SXR* siRNA or control luciferase siRNA at the indicated concentrations for 24 h. *D*, effects of *SXR* siRNA on ligand-induced up-regulation of *TSK*, *MATN2*, and *CD14* mRNA in MG63 cells. After 48-h treatment with siRNA (14 or 70 nM), cells were stimulated with vitamin K₂ (1 or 10 μM) or RIF (10 μM) for 12 h. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (by Student's *t* test). *WB*, Western blotting.

accumulation of collagen in parental MG63 cells compared with luciferase siRNA (Fig. 6, *B* and *C*).

Taken together, our results indicate that vitamin K₂ promotes collagen accumulation in osteoblastic cells via the SXR signaling pathway.

DISCUSSION

In the present study, we identified novel SXR target genes that are up-regulated by both vitamin K₂ and RIF in osteoblastic cells using oligonucleotide microarrays. The effectiveness of vitamin K₂ and RIF treatment was evident by their ability to up-regulate mRNA levels for the well known SXR target gene MDR1. SXR-dependent induction of TSK, MATN2, and CD14 has not been previously reported. Functional analyses indicated that vitamin K₂ can enhance collagen accumulation in osteoblastic cells and that SXR may play a role in the collagen assembly mechanism. Taken together, these results provide important evi-
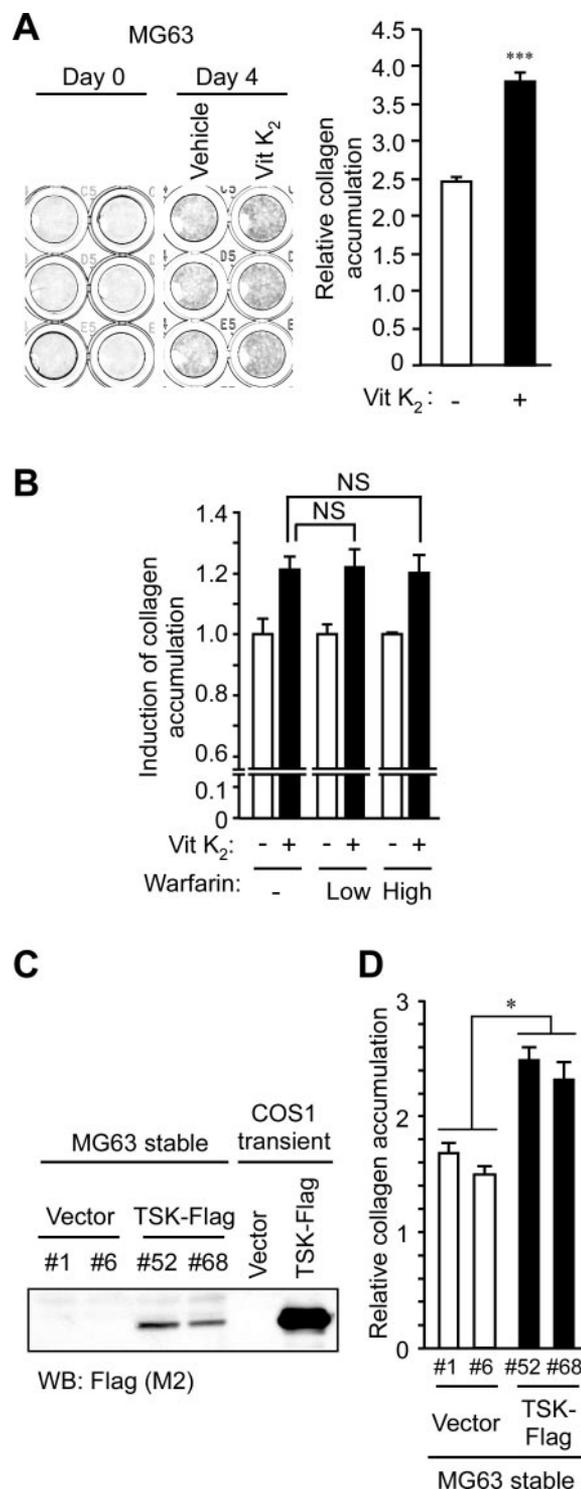
A

B

C

D

FIGURE 5. **Vitamin K₂ (*Vit K₂*) and TSK expression promote collagen accumulation in osteoblastic cells.** *A*, MG63 cells at confluence were maintained in osteoblast differentiation medium containing ascorbic acid and β-glycerophosphate in the presence or absence of vitamin K₂ (1 μM) for 4 days, and collagen accumulation was analyzed by Sirius red staining (*left panel*). The collagen level was quantified by the absorbance at 550 nm (*right panel*). ***, $p < 0.001$ (by Student's *t* test). *B*, the vitamin K₂-stimulated collagen accumulation was not affected by a γ-carboxylase inhibitor warfarin. Cells at confluence were pretreated with vehicle or warfarin (low, 5 μM; high, 25 μM) for 1 day and incubated with vehicle or vitamin K₂ (1 μM) for another 3 days (warfarin final concentration; low, 2.5 μM; high, 12.5 μM). *NS*, not significant. *C*, generation of MG63 cells stably expressing TSK-FLAG construct. Expression of TSK protein in MG63/TSK-FLAG clones #52 and #68 was immunodetected by anti-FLAG antibody. *D*, TSK overexpression augments collagen accumulation in MG63 cells. Cells at confluence were maintained in the differentiation medium for 7 days, and collagen accumulation was analyzed by Sirius red staining as described for *A*. *, $p < 0.05$ (by Student's *t* test). *WB*, Western blotting.
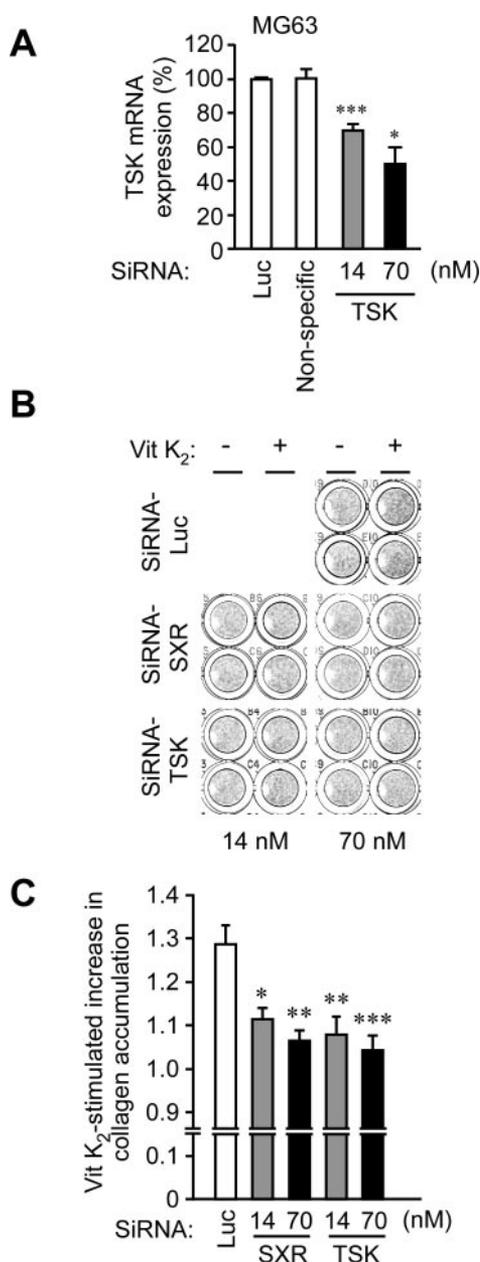
FIGURE 6. **Involvement of SXR and TSK in vitamin K$_2$ (*Vit K$_2$*)-mediated collagen accumulation.** *A*, effects of *TSK*-specific siRNA on *TSK* mRNA level in MG63 cells. Cells were transfected with luciferase (*Luc*) siRNA, nonspecific siRNA, or *TSK* siRNA (14 or 70 nM) for 48 h. *B*, *SXR* and *TSK* siRNAs reduce vitamin K$_2$-mediated collagen accumulation in MG63 cells. Confluent cells in differentiation medium were transfected with siRNA at the indicated concentrations twice every 2 days. Collagen accumulation in 4-day culture in the presence of vitamin K$_2$ (1 μM) or vehicle was determined by Sirius red staining. *C*, vitamin K$_2$-stimulated increase in collagen accumulation in *B* was quantified by the absorbance at 550 nm. Each column shows fold induction by vitamin K$_2$ normalized to that by vehicle and indicated siRNA treatment. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (by Student's *t* test, using the fold induction by vitamin K$_2$ in Luc siRNA-treated cells as a control).

dence that vitamin K$_2$ directly activates SXR to promote extracellular matrix formation in osteoblastic cells.

While vitamin K has been well characterized as a cofactor of γ-carboxylase, we have previously showed that vitamin K$_2$ could have an anabolic effect on osteoblasts by up-regulating the mRNA levels for bone marker genes through SXR (13). Our present findings that vitamin K$_2$ promotes extracellular matrix formation by activating SXR to up-regulate the *TSK* mRNA level provides further evidence that vitamin K$_2$ stimulates bone formation via altering gene expression.

The vitamin K$_2$-stimulated collagen accumulation through the activation of SXR signaling may be beneficial to decrease bone fractures. Since bone collagen content is reduced in aged and osteoporotic bones (24), the amount and quality of collagen fibrils may be important for maintaining bone strength. Therefore, in addition to its role as an enzymatic cofactor that facilitates γ-carboxylation of bone Gla proteins, vitamin K$_2$ may serve as a critical factor regulating bone matrix formation.

The identification of new SXR-mediated vitamin K$_2$ target genes in bone cells has implications for bone homeostasis. Human TSK is an ortholog of chicken TSK, which was recently identified as a bone morphogenic protein-binding protein that plays a role in the development of primitive streak and Hensen's node formation during chick gastrulation (21). TSK, like other small leucine-rich proteoglycans, may play a role in bone formation. Small leucine-rich proteoglycans such as biglycan, decorin, and chondroadherin have been characterized as collagen-binding proteins in bone tissues (25–28). Biglycan-deficient mice exhibit reduced bone mass (29), and biglycan/decorin double-deficient mice show a more severe phenotype of osteoporosis (30).

MATN2 is expressed in various osteoblastic cells as well as mouse primary osteoblasts (31, 32), and it was shown to interact with collagen I (33). The involvement of matrilin proteins together with small leuicine-rich proteoglycans in the collagen assembly is exemplified by the complex of matrilin-1 and biglycan/decorin that act as a linkage between the collagen II and collagen VI fibrils (22).

The CD14 antigen is a lipopolysaccharide-binding protein expressed in monocytes where it initiates the innate immune response to bacterial invasion (34). The soluble form of CD14 is an inducer of B-lymphocyte growth and differentiation (35), and B-lymphocyte lineage cells regulate osteoclastogenesis by expressing receptor activator of NF-κB ligand (RANKL) and serving as osteoclast progenitor cells (36). This suggests a role for CD14 in osteoclastogenesis through B-lymphocyte lineage cells. A role for CD14 in bone formation is also suggested by a report showing that the antigen was up-regulated during the differentiation of mouse primary osteoblasts (37). Because osteoclastic resorption and osteoblast formation are coupled in the bone remodeling process, CD14 may play a role as a "coupling factor" between the two functions. In this context, it is interesting that CD24 was identified as an up-regulated gene by both vitamin K$_2$ and RIF in osteoblastic cells in our microarray analysis because CD24 is also a cell surface antigen predominantly expressed in B-cell lineage cells and it has been implicated in both activation and differentiation of B lymphocytes (38).

In summary, we conclude that SXR mediates vitamin K$_2$-activated transcription of extracellular matrix-related genes as well as cell surface markers of B-lymphoid lineage cells that may be involved in both osteoblastogenesis and osteoclastogenesis. These results would provide new insight into vitamin K$_2$ and SXR action on bone homeostasis and osteoporosis treatment and further support the idea that vitamin K$_2$ acts as a transcriptional mediator of gene expression in bone cells, in addition to its well known role as an enzymatic cofactor.

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