

Vitamin K₂ induces phosphorylation of protein kinase A and expression of novel target genes in osteoblastic cells

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Abstract

Vitamin K is known as a critical nutrient required for bone homeostasis and blood coagulation, and it is clinically used as a therapeutic agent for osteoporosis in Japan. Besides its enzymatic action as a cofactor of vitamin K-dependent γ -glutamyl carboxylase (GGCX), we have previously shown that vitamin K₂ is a transcriptional regulator of bone marker genes and extracellular matrix-related genes, by activating the steroid and xenobiotic receptor (SXR). To explore a novel action of vitamin K in osteoblastic cells, we identified genes up-regulated by a vitamin K₂ isoform menaquinone-4 (MK-4) using oligonucleotide microarray analysis. Among these up-regulated genes by MK-4, growth differentiation factor 15 (GDF15) and stanniocalcin 2 (STC2) were identified as novel MK-4 target genes independent of GGCX and SXR pathways in human and mouse osteoblastic cells. The induction of GDF15 and STC2 is likely specific to MK-4, as it was not exerted by another vitamin K₂ isoform MK-7, vitamin K₁, or the MK-4 side chain structure geranylgeraniol. Investigation of the involved signaling pathways revealed that MK-4 enhanced the phosphorylation of protein kinase A (PKA), and the MK-4-dependent induction of both *GDF15* and *STC2* genes was reduced by the treatment with a PKA inhibitor H89 or siRNA against PKA. These results suggest that vitamin K₂ modulates its target gene expression in osteoblastic cells through the PKA-dependent mechanism, which may be distinct from the previously known vitamin K signaling pathways.

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Introduction

Vitamin Ks are fat-soluble 2-methyl-1,4-naphthoquinone-related compounds, including natural phyloquinone (K₁) and menaquinones (K₂). Vitamins K₁ and K₂ differ only in the substituent group. Vitamin K₁ possesses a phytol group (partially saturated polyisoprenoid group), whereas K₂ possesses a repeating, unsaturated *trans*-polyisoprenyl group. Menaquinones include a range of related forms generally designated as menaquinone-*n* (MK-*n*), where *n* is the number of isoprenyl groups. Vitamin Ks play a role in the bone-building process as well as classic blood coagulation pathway. Indeed, clinical studies have demonstrated that vitamin K₂ is an effective treatment for osteoporosis and preventing fractures (Booth *et al.* 2000, Shiraki *et al.* 2000). Menaquinone-4 (MK-4), one of the vitamin K₂ containing four isoprene units, is frequently prescribed for osteoporosis in Japan.

One of the notable molecular functions of vitamin K is as a cofactor for vitamin K-dependent γ -glutamyl carboxylase (GGCX). GGCX catalyzes the post-translational modification of specific glutamates to γ -carboxyglutamate (Gla) in a number of proteins.

Most vitamin K-dependent proteins are involved in the hemostatic process and are associated with bone metabolism. Osteocalcin (bone Gla protein), and matrix Gla protein (MGP) are two major Gla proteins in bone and γ -carboxylated proteins are important in bone metabolism. Osteocalcin serves as a good biochemical marker of the metabolic turnover of bone because osteocalcin lacking Gla residues cannot bind to hydroxyapatite, one of the major components of bone matrix (Nishimoto & Price 1985, Vergnaud *et al.* 1997). Moreover, levels of undercarboxylated osteocalcin increase during aging and significantly correlate with fracture risk (Vergnaud *et al.* 1997).

MGP is predominantly expressed and produced in chondrocytes and vascular smooth muscle cells (Luo *et al.* 1997, Shanahan & Weissberg 1998). Data from rodent studies revealed that MGP plays a key role in the inhibition of tissue calcification. Luo *et al.* (1997) reported that MGP-deficient mice showed excessive cartilage formation and growth plate mineralization, resulting in impaired growth of the long bones. Thus, vitamin K plays a significant role in bone homeostasis through γ -carboxylated proteins. On the other hand, we previously reported that vitamin K₂ has a

transcriptional regulatory function in addition to its role as an enzyme cofactor (Tabb *et al.* 2003). Vitamin K₂ was a ligand of the steroid and xenobiotic receptor (SXR), and both vitamin K₂ and the known SXR ligands rifampicin (RIF) up-regulated expression of the prototypical SXR target gene cytochrome P450 (CYP) 3A4 and bone marker genes, such as alkaline phosphatase and osteoprotegerin (Tabb *et al.* 2003). Furthermore, we identified SXR-dependent vitamin K₂ target genes that participated in extracellular matrix formation in osteoblastic cells (Ichikawa *et al.* 2006). These findings suggested an important role for vitamin K₂-dependent transcriptional regulation in bone homeostasis. Meanwhile, during our microarray analyses that identify the SXR target genes using osteoblastic cells treated with RIF or vitamin K₂, we found that a number of genes were specifically up-regulated by vitamin K₂ but not RIF. This observation suggested that potentially novel mechanism could be associated with the up-regulation of these genes by vitamin K₂.

In the present study, we screened for genes induced by MK-4 in osteoblastic MG63 cells using microarray analysis, and identified several vitamin K₂-target genes. Here, we focused on the growth differentiation factor 15 (GDF15) and stanniocalcin 2 (STC2) genes as MK-4 targets. We found that other vitamin Ks, geranylgeraniol (GGO), and SXR agonists failed to induce the

expression of *GDF15* and *STC2* genes, although a protein kinase A (PKA) activator forskolin (FSK) induced the expression of both genes. Our findings indicate that GDF15 and STC2 are regulated by a PKA-dependent, GGCX- and SXR-independent pathways.

Materials and methods

Materials

RIF was purchased from Nacalai Tesque (Kyoto, Japan). Vitamin K₁, GGO, and FSK were purchased from Sigma. MK-4 and MK-7 were gifts of Eisai Co. Ltd (Tokyo, Japan). H89 was obtained from BioMol (Plymouth Meeting, PA, USA).

Cell culture and generation of stable cell lines expressing SXR

MG63 human osteosarcoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 µg/ml streptomycin. Mouse MC3T3-E1 osteoblastic cells were maintained in α -MEM supplemented with 10% FBS, 50 U/ml penicillin, and 50 µg/ml streptomycin. Prior to vitamin K treatment, cells were cultured in phenol red-free media containing 10%

Table 1 Common up-regulated genes by 48-h treatment with menaquinone-4 (MK-4; 10 µM) in both MG63/vector and MG63/Flag-VP16C-SXR stable cell lines identified by GeneChip analysis

Probe set ID	Ensemble gene ID	Gene symbol	Description	Fold change	
				MG63/vector	MG63/Flag-VP16C-SXR
221577_x_at	ENSG00000130513	<i>GDF15</i>	Growth differentiation factor 15	8.00	5.66
218145_at	ENSG00000101255	<i>TRIB3</i>	Tribbles homolog 3 (<i>Drosophila</i>)	6.50	4.29
207145_at	ENSG00000138379	<i>GDF8</i>	Growth differentiation factor 8	4.29	2.00
219270_at	ENSG00000128965	<i>MGC4504</i>	Hypothetical protein MGC4504	3.03	3.48
202847_at	ENSG00000100889	<i>PCK2</i>	Phosphoenolpyruvate carboxykinase 2 (mitochondrial)	2.46	2.00
205047_s_at	ENSG00000070669	<i>ASNS</i>	Asparagine synthetase	2.46	3.25
203665_at	ENSG00000100292	<i>HMOX1</i>	Heme oxygenase (decycling) 1	2.30	2.46
Q6 206026_s_at	ENSG00000123610	<i>TNFAIP6</i>	Tumor necrosis factor, α -induced protein 6	2.30	2.00
203438_at	ENSG00000113739	<i>STC2</i>	Stanniocalcin 2	2.14	2.00
203477_at	ENSG00000204291	<i>COL15A1</i>	Collagen, type XV, α 1	2.14	2.14
206025_s_at	ENSG00000123610	<i>TNFAIP6</i>	Tumor necrosis factor, α -induced protein 6	2.14	2.46
209921_at	ENSG00000151012	<i>SLC7A11</i>	Solute carrier family 7, member 11	2.14	2.64
202887_s_at	ENSG00000168209	<i>DDIT4</i>	DNA-damage-inducible transcript 4	2.00	2.64
204422_s_at	ENSG00000138685	<i>FGF2</i>	Fibroblast growth factor 2 (basic)	2.00	2.30
220892_s_at	ENSG00000135069	<i>PSAT1</i>	Phosphoserine aminotransferase 1	2.00	2.00

MG63/vector and MG63/Flag-VP16C-SXR cells are stably expressing Flag-pcDNA3 vector and pcDNA3-Flag-VP16C-SXR respectively. Twofold or more up-regulated genes by MK-4 over vehicle in both cells were selected, except SXR-dependent up-regulated ones with the ratios of fold change in MG63/Flag-VP16C-SXR versus MG63/vector by ≥ 1.5 -fold. Gene annotation was determined based on the probe set ID by the Array Finder on the Affymetrix web site (<http://www.affymetrix.com>).

dextran–charcoal stripped FBS (dccc-FBS). N-terminally Flag-tagged pcDNA3 (Invitrogen) plasmids containing VP16C-SXR (pcDNA3-Flag-VP16C-SXR) were previously described (Ichikawa *et al.* 2006). VP16C-SXR contained 20 amino acids from the C-terminus of VP16 activation domain upstream of SXR. Generation of MG63 cell lines stably expressing pcDNA3-Flag-VP16C-SXR (MG63/Flag-VP16C-SXR) or the empty vector Flag-tagged pcDNA3 (MG63/vector) were previously described (Ichikawa *et al.* 2006).

Western blot analysis

- Q3** 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 MgCl₂, 1 mM sodium orthovanadate, 10 µg/ml aprotinin, and 10 µg/ml leupeptin). Protein concentrations were analyzed using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Proteins were resolved by SDS-PAGE and electroblotted onto Immobilon-P Transfer Membrane (Millipore, Billerica, MA, USA). The antibody–antigen complexes were detected using the Western Blotting Chemiluminescence Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Antibodies used included anti-phospho PKA and anti-PKA (Cell Signaling Technology, Danvers, MA, USA).

Q4

Preparation of cRNA and oligonucleotide array hybridization

Total RNA was extracted from MG63/vector and MG63/Flag-VP16C-SXR cells treated with vehicle (0.1% ethanol) and MK-4 (10 µM) for 48 h. The methods for preparation of cRNA and subsequent steps leading to hybridization and scanning of the U133A GeneChip Arrays Station (Affymetrix, Santa Clara, CA, USA) were performed as described previously (Ichikawa *et al.* 2006). Data analysis was performed using Affymetrix Microarray Suite software. For comparing arrays, normalization was performed using data from all probe sets.

Quantitative reverse transcription PCR analysis

Osteoblastic cells were treated with RIF, MK-4, MK-7, vitamin K₁, GGO, FSK, 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 using the SuperScript II Reverse Transcriptase (Invitrogen) and oligo-dT₂₀ primer. For PCR amplification, the primer sequences were: human GDF15, 5'-GAAACGCTACGAGGACCTGCTA-3' and 5'-ACGAGGTCGGTGTTCGAATC-3'; human STC2, 5'-TGGGATTTGCATGACTTTTCTG-3' and 5'-GGCGTC-TTTGATGAATGACTTG-3'; human GGCX, 5'-CTTGA-GACCTTTGAGGCAGTT-3' and 5'-GGATTGACTCA-GGAGGATTAGAATG-3'; human CYP3A4, 5'-TTCAGCC-CATCTCCTTTTCATATTT-3' and 5'-CAGTTGGGTGTT-GAGGATGGA-3'; human α -catalytic subunit of PKA (PRKACA), 5'-CAACTTTCCGTTCTCTCGTCAA-3' and 5'-CGCCGGGCACGTACTC-3'; human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-GCCTGCCT-GACCAAATGC-3' and 5'-GTGGTTCGTTGAGGGCAATG-3'; mouse GDF15, 5'-AGACTGTGCAGGCAACTCTTGA-3' and 5'-ACACTCGCCCACGCACAT-3'; mouse STC2, 5'-CAACTCTTGTGAAATCCAGGGTTT-3' and 5'-CATC-GAATTTCCAGCGTTGT-3'; and mouse GAPDH, 5'-GCATGGCCTTCCGTGTTC-3' and 5'-TGTCATCAT

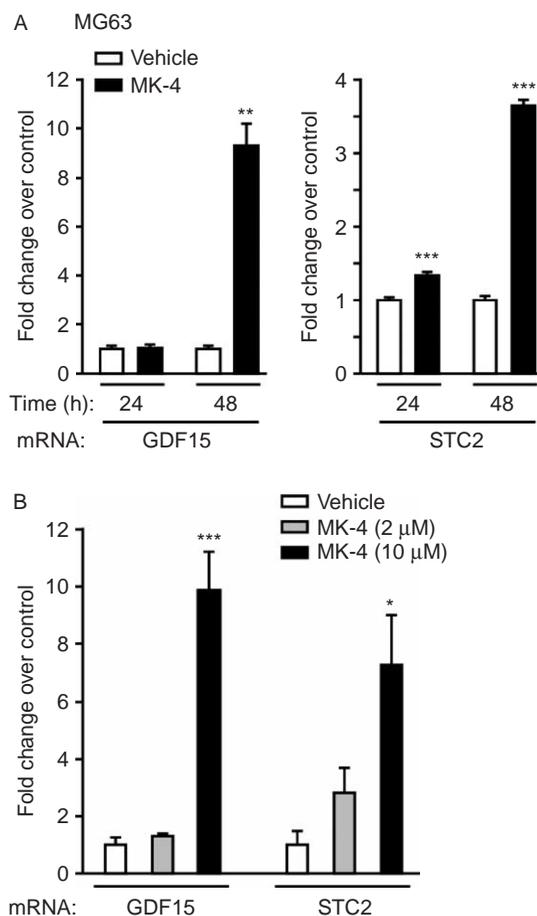


Figure 1 Up-regulation of growth differentiation factor 15 (GDF15) and stanniocalcin-2 (STC2) mRNA levels by a vitamin K₂ isoform menaquinone-4 (MK-4) in osteoblastic cells. Human osteoblastic MG63 cells were treated with MK-4 (10 µM) or vehicle for the indicated times (A), and mouse osteoblastic MC3T3-E1 cells were treated with MK-4 (2 or 10 µM) or vehicle for 48 h (B). mRNA levels for GDF15 and STC2 were determined by quantitative RT-PCR (qRT-PCR). Data are representative of experiments with similar results, each performed in triplicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ when compared with control cells treated with vehicle.

ACTTGGCAGGTTTCT-3'. mRNAs were quantified by real-time PCR using SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) and the ABI Prism 7000 system (Applied Biosystems) as previously described (Ichikawa *et al.* 2006).

RNA interference

Small interfering RNA (siRNA) duplexes to target human GGCX (D-009856-02) and PRKACA (D-004649-01) were purchased from Dharmacon Research Inc. (Lafayette, CO, USA). An siRNA specific to luciferase gene (Luciferase GL2 Duplex, Dharmacon) was used as a control. Cells were transfected with siRNA using GeneSilencer reagent (Genlantis, San Diego, CA, USA) for indicated times in the culture medium containing 10% dcc-FBS in the presence or absence of MK-4.

Statistical analysis

Differences between two groups were analyzed using two-sample, two-tailed Student's *t*-test. A *P* value < 0.05 was considered to be significant. All data are presented in the text and figures as the mean ± s.d.

Results

Identification of genes up-regulated by MK-4 in osteoblastic cells by microarray analysis

To identify up-regulated genes by MK-4 treatment in osteoblastic cells, we prepared biotin-labeled cRNA samples from MG63 cells expressing empty Flag-pcDNA3 (MG63/vector) or Flag-VP16C-SXR (MG63/Flag-VP16C-SXR) treated with vehicle (0.1% ethanol) or MK-4 (10 μM). The Affymetrix U133A GeneChip array represents more than 18 000 human transcripts from ~14 000 genes. Gene expression analysis for the MG63 samples was performed by hybridizing aliquots of cRNA (10 μg each) to the GeneChip arrays. Eighty-five transcripts were induced twofold or greater by MK-4 in MG63/vector cells, whereas 77 transcripts were induced in MG63/Flag-VP16C-SXR cells. In the present study, we focused on the SXR-independent gene expression in osteoblastic cells, by screening the common up-regulated genes (greater than or equal to twofold) in both vector and Flag-VP16C-SXR-transfected MG63 cells. In this population, we excluded genes up-regulated with the ratios of fold change in Flag-VP16C-SXR cells versus vector cells by ≥ 1.5-fold, as we considered that such genes were SXR dependent. Flag-

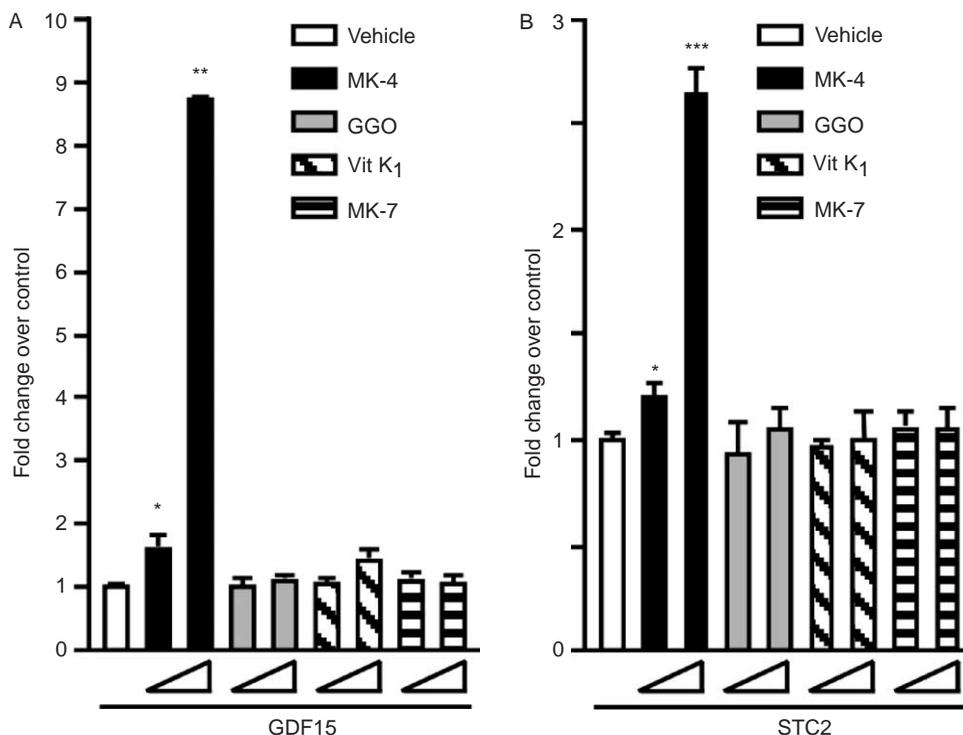


Figure 2 Specificity of MK-4 for induction of GDF15 and STC2 mRNA expression in osteoblastic cells. MG63 cells were treated with 5 or 10 μM of vitamin K₁, MK-4, MK-7, or geranylgeraniol (GGO) for 72 h. mRNA levels for GDF15 (A) and STC2 (B) were determined by qRT-PCR. Data are representative of experiments with similar results, each performed in triplicate. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 when compared with control cells treated with vehicle.

VP16C-SXR was constructed previously that could provide more intense SXR ligand-dependent signals when compared with the original SXR plasmid (Ichikawa *et al.* 2006). We used Flag-VP16C-SXR as it was useful to exclude SXR-dependent genes from the SXR-independent gene group. Through this procedure, 15 transcripts from 14 distinct genes were selected as MK-4 targets that were potentially independent of SXR pathways (Table 1). We focused on GDF15 and a secreted peptide hormone STC2 as putative bone-related genes for further experiments.

Induction of GDF15 and STC2 specifically by MK-4 in osteoblastic cells

We validated whether mRNA expression levels for these two genes could be modulated by MK-4 in parental MG63 cells using quantitative RT-PCR (qRT-PCR) analysis. In proliferating culture of MG63 cells, the significant induction of both GDF15 and STC2 was detected after 48-h treatment with MK-4 (Fig. 1A). GDF15 and STC2 were also induced in mouse osteoblastic MC3T3-E1 cells after 48-h treatment with MK-4 (Fig. 1B).

We next investigated whether GDF15 and STC2 were induced by other vitamin Ks or the MK-4 side chain structure GGO. Induction of GDF15 and STC2 mRNA by vitamin K₁, MK-7, or GGO was compared with that by MK-4. MK-4 of 5 and 10 μ M significantly up-regulated the MK-4 target genes, whereas the others had no effect (Fig. 2).

Q5

Up-regulation of MK-4 target genes in a GGCX and SXR-independent manner

Since MK-4 target genes were not induced by vitamin K₁ or MK-7, we next sought to verify that a GGCX-mediated pathway does not participate in the induction of these MK-4 target genes. We investigated the effects of a siRNA against GGCX on the ligand-dependent induction of gene expression. Ninety-six-hour treatment with the specific siRNA duplex against GGCX (70 nM), but not with a control siRNA directed against luciferase, reduced the GGCX mRNA level by more than 80% in MG63 cells (Fig. 3A). In that cell system, the GGCX siRNA had no effects in MK-4-activated mRNA expression for GDF15 and STC2 (Fig. 3B). We next examined whether the SXR pathway was involved in the regulation of GDF15 and STC2 mRNA expression using MG63/Flag-VP16C-SXR cells. In cells stably expressing SXR, mRNA encoding the SXR target gene *CYP3A4* was induced in 24 h by treatment with MK-4 or the SXR agonist RIF. In contrast, GDF15 and STC2 were up-regulated after 48-h treatment with MK-4 but not with RIF (Fig. 4A and B).

PKA is an activator for the induction of MK-4 target genes in osteoblastic cells

It has been shown that the modulation of transcriptional activities by PKA phosphorylation is one of the key events in osteoblasts, such as through parathyroid hormone or β 2-adrenergic receptor pathways (Selvamurugan *et al.* 2000, Elefteriou *et al.* 2005). There is a report that MK-4 might modulate gene expression and activate transcriptional factor activities in a PKA-dependent manner in a hepatocellular carcinoma cell line (Otsuka *et al.* 2004). Thus, we questioned whether PKA activity was responsible for the up-regulation of GDF15 and STC2 by MK-4 in osteoblastic cells. MK-4 (10 μ M) markedly induced phosphorylation of PKA in MG63 cells from 2 to 24 h after the stimulation (Fig. 5A). In experiments of PKA activation by FSK (10 nM), the mRNA expression of GDF15 and STC2 was significantly up-regulated after 48-h treatment (Fig. 5B).

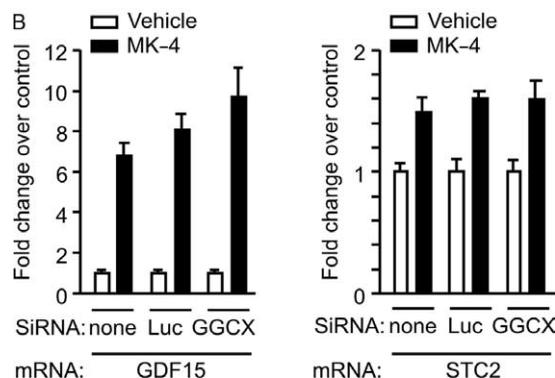
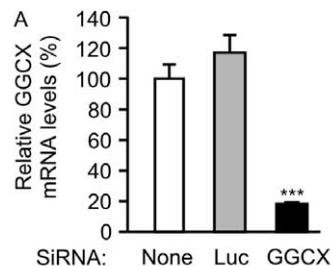


Figure 3 GDF15 and STC2 were up-regulated by a GGCX-independent pathway in osteoblastic cells. (A) MG63 cells were transfected with GGCX siRNA (70 nM) for 96 h, and mRNA expression of GGCX was determined by qRT-PCR. Data represent percentages of mRNA levels using the value with Luc siRNA treatment as 100%. (B) Effects of GGCX siRNA on MK-4-induced up-regulation of GDF15 and STC2. At 24 h after transfection of GGCX siRNA, MG63 cells were treated with MK-4 (10 μ M) for 72 h and mRNA expression was determined by qRT-PCR. Data represent fold changes in mRNA over vehicle treatment. *** $P < 0.001$ when compared with control cells with no siRNA treatment.

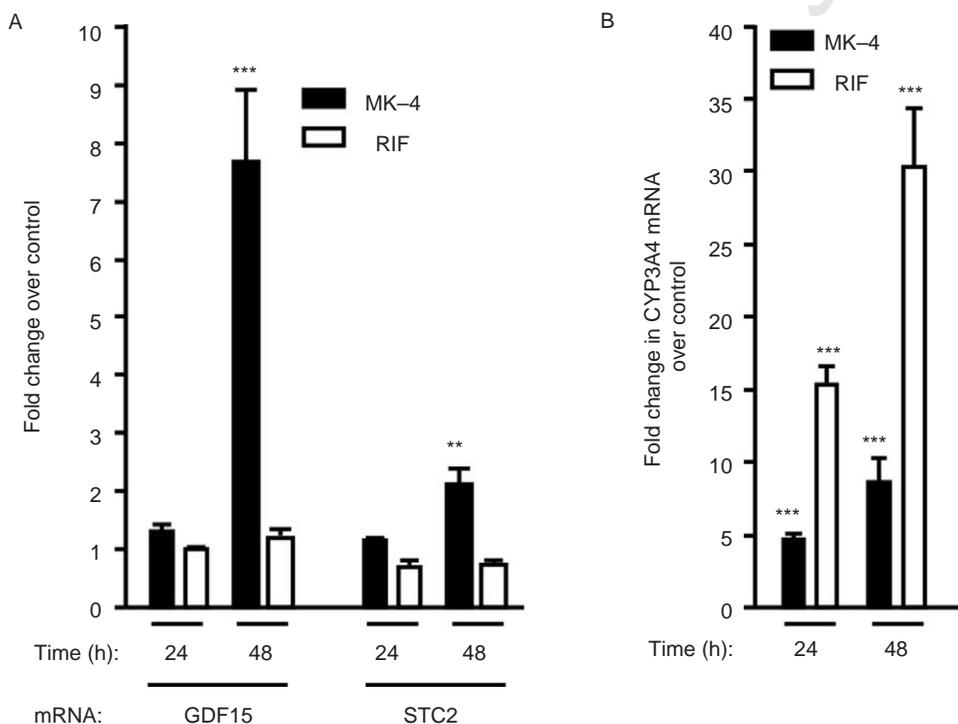


Figure 4 GDF15 and STC2 were up-regulated in an SXR-independent manner in osteoblastic cells. MG63/Flag-VP16C-SXR cells were treated with MK-4 (10 μ M), rifampicin (RIF, 10 μ M), or vehicle for 24 or 48 h, and mRNA expression was determined by qRT-PCR. (A) GDF15 and STC2 mRNA levels. (B) CYP3A4 mRNA levels. Data are representative of experiments with similar results, each performed in triplicate. ** $P < 0.01$, *** $P < 0.001$ when compared with control cells treated with vehicle.

Because it was likely that PKA activity was related to the transcriptional regulation of GDF15 and STC2, we further investigated whether the loss of function of PKA might affect the expression of GDF15 and STC2. Using a specific PKA inhibitor H89 (10 μ M) for 72 h, MK-4-dependent up-regulation of these two genes was reduced by ~30% (Fig. 6A). We also performed the knockdown study of PRKACA in MG63 cells using a specific siRNA duplex (Fig. 6B and C). In a condition of >60% reduction of PRKACA mRNA levels by siPKA Q6 compared with the control siRNA against luciferase, MK-4-dependent up-regulation of GDF15 and STC2 was reduced by 45 and 30% respectively.

Discussion

We previously reported that SXR mediated the transcriptional regulation of several osteoblastic marker genes by the vitamin K₂ congener MK-4 (Tabb *et al.* 2003). In the present study, we identified GDF15 and STC2 as novel vitamin K₂ target genes up-regulated only by MK-4 in an SXR-independent manner in osteoblastic cells. The expression of both *GDF15* and *STC2* genes was markedly induced by MK-4 after 48-h treatment,

whereas vitamin K₁, MK-7, GGO, and the SXR agonist RIF were not effective in inducing the expression of the genes. Furthermore, siRNA against GGCX did not affect the up-regulation of *GDF15* and *STC2* gene expression by MK-4. These results suggested that induction of GDF15 and STC2 was specific for MK-4 in osteoblastic cells, and that this induction was mediated by SXR- and GGCX-independent pathway(s).

GDF15 belongs to the superfamily of transforming growth factor- β , and it is generated as a 40 kDa propeptide from which the N-terminus is cleaved and a 30 kDa disulfide-linked dimeric protein is secreted as the active form (Bootcov *et al.* 1997). It was first named macrophage-inhibiting cytokine 1 (Bootcov *et al.* 1997) or placental bone morphogenetic protein (Hromas *et al.* 1997), and also later named like prostate-derived factor (Paralkar *et al.* 1998) or non-steroidal anti-inflammatory drug-activated gene (Baek *et al.* 2001). GDF15 is abundantly expressed in placenta and mildly expressed in liver and prostate at baseline, but many tissues show the dramatic induction of expression following various stimuli such as cytokine, growth factor, or hypoxia (Bootcov *et al.* 1997, Albertoni *et al.* 2002, Nazarova *et al.* 2004, Schlittenhardt *et al.* 2004). It has been shown that several kinases and transcriptional factors may contribute

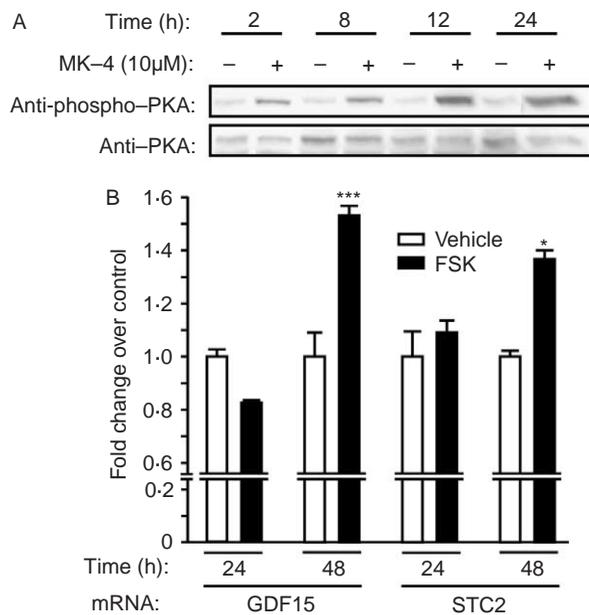


Figure 5 Phosphorylation of protein kinase A (PKA) by MK-4 and induction of GDF15 and STC2 mRNA by a PKA activator. (A) MG63 cells were treated with MK-4 (10 μM) or vehicle (MK-4) for the indicated times. Whole cell lysates were separated by SDS-PAGE, and phosphorylated PKA was detected by immunoblot analysis using anti-phospho-PKA antibody. The identical membrane was re-probed with whole PKA antibody (anti-PKA). (B) MG63 cells were treated with forskolin (FSK, 10 nM) or vehicle (0.1% DMSO) for the indicated times. mRNA levels for GDF15 and STC2 were determined by qRT-PCR. Data are representative of experiments with similar results, each performed in triplicate. * $P < 0.05$, *** $P < 0.001$ when compared with control cells treated with vehicle.

to the regulation of GDF15 expression, including p53 (Li *et al.* 2000, Tan *et al.* 2000), protein kinase C (Shim & Eling 2005), phosphatidylinositol 3-kinase/AKT (Yamaguchi *et al.* 2004), or EGR-1 (Baek *et al.* 2005). The transcriptional regulation by PKA has not been definitely shown previously, yet it is likely that PKA is involved in GDF15 regulation as the gene expression was induced by dibutyl cAMP in murine preadipocytes (Uldry *et al.* 2006). Although PKA sometimes phosphorylates p53, it is unlikely that p53 is responsible for the modulation of GDF15 expression in MG63 cells as the cell line has been shown to lack p53 (Masuda *et al.* 1987, Diller *et al.* 1990).

Regarding the physiological significance of GDF15 in bone-related tissues, the gene may play a role in the developmental stage as it has been shown to be expressed in the skin and in the cartilaginous tissue in the 18-dpc rat embryos (Paralkar *et al.* 1998). Similar to the function of bone morphogenic proteins, s.c. implantation of recombinant GDF15 protein ectopically induced the cartilage and immature endochondral bone formation (Paralkar *et al.* 1998). In prostate cancer, GDF15 expression was expressed only in osseous metastatic lesions, while it was reduced

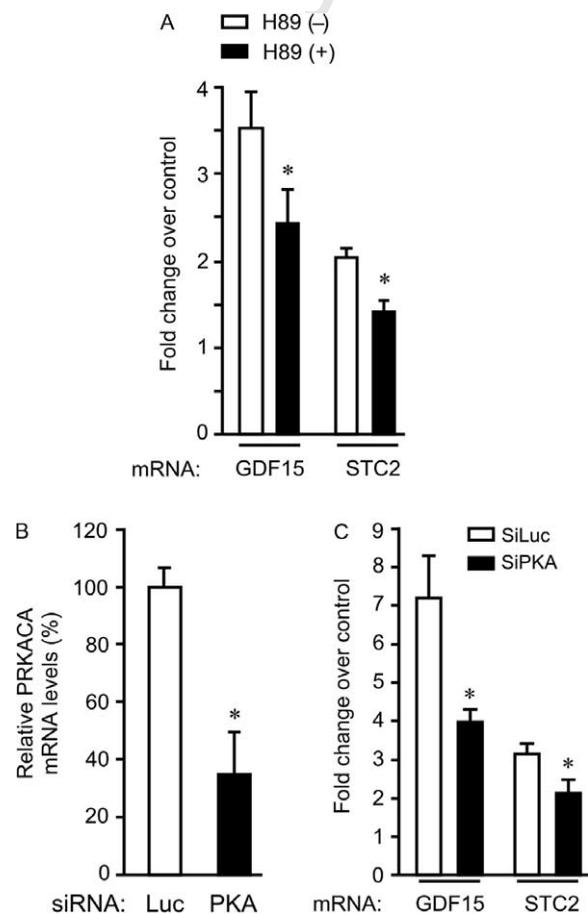


Figure 6 MK-4 induces GDF15 and STC2 in a PKA-dependent manner. (A) MG63 cells were treated with MK-4 (10 μM) or vehicle in the presence or absence of H89 (10 μM) for 72 h. mRNA levels for GDF15 and STC2 were determined by qRT-PCR. (B) MG63 cells were transfected with siRNAs (14 nM) that target either α-catalytic subunit of PKA, PRKACA (siPKA), or luciferase (siLuc) for 72 h. PRKACA mRNA expression was determined by qRT-PCR. (C) Effects of siPKA on MK-4-dependent up-regulation of GDF15 and STC2. Cells were transfected with siRNAs (14 nM) and incubated with MK-4 (10 μM) or vehicle for 72 h. Data are representative of experiments with similar results, each performed in triplicate. * $P < 0.05$ when compared with control cells treated with vehicle test.

or absent in primary tumor, suggesting that GDF15 expression might be linked to the osteoblastic phenomena associated with bone metastasis (Thomas *et al.* 2001). GDF15 also has modulating effects on cell adhesion or proliferation (Li *et al.* 2000, Tan *et al.* 2000, Nazarova *et al.* 2004). As MK-4 has been known as a modulator of cell proliferation in osteoblastic cells (Akedo *et al.* 1992) and hepatocellular carcinoma cells (Otsuka *et al.* 2004), GDF15 might be also involved in this growth-regulatory function of MK-4.

STCs represent a small family of secreted glycoprotein hormones, consisting of STC1 and STC2, which has been conserved from fish to mammals. It was

assumed that mammalian STC would mimic the function of fish STC-1 in mineral homeostasis (Olsen *et al.* 1996, Wagner *et al.* 1997, Madsen *et al.* 1998). STC1 is expressed in osteoblastic cells and could be induced during osteoblastic differentiation of rat fetal calvaria cells (Yoshiko *et al.* 1999, 2003). Overexpression and knockdown study of STC1 led to the acceleration and retardation of osteogenic development respectively (Yoshiko *et al.* 2003). STC1 is also expressed in chondrocytes and may also stimulate chondrocyte proliferation in cartilage matrix (Jiang *et al.* 2000, Filvaroff *et al.* 2002). In transgenic mice expressing human STC2, growth restriction and developmental retardation were observed in both prenatal and postnatal stages, and ossification was reduced in different endochondral skeletal elements (Gagliardi *et al.* 2005). Systemic overexpression of human STC1 in transgenic mice, however, also exhibited postnatal growth retardation (Varghese *et al.* 2002) and reduced ossification in cranial bones (Gagliardi *et al.* 2005), suggesting the discrepancy between animal models and culture cells. More specific studies targeting osteoblast and chondrocyte lineage will be required for better understanding of the STC physiology in bone development and formation, yet STC could be one of the key factors for the regulation of bone homeostasis. Interestingly, it has been shown that STC2 is closely related to steroid hormone physiology, as its expression is induced by estrogen (Charpentier *et al.* 2000, Bouras *et al.* 2002), or it has an inhibitory effect on FSH-induced progesterone production in rat ovary granulosa cells (Luo *et al.* 2005). It is likely that STC2 could play a paracrine role also in bone formation.

In this study, we investigated the effects of PKA on GDF15 and STC2 expression in osteoblastic cells and demonstrated that PKA was responsible, at least in part, for the MK-4-dependent up-regulation of these two genes. It has been reported that MK-4 activated transcriptional factors AP-2, USF, and CREB, and PKA activity might be stimulated by MK-4 in hepatocellular carcinoma cell line HepG2 (Otsuka *et al.* 2004). The activation of PKA by FSK in the present study mimicked the MK-4-induced up-regulation of GDF15 and STC2 in osteoblastic cells, although it is not clear whether MK-4 directly increases cyclic AMP production. It is also possible that PKA is phosphorylated by MK-4 through a cAMP-independent pathway, such as the one mediated by sphingosine (Ma *et al.* 2005). Future study will reveal the molecular details of MK-4-induced PKA activity in osteoblastic cells. It might be also interesting to investigate whether the MK-4-dependent PKA activation is important in other non-osteoblastic cells.

Here, we have shown that the PKA-dependent pathway is involved in the regulation of MK-4 target genes in osteoblastic cells in an SXR- and GGCX-independent manner. Our data suggest that GDF15 and STC2 are novel

MK-4 target genes up-regulated by the PKA-dependent pathway. Induction of GDF15 and STC2 at proliferation stage and post-confluent phase in osteoblastic cells might affect osteogenesis and chondrogenesis via autocrine or paracrine mechanisms.

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Disclosure

The authors have nothing to declare.

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