Farnesoid X Receptor Activation Prevents the Development of Vascular Calcification in ApoE\(^{-/-}\) Mice With Chronic Kidney Disease

Shinobu Miyazaki-Anzai, Moshe Levi, Adelheid Kratzer, Tabitha C. Ting, Linda B. Lewis, Makoto Miyazaki

Rationale: Vascular calcification is highly associated with cardiovascular morbidity and mortality, especially in patients with chronic kidney disease. The nuclear receptor farnesoid X receptor (FXR) has been implicated in the control of lipid, carbohydrate and bile acid metabolism in several cell types. Although recent studies have shown that FXR is also expressed in vascular smooth muscle cells, its physiological role in vasculature tissue remains obscure.

Objective: Here, we have examined the role of FXR in vascular calcification.

Methods and Results: The FXR gene, a bile acid nuclear receptor, was highly induced during osteogenic differentiation of bovine calcifying vascular cells (CVCs) and in the aorta of apolipoprotein (Apo)E\(^{-/-}\) mice with chronic kidney disease which are common tissue culture and mouse model, respectively, for aortic calcification. FXR activation by a synthetic FXR agonist, 6a-ethyl chenodeoxycholic acid (INT-747) inhibited phosphate induced-mineralization and triglyceride accumulation in CVCs. FXR dominant negative expression augmented mineralization of CVCs and blocked the anticalcific effect of INT-747 whereas VP16FXR that is a constitutively active form reduced mineralization of CVCs. INT-747 treatment also increased phosphorylated c-Jun N-terminal kinase (JNK). SP600125 (specific JNK inhibitor) significantly induced mineralization of CVCs and alkaline phosphatase expression, suggesting that the anticalcific effect of INT-747 is attributable to JNK activation. We also found that INT-747 ameliorates chronic kidney disease induced-vascular calcification in 5/6 nephrectomized ApoE\(^{-/-}\) mice without affecting the development of atherosclerosis.

Conclusions: These observations provide direct evidence that FXR is a key signaling component in regulation of vascular osteogenic differentiation and, thus representing a promising target for the treatment of vascular calcification. (Circ Res. 2010;106:1807-1817.)

Key Words: farnesoid X receptor ▪ vascular calcification ▪ chronic kidney disease

Vascular calcification is very common in subjects with chronic kidney disease (CKD) and is an independent predictor of cardiovascular mortality.\(^1-6\) Accumulation of calcium-phosphate complex in vascular wall decreases aortic elasticity and flexibility, which impairs cardiovascular hemodynamics, resulting in substantial morbidity and mortality. This process was considered to be passive. However, recent studies have shown that it is a highly orchestrated process that entrains a repertoire of transcription factors including msh homeo box (Msx)2,\(^7\) osterix,\(^6\) and runt-related transcription factor (Runx)2\(^8\) and involves the activation of an osteogenic program that recapitulates the molecular fingerprints seen in bone formation. Vascular calcified cells express many bone-related proteins, including alkaline phosphatase (ALP) and type I collagen (COL1A1).\(^5-7\) In addition, in vitro and in vivo models of vascular calcification have implicated a variety of factors in the pathogenesis of calcification, including osteoprotegrin, osteopontin, osteocalcin (OCL), matrix \(\gamma\)-carboxyglutamic acid protein (MGP), phosphate, inflammatory cytokines, lipids and reactive oxygen species.\(^6\) Despite these insights, it is still not fully known how vascular calcification is regulated and potential treatment modalities for this disease remain elusive.

In addition to their detergent effects on dietary lipids and fat-soluble vitamins absorption, bile acids exert several biological functions via a number of nuclear and plasma membrane receptors, including farnesoid X receptor (FXR), TGR5, vitamin D receptor, and pregnane X receptor.\(^8-11\) Thus, bile acids are signaling molecules governing not only bile acid synthesis, conjugation and transport, but also lipid, carbohydrate and energy metabolism.
FXR is a member of the nuclear receptor family of transcription factors activated by bile acids, the most potent endogenous ligand being chenodeoxycholic acid.\(^\text{12,13}\) FXR is highly expressed in tissues in which bile acids are present at high concentration, such as liver, kidney and intestine. Analysis of FXR function using genetically deficient mice and synthetic agonists has established the important role of this receptor in the control of bile acid, lipid and carbohydrate metabolism.\(^\text{14–16}\) Recent studies have also shown that functional FXR is expressed in vascular cells, including vascular smooth muscle cells and endothelial cells.\(^\text{17–19}\) FXR expression is also observed in the atherosclerotic lesions of human aorta.\(^\text{17}\) FXR directly and indirectly regulates the transcription of genes involved in lipoprotein metabolism such as SR-BI, apolipoprotein (Apo)AI, ApoCII, ApoCIII, phospholipid transfer protein, sterol regulatory element binding protein (SREBP)-1c, and very-low-density lipoprotein receptor.\(^\text{8,12,15,16}\) Studies using FXR\(^-/-\) mice have shown that FXR deficiency increases plasma triglycerides, cholesterol and high-density lipoprotein cholesterol, whereas administration of FXR agonists reduces plasma triglycerides.\(^\text{20–22}\) However, the role of FXR in modulation of atherosclerosis is controversial.\(^\text{24–28}\) In addition the role of FXR in vascular calcification development has not yet been studied.

In the present study, we have found that FXR is highly upregulated during osteogenic differentiation in bovine calcifying vascular cells (CVCs) and in the aorta of ApoE\(^-/-\) mice with CKD. In addition, FXR activation with the specific agonist 6\(^\alpha\)-ethyl chenodeoxycholic acid (INT-747) blocks mineralization, lipid accumulation and osteogenic differentiation in CVCs. We also have examined the effects of INT-747 administration to apolipoprotein E-deficient (ApoE\(^-/-\)) mice with CKD induced by 5/6 nephrectomy. INT-747 treatment ameliorates CKD-induced vascular calcification in ApoE\(^-/-\) mice without affecting the development of atherosclerosis. These observations suggest that FXR activation may represent a promising therapeutic agent for intervention in CKD-induced vascular calcification.

### Methods

An expanded Methods section is available in the Online Data Supplement at [http://circres.ahajournals.org](http://circres.ahajournals.org).

Both 5/6 nephrectomized and sham-operated ApoE\(^-/-\) mice were purchased from Jackson Laboratory. At 8 weeks of age, animals were fed a Western diet with or without INT-747 for 12 weeks. CVCs were cultured in DMEM containing 15% FBS with 2.0 mmol/L phosphate.

### Results

**FXR Is Highly Induced in the In Vitro and In Vivo Models of Vascular Calcification**

Members of the nuclear hormone receptor superfamily are transcription factors that regulate diverse pathways of metabolism. In addition to acting as molecular sensors of lipid and carbohydrate homeostasis, several members of the nuclear receptor family (eg, erosoxime proliferator-activated receptor, retinoid X receptors, and liver X receptors [LXRs]) also exert beneficial pleiotropic effects to ameliorate atherosclerosis and its complications. However, the role of nuclear receptors in the development of vascular calcification is still unclear. To examine which nuclear receptors may be involved in vascular calcification and to identify nuclear receptor genes that were altered during osteogenic differentiation of vascular cells, we used a quantitative RT-PCR array to analyze all nuclear receptor genes with exception of EAR2, whose cDNA sequence is currently not available. To induce calcification, bovine vascular cells at 90% to 95% confluence were cultured with 2 mmol/L phosphate (high phosphate) for 14 days. High phosphate treatment promoted mineralization (Figure 1A and 1C), consistent with previous reports. Interestingly, calcified nodules accumulate neutral lipids stained by Oil Red O (Figure 1B). Thin layer chromatography and quantitative analysis showed that triglycerides (Figure 1D and 1E) and total cholesterol (Figure 1F) levels were increased by 7.2-fold and 1.3-fold, respectively, in CVCs treated with 2 mmol/L phosphate. RNA from these cells was subjected to quantitative RT-PCR analysis. We identified 15 nuclear receptor genes with distinct levels of expression, 7 of which were markedly induced during osteogenic calcification of CVCs (Table). The gene encoding mineralocorticoid receptor (MR) ranked the highest in this analysis, followed by FXR, HNF4\(\alpha\), COUP-TFI, RXR\(\alpha\), VDR, and RAR\(\gamma\) gene. In this study, we focused on determining the role of FXR in the pathogenesis of vascular calcification because MR has already been reported to promote vascular calcification.\(^\text{29}\) FXR mRNA and protein levels were time-dependently induced during vascular osteogenic differentiation and increased by 14.5-fold in calcified cells at day 21 of culture compared to noncalcified cells (Figure 2A and 2A, inset). As expected, osteogenic markers such as ALP (Figure 2B), MGP (Figure...
2C), and OCL (data not shown) mRNAs were also induced during osteogenic differentiation.

We then examined aortic FXR expression in mouse models of vascular calcification. Consistent with previous reports, 5/6 nephrectomized ApoE−/− mice fed a Western diet for 24 weeks exhibited a significant acceleration of atherosclerosis and vascular calcification compared to ApoE−/− mice with normal 2 kidneys. Atherosclerotic plaque size was increased by 84% in 5/6 nephrectomized ApoE−/− (Online Figure I, B) fed control and Western diet, respectively. Aortic calcium content was increased by 2.7-fold (Online Figure I, C) compared to ApoE−/− control fed Western diet. Real-time PCR analysis showed that 5/6 nephrectomy induced aortic FXR expression in ApoE−/− mice by 5.8-fold under control diet and 3.2-fold higher on Western diet (Figure 2D). mRNA expression of the osteogenic markers ALP and OCL was significantly higher in 5/6-nephrectomized ApoE−/− mice fed with either Western or control diet (Figure 2E and 2F).

**Figure 1.** The calcified nodules in CVCs accumulate not only calcium but also neutral lipids. At 90% to 95% confluence, CVCs were treated with osteogenic medium containing 2.0 mmol/L phosphate. After 14 days in culture, cells were stained with alizarin red (A) and oil red O (B), which identify calcium mineral and neutral lipid as red, respectively. Arrows indicate calcified nodules. C, Ca content. D, Thin layer chromatographic analysis. E, Triglycerides. F, Cholesterol levels. **P<0.01 and ***P<0.001 vs undifferentiated CVC (day 0, 1 mM Pi).

### Table. Induced Nuclear Receptor Genes During Osteogenic Differentiation of CVC

<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>Gene</th>
<th>Abbreviation</th>
<th>Fold Change</th>
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</thead>
<tbody>
<tr>
<td>1. NR3C2</td>
<td>Mineralocorticoid receptor</td>
<td>MR</td>
<td>88.1</td>
</tr>
<tr>
<td>2. NR1H4</td>
<td>Farnesoid X receptor</td>
<td>FXR</td>
<td>11.3</td>
</tr>
<tr>
<td>3. NR2A</td>
<td>Hepatocyte nuclear factor 4α</td>
<td>HNF4α</td>
<td>2.8</td>
</tr>
<tr>
<td>4. NR2A1</td>
<td>Chicken ovalbumin upstream promoter TF1</td>
<td>COUP-TF1</td>
<td>2.2</td>
</tr>
<tr>
<td>5. NR2B1</td>
<td>Retinoid X receptor α</td>
<td>RXRα</td>
<td>2.2</td>
</tr>
<tr>
<td>6. NR1I1</td>
<td>Vitamin D receptor</td>
<td>VDR</td>
<td>2.1</td>
</tr>
<tr>
<td>7. NR1B3</td>
<td>Retinoic acid receptor γ</td>
<td>RARγ</td>
<td>2.0</td>
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Bovine calcifying vascular cells were cultured for 14 days after confluence. The gene expression of nuclear receptor was determined by a customized PCR array. Data are presented as mRNA expressions relative to day 0 control. The average Ct at day 14: MR, 29.32; FXR, 19.55; HNF4α, 27.24; COUP-TF1, 17.61; RXRα, 19.32; VDR, 18.95; RARγ, 19.34. TF indicates transcription factor.

**FXR Activation Blocks Phosphate-Induced Mineralization and Triglyceride Accumulation in Bovine Vascular Cells**

Because FXR mRNA was highly induced in both in vivo and in vitro vascular calcification models, we examined the effect of FXR activation in vascular calcification. CVCs were
treated with an FXR agonist INT-747 to test whether FXR activation influences phosphate induced-mineralization. Alizarin red staining revealed that the treatment with INT-747 dose-dependently reduced phosphate induced-mineralization of CVCs (Figure 3A). Calcium content of CVCs was increased by 18.2-fold in response to 2 mmol/L phosphate compared to 1 mmol/L phosphate. Consistent with Alizarin red staining, INT-747 treatment dose-dependently decreased calcium content in CVCs under both high and normal phosphate conditions. A concentration of 3 μmol/L INT-747 reduced calcium content by 79% in normal phosphate and 83% in high phosphate conditions (Figure 3B). INT-747 treatment also reduced triglyceride content but not cholesterol content. At the 3 μmol/L concentration, triglyceride level was reduced by 38% and 64% in the presence of 1 mmol/L and 2 mmol/L phosphate, respectively (Figure 3C). To determine whether the anticalcific effect of INT-747 is via FXR activation, we overexpressed FXR dominant negative (DN)31 and wild-type FXR in CVCs using Lenti-X lentiviral expression system. Figure 3D and 3E shows that FXR DN expression significantly increased calcium content in CVCs but also blocked the anti-calcific effect of INT-747. Wild-type FXR overexpression did not affect mineralization of CVCs and the anticalcific effect of INT-747 (Figure 3E and 3G). Because wild-type FXR overexpression was not effective to inhibit mineralization of CVCs, we treated CVCs with adenovirus expressing VP16FXR that is a constitutively active form. The overexpression of VP16FXR was able to reduce mineralization of CVCs by 54% (Figure 3F and 3G) consistent with FXR activation by INT-747. We then analyzed genes encoding osteogenic markers such as ALP, COL1A1, and MGP to test whether INT-747 affects osteogenic differentiation. As shown in Online Figure II, the gene expression of ALP, COL1A1 and MGP were reduced in CVCs treated with 3 μmol/L INT-747 in the presence of high phosphate for 14 days, suggesting that FXR activation inhibits osteogenic differentiation. In addition, INT-747 decreased mRNA levels of Msx2 and osterix but not Runx2, which are 3 major transcription factors involved in osteogenic differentiation (Online Figure II). Consistent with reduced triglyceride content (Figure 3B), INT-747 treatment decreased mRNA abundance of transcription factors and enzymes involved in lipogenesis including SREBP-1, SREBP-2, fatty acid synthase, and acetyl-CoA carboxylase (ACC)1. As expected, long-term treatment (14 days) with 3 μmol/L INT-747 significantly induced FXR target genes, small heterodimer partner (SHP) and angiotensin type II receptor (AT2R)18,32 (Online Figure II). In addition, short-term treatment (24 hours) with INT-747 dose-dependently increased SHP mRNA level (Online Figure III). FXRDN also completely inhibited the induction of SHP and the reduction of ALP, COL1A1, MGP, Msx2 and osterix by INT-747 treatment (Online Figure IV). Consistent with FXR activation by INT-747, VP16FXR expression significantly reduced Msx-2, osterix, ALP and COL1A1 mRNA levels (Online Figure V).

The Anticalcific Effect of INT-747 is Attributable to Activation of c-Jun N-Terminal Kinase

To understand the mechanisms by which FXR activation blocks mineralization of CVCs, we examined whether FXR activation influences a cell signaling involved in vascular calcification. Previous studies have shown that mitogen-activated protein kinase (MAPK) and AKT path-
ways are involved in osteogenic differentiation and mineralization of vascular cells.\textsuperscript{11,13} INT-747 treatment significantly increased phosphorylated c-Jun N-terminal kinase (p-JNK) level by 3.1-fold. Interestingly, total JNK protein was significantly lowered in INT-747–treated CVCs; p-extracellular signal-regulated kinase (ERK), total ERK, p-p38 MAPK, p-Akt, and GAPDH were not different (data not shown).

**Figure 3.** FXR agonist, 6a-ethyl chenodeoxycholic acid (INT-747) reduced mineralization in CVCs under normal and high phosphate conditions. A, Alizarin staining: CVCs were treated with different concentration of INT-747 for 14 days in the presence of high (2.0 mmol/L) phosphate and stained with alizarin red. B, Calcium content. C, Triglyceride content was analyzed by enzymatic analysis. ##P<0.01 vs 1 mM Pi–vehicle; *P<0.05 and **P<0.01 vs 2 mM Pi–vehicle. D, Western blot analysis of FXR DN and wild-type (wt) FXR. FXR DN and wild-type FXR proteins were detected using anti-FLAG antibody. E, FXR DN but not wild-type FXR expression augments mineralization of CVCs and inhibits the anticalcific effect of INT-747. CVCs were pretreated overnight with lentivirus expressing Mock (empty), FXR DN, and wild-type FXR at 10 multiplicities of infection and incubated with INT-747 (3 µmol/L) for 14 days. **P<0.01 vs mock–vehicle. F and G, Western blot analysis of wild-type FXR and VP16FXR (F) and Ca content in VP16FXR-treated CVCs (G). Wild-type FXR and VP16FXR protein were detected using anti-Flag and anti-VP16 antibody, respectively. CVCs were pretreated overnight with adenoviruses expressing Mock (empty), wild-type FXR, and VP16FXR at 40 multiplicities of infection and incubated for 14 days in the presence of high phosphate. **P<0.01 vs mock.

Msx2 and Osterix Augments Mineralization and Osteogenic Differentiation of CVCs

To determine whether Msx2 and osterix mediate phosphate-induced calcification, Msx2 and osterix were overexpressed using adenoviral expression system. CVCs were pretreated adenovirus expressing Msx2 and osterix at 40 multiplicities of infection for overnight and then cultured for 7 days in the presence of high phosphate. Msx2 and osterix overexpression (Online Figure VI) increased calcium levels in CVCs by 9.3-fold and 3.7-fold, respectively (Online Figure VI, B and C). In addition, Msx2 and osterix overexpression induced ALP expression by 10.7-fold and 5.7-fold, respectively and COL1A1 by 8.0-fold and 7.4-fold, respectively (Online Figure VI, D).
FXR Activation Prevents the Development of Vascular Calcification in ApoE−/− With Chronic Kidney Disease

To examine whether FXR activation protects against in vivo vascular calcification, we treated 5/6 nephrectomized ApoE−/− mice with INT-747 at either 10 or 20 mg/kg for 12 weeks. There were no significant differences in body weight and food intake among the experimental groups. Blood cholesterol, triglyceride and phosphorus levels were significantly higher in ApoE−/− mice compared to C57BL/6 control mice. Blood urea nitrogen and serum phosphorus levels were higher in ApoE−/− mice with 5/6 nephrectomy than 2 kidney control, confirming that ApoE−/− mice developed CKD induced by 5/6 nephrectomy. Blood urea nitrogen and phosphorus levels were unaffected by INT-747 treatment. Twelve week treatment with INT-747 at either dose reduced both serum and hepatic triglycerides in ApoE−/− mice with CKD. Hepatic cholesterol was reduced in the liver of ApoE−/− mice with CKD treated with INT-747 at the higher 20 mg/kg concentration. Serum cholesterol, serum blood urea nitrogen, serum phosphorus, serum calcium, serum glucose and aspartate aminotransferase levels were unaffected by INT-747 treatment (Online Table I).

Atherosclerotic lesions in male ApoE−/− mice were quantified by en face analysis of aortas after 12 weeks on a Western diet in the presence or absence of the FXR agonist. Quantification of Sudan IV-stained en face preparations of aortas showed
that atherosclerosis developed in ApoE<sup>−/−</sup> mice with 2 kidneys and was accelerated by 5/6 nephrectomy. No atherosclerotic plaque formation was observed in C57BL/6 mice. Unexpectedly, however, INT-747 treatment at both 10 and 20 mg/kg doses had no effect on atherosclerotic plaque formation in ApoE<sup>−/−</sup> mice with CKD (Figure 5A). In contrast, 5/6 nephrectomized ApoE<sup>−/−</sup> mice receiving the LXR agonist GW3965 at 5 mg/kg as a positive control exhibited a statistically significant 84% reduction in average lesion area (S.M., M.L., and M.M., unpublished data, 2010). To examine the effectiveness of INT-747 in influencing aortic calcification, the calcium content in the whole aorta was analyzed. Consistent with Online Figure I, Figure 5B shows that aortic calcium content was significantly increased in ApoE<sup>−/−</sup> mice and was further increased by CKD. The groups receiving both 10 and 20 mg/kg INT-747 showed a statistically significant 61% and 66% reduction in aortic calcium content compared to untreated ApoE<sup>−/−</sup> mice with CKD. To further demonstrate the anti–vascular calcific effect, aortic sinus lesions from ApoE<sup>−/−</sup> mice with CKD were analyzed with Von Kossa staining. Quantification of calcified lesion revealed that a significant 81% reduction in calcified lesion area in mice treated with 10 mg/kg INT-747 compared with untreated controls (Figure 5C and 5D). No significant difference was observed in atherosclerotic lesion between vehicle and 10 mg/kg INT-747 groups, consistent with en face analysis (1 704 582 ± 61 545 versus 1 733 145 ± 53 813 µm<sup>2</sup>).

Because vascular calcification is known to be associated with increased expression of genes involved in osteogenesis, we examined whether INT-747 affects aortic osteogenic gene expression in ApoE<sup>−/−</sup> mice with CKD. Consistent with previous reports, CKD induced osteogenic marker genes such as ALP, osteopontin, OCL, MGP and genes encoding osteogenic transcription factors including Msx2 and osterix. Treatment with INT-747 reduced aortic mRNA levels of osteogenic markers (ALP, OPN, OCL and MGP) and osteogenic transcription factors (Msx2 and osterix). Aortic mRNA
level of bone morphogenetic protein 2 was unchanged by INT-747 treatment (data not shown). The FXR target gene SHP was induced by INT-747 treatment, whereas SREBP-1c and its target genes including fatty acid synthase and ACC1 were reduced in ApoE<sup>-/-</sup> mice treated with INT-747 (Figure 6).

**Discussion**

Members of the nuclear hormone receptor superfamily are transcription factors that regulate diverse pathways of metabolism. In addition to acting as molecular sensors of lipid, carbohydrate and mineral homeostasis, several members of the nuclear receptor family (eg, peroxisome proliferator-activated receptors and LXR<sub>α</sub>) also exert beneficial pleiotropic effects to ameliorate atherosclerosis and its complications. In this study, we examined which nuclear receptor(s) is involved in the development of vascular calcification, a major complication in subjects with CKD and a strong predictor of cardiovascular mortality. We first analyzed gene expression of 47 nuclear receptors in CVCs, an in vitro model of vascular calcification. We found that 8 genes encoding nuclear receptors, including MR and FXR, were dramatically induced during osteogenic differentiation of CVCs (Table I). Because it has been already reported that activation of MR with

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**Figure 6.** INT-747 reduced aortic gene expression of osteogenic transcription factors, osteogenic markers, and lipogenic enzyme genes in ApoE<sup>-/-</sup> mice with CKD. Male mice were fed a Western diet for 12 weeks. A, Osteogenic transcription factors: Msx2, Runx2, and osterix. B, Osteogenic markers: ALP, OPN, OCL, and MGP. C, FXR and its target SHP. D, Lipogenic genes: SREBP-1c, fatty acid synthase (FAS), and ACC1. *P<0.05 and **P<0.01 vs 2KD ApoE<sup>-/-</sup>/vehicle; #P<0.05 and ##P<0.01 vs 5/6NX ApoE<sup>-/-</sup>/vehicle.
aldosterone promotes vascular osteogenic differentiation and mineralization of CVCs, we focused our studies to determine the role of FXR activation in the development of vascular calcification. This study demonstrates that a synthetic FXR agonist, INT-747 inhibits mineralization and osteogenic differentiation of cultured vascular cells. The anticalcific effect of INT-747 was blocked in CVCs expressing FXR DN. Consistent with FXR activation by INT-747 treatment, VP16FXR expression also inhibits mineralization of CVCs. In addition, FXR activation increased phospho-JNK in CVCs. SP600125 (JNK-specific inhibitor) significantly increased phospho induced-mineralization of CVCs. These results suggest that the anticalcific effect of INT-747 is attributable to JNK activation. We also found that FXR activation prevents the development of CKD induced vascular calcification in ApoE−/− mice without affecting the development of accelerated atherosclerosis. These observations suggest that FXR mediates inhibitory signaling in vascular calcification, similar to be known inhibitory signaling factors such as osteocalcin, osteoprotegrin and osteopontin that are also increased in the osteogenic process. 

Our observations from in vivo and in vitro experiments suggest that direct effects of FXR agonist on vascular smooth muscle cells in the artery wall are responsible for the anti-vascular calcific effects. Consistent with this hypothesis, we found that reduction of mineralization by INT-747 correlates with the reduction of genes involved in osteogenic differentiation. INT-747 also reduces crucial transcription factors involved in osteogenic differentiation such as Mxs2 and osterix in the both CVCs and aorta of ApoE−/− mice. These results suggest that these transcription factors are targets of FXR and that the reduction of these osteogenic transcription factors may contribute to the anticalcific effects of INT-747. Consistent with the reduction of mineralization and osteogenic transcription factor expression, other osteogenic markers including ALP, OCL, MGP, and COL1A1 were reduced in both aorta of CKD-ApoE−/− mice and CVCs treated with INT-747. In addition, SP600125 treatment induced Mxs2 and osterix expression, suggesting that JNK activation mediates the reduction of Mxs2 and osterix expression by INT-747. Taken together, these results suggest that FXR is a key regulator in regulation of osteoblastic differentiation of vascular cells.

Inflammatory cytokines and oxidative stress induced by macrophages are also known to contribute to the development of vascular calcification and atherosclerosis. ApoE−/− mice with CKD show increased macrophage infiltration (CD68 and F4/80) and inflammatory maker gene (TNFα and IL-1β) expression compared to normal ApoE−/− mice (Online Figure VII). However, INT-747 treatment did not affect macrophage infiltration or inflammatory cytokine expression induced by CKD. In addition to the evidence showing absence of expression of FXR in macrophages, these results support our hypothesis that INT-747 directly inhibits osteogenic differentiation in vascular smooth muscle cells. We cannot completely exclude the possibility that FXR activation in tissues other than vascular smooth muscle cells (ie, liver, small intestine, and kidney) contributes to the anticalcific effect of INT-747. In fact, we found that INT-747 treatment reduced hepatic neutral lipids (triglyceride and cholesterol) and altered hepatic (SHP, CYP7Al, and SREBP-1c) gene and intestinal gene (FGF-15) expression, consistent with previous reports. The reduction in vascular calcification is also accompanied by a reduction in circulating triglycerides but not cholesterol. Because circulating triglyceride level is a risk factor for vascular diseases, the reduction of circulating triglycerides may be involved in the reduction of vascular calcification. Studies with tissue-specific FXR knock out and transgenic mice would be of considerable interest to determine the contribution of systemic effects of alterations in serum lipid levels versus the direct effect of FXR in the vasculature.

This is the first report showing that calcified vascular cells accumulate not only minerals but also neutral lipids (Figure 1). Quantitative lipid analysis showed that the accumulated neutral lipid was triglyceride, which was reduced by INT-747 treatment. The data suggests that the accumulation of lipids contributes to mineralization and osteogenic differentiation of CVCs. Furthermore, in both CVCs and aorta of ApoE−/− mice with CKD, INT-747 treatment increased expression of a major FXR target gene, SHP, which is required for the antilipogenic and the triglyceride-lowering effect of FXR agonists in the liver by interacting with LXR. We also found that INT-747 reduced SREBP-1 gene and its target lipogenic genes including fatty acid synthase and ACC in CVCs and also in aorta of ApoE−/− mice consistent with reduced triglyceride level. A recent study reported that LXR activation by synthetic LXR agonists accelerates protein kinase A induced-mineralization in mouse vascular smooth muscle cells. Consistent with this report, we also found that LXR activation increased SREBP-1c expression and triglyceride level in CVCs, thereby accelerating phosphate-induced mineralization of CVCs (unpublished data, S.M.-A. and M.M.). The inhibition of LXR-SREBP1c pathway; thus, antilipogenic program by FXR agonist may also be playing an important role in the anticalcific effect. Additional studies to ascertain the involvement of SHP and SREBP-1 in the anticalcific effect of FXR agonist and the development of vascular calcification are currently underway.

The influence of FXR on the development of atherosclerosis has been controversial. In male ApoE−/− mice, FXR deficiency increased atherosclerotic lesions in mice fed a Western diet containing 1.25% cholesterol. In contrast to males, female FXR−/−; ApoE−/− double mutant mice fed a high fat cholesterol-free diet showed a significant reduction of atherosclerotic lesion formation. In LDLR−/− mice, loss of FXR reduced atherosclerotic lesion size in male but not in female mice. In addition, 2 recent publications showed that FXR activation by synthetic FXR agonists including INT-747 prevents atherosclerotic lesion formation in LDLR−/− and ApoE−/− mice. Males were used in this study because males are more sensitive to CKD-induced vascular diseases than females and the antiatherogenic effect of INT-747 was previously studied in males. In the present study, INT-747 treatment did not prevent CKD-induced atherosclerosis in ApoE−/− mice. The differential response of INT-747 to atheroscle-
rosis between this study and the study by Mencarelli et al is probably attributable to the differences in kidney function, although the administration method (oral gavage versus dietary administration) is also different. A recent study has shown that FXR activation improves diabetic nephropathy. These data suggest that the normal kidney function may be required for the antiatherosclerotic effect of FXR agonists and that FXR activation is not effective in preventing CKD-induced atherosclerosis.

In summary, FXR expression is increased during osteogenic differentiation of vascular cells which likely plays an inhibitory role in vascular calcification. Indeed, we have provided evidence that FXR activation inhibits aortic calcification in ApoE−/− with CKD without affecting the development of atherosclerosis. FXR activation also directly inhibits mineralization of vascular cells. Clinical studies have demonstrated that more than half of patients with chronic kidney disease die of cardiovascular diseases, including advanced calcific arterial disease. However, no pharmacological therapies have yet been shown to prevent disease progression. Therefore, the present data will be valuable in directing future efforts in the development of FXR agonists to treat CKD induced-vascular calcification.

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Disclosures

None.

References

Vascular calcification is the leading cause of death in patients with chronic kidney disease (CKD).

- The bile acid nuclear receptor farnesoid X receptor (FXR) controls bile acid, lipid, and glucose metabolism and is expressed in vascular cells including vascular smooth muscle cells and endothelial cells.
- FXR activation by synthetic agonists protects mice from a variety of metabolic diseases such as liver steatosis, insulin resistance, hyperlipidemia, cholestasis, diabetic nephropathy, and atherosclerosis; however, its role in vascular calcification is unknown.

What New Information Does This Article Contribute?

- Vascular FXR is highly induced during vascular calcification.
- Pharmacological activation of FXR reduces mineralization of vascular cells as well as vascular calcification in hyperlipidemic mice with CKD.
- Activation of FXR reduces osteogenic differentiation of vascular cells by reducing expression of osteogenic transcription factors and activating c-Jun N-terminal kinase (JNK).

Although more than half of the death in patients with CKD is attributable to cardiovascular diseases, particularly vascular calcification, the efficient treatment and the etiology for this disease is unknown. FXR plays a central role in regulation of bile acid, cholesterol, and glucose metabolism but its specific role in vascular diseases in CKD has not been studied. In this study, we have identified that FXR is highly induced in vascular calcification of both cell culture and animal models. In addition, FXR activation by genetic and pharmacological modification blocks vascular calcification in vitro and in vivo. We also found that JNK activation and reduced osteogenic transcription factors such as Msx2 and osterix contribute to the anticalcific effect of the FXR activation. This is the first study to demonstrate that FXR plays an important role in a feedback loop of vascular calcification. Furthermore, FXR activation might be a promising tool for prevention and treatment for vascular calcification in CKD.
Farnesoid X Receptor Activation Prevents the Development of Vascular Calcification in ApoE−/− Mice With Chronic Kidney Disease
Shinobu Miyazaki-Anzai, Moshe Levi, Adelheid Kratzer, Tabitha C. Ting, Linda B. Lewis and Makoto Miyazaki

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Supplemental Material

Miyazaki-Anzai et al.

FXR activation prevents the development of vascular calcification in ApoE⁻/⁻ mice with chronic kidney disease

1. Extended Materials and Methods

Animal Studies: 5/6 nephrectomized and sham-operated ApoE⁻/⁻ mice were purchased from Jackson Laboratory (Bar Harbor, ME). Details of 5/6 nephrectomy are described in the Supplemental Methods. At 8 weeks of age, animals were fed either a high-fat/high-cholesterol diet (Western diet) (Harlan Teklad, 000750, containing 21% fat, 0.15% cholesterol [w/w]) with or without INT-747 for 12 weeks. For Figure 2 and S1, animals were fed western diet for 24 weeks. Where indicated, diets were supplemented with INT-747 [EC₅₀ 85 nM on FXR], at a level sufficient to provide 10 or 20 milligrams per kilogram body weight (mpk) dose on consumption of a 3-g diet by a 25-g mouse per day.¹² Animal experiments were approved by the Institutional Animal Care and Research Advisory Committee of the University of Colorado at Denver.

Cell culture studies: Bovine calcifying vascular cells (CVC) were cultured in DMEM containing 15% FBS with either 1.2 mM phosphate (normal-phosphate concentration) or 2.0 mM phosphate (high-phosphate concentration). CVC were treated with several concentrations of 6α-ethyl-chenodeoxycholic acid (INT-747, Intercept Pharmaceuticals Inc., New York) and the medium were changed every 2-3 days.¹ At 14 days of the confluence, cells were stained with either Alizarin red which identifies calcium deposit or Oil red O which identifies neutral lipid deposit.

Calcium content in cultured cells and aorta: Calcium deposition in the plates was quantified as previously described.³ Cells were decalcified using 0.6M HCl solution. After collecting the supernatant, the cells were washed with PBS and solubilized with 0.1N NaOH/0.1% SDS solution for protein quantification. The aortas were removed and frozen at -20°C until analysis. The dried aorta was defatted with chloroform and methanol (2:1) for 48 hours and dehydrated by acetone for three hours. The dried samples were incinerated to ashes at
600°C for 24 hours using an electric muffle furnace (Thermo Scientific), then extracted with HCl and diluted with distilled water. The levels of calcium in the aorta were determined and represented as the weight of calcium per dry weight of aorta. The calcium content was quantified calorimetrically using the o-cresolphthalein method. The protein content was measured using a BCA protein assay kit.

**Histology and lesion analysis:** For *en face* analysis, mice were euthanized and the aorta dissected out, opened longitudinally from heart to the iliac arteries, and stained with Sudan IV to determine lesion area. Images were captured by use of a Zeiss Axiocam-CCD video camera and analyzed by a single technician who was blinded to the study protocol and used AxioVision image analysis software. The extent of lesion formation is expressed as the percentage of the total aortic surface area covered by lesions. Atherosclerotic lesions at the aortic valve were analyzed as described. The upper portion of the heart and proximal aorta were obtained, embedded in OCT compound, and stored at −80°C. 10-μm sections were analyzed for a distance of 800 μm. Sections were stained with Von Kossa and Oil Red O. The lipid-staining and calcified areas on 25 sections were determined in a blinded fashion by light microscopy. The mean value of lesion area of aortic wall per section was then calculated.

**RNA analysis:** Total RNA was isolated by using Tri reagent coupled with RNAeasy kit. Real-time quantitative PCR assays were performed by using an Applied Biosystems StepOne qPCR instrument. In brief, 1 μg of total RNA was reverse transcribed with random hexamers by using High Quality Reverse Transcription Reagents Kit (Applied Biosystems). Each amplification mixture (10 μl) contained 25 ng cDNA, 900 nM forward primer, 900 nM reverse primer and 5 μl of Universal fast PCR Master Mix. Samples were analyzed for 18S rRNA expression in parallel in the same run. Quantitative expression values were calculated from absolute standard curve method using the plasmid template for each target gene. Primer sequences are available upon request.

**Lentiviral and adenoviral transduction for CVC:** Lentiviral CMV vectors (Lenti-X, Clontech) expressing mouse FXR dominant negative (FXRDN) with a FLAG tag were generated as previously described. The dominant negative FXRα, lacking the C-terminal 10 amino acids and the AF-2 subdomain was prepared by a mutagenesis kit (Stratagene). Lentiviral stocks were obtained by transfection of 5 × 10⁶ 293T cells with Lenti-X HT Packing system (Clontech). The
titers were analyzed by Lenti-X qRT-PCR Titration kit (Clontech). Cells were infected with recombinant lentiviruses and adenoviruses at a multiplicity of infection (MOI) of 10 and 40, respectively. Adenovirus expressing Msx2 with a c-myc tag, osterix with a FLAG tag and VP16FXRα (VP16 domain was inserted into the N-terminus of FXRα) were generated in the Vector Core in the Diabetes Endocrinology Research Center (DERC), University of Colorado-Denver or by RAPAd.I system (Cell Biolabs). The adenoviral cosmid vector contains IRES-GFP to check the transfection efficiency. CVC was infected with either lentivirus or adenovirus in DMEM 15% FBS containing 5 µg/ml Polybrene® (Sigma). After 24 or 48 h, the infected cells were treated with media containing 2mM phosphate.

**Western blotting:** The cells were washed four times with ice-cold PBS and the nuclear extracts were isolated using a nuclear extract kit (Active Motif). These samples were separated by SDS-PAGE, transferred to nitrocellulose membrane, immunoblotted with a FXR monoclonal antibody (R&D systems), FLAG monoclonal antibody (Sigma), GFP antibody (Santa Cruz), myc monoclonal antibody (Covance), phospho-AKT antibody (Cell Signaling), phospho-JNK antibody (Cell Signaling), psopho-p38 MAPK antibody (Cell Signaling), phospho-ERK antibody (Cell Signaling), total JNK antibody (Cell Signaling) or total ERK antibody (Cell Signaling) and visualized with horseradish peroxidase coupled to anti-mouse secondary antibody with enhancement by ECL detection kits.

**5/6 nephrectomy:** Under anesthesia with ketamine/Xylazine, a two-step procedure was performed. In the first step of surgery the left kidney was decapsulated and 1/3rd of each kidney pole ligated with a silk suture. The kidney tissue from each pole was then excised with scissors. The muscle and skin layers of the incision are then closed with sterile surgical staples. The animals recovered for one week before the second surgery. In the second surgery, the entire right kidney was decapsulated and surgically removed by ligating with silk suture at the bottom of the renal artery and then excising the tissue with scissors to complete the 5/6 nephrectomy.

**Tissue lipid composition:** Hepatic and CVC cholesterol and triglycerides were analyzed using gas chromatography as previously described. 7, 8
**Blood chemistry:** Total serum cholesterol, triglycerides, calcium and phosphate, blood urea nitrogen, were determined enzymatically in the serum or plasma of mice.

**Statistical analysis:** Statistical analyses for multiple groups were performed with 1-way ANOVA with Tukey post hoc test (GraphPad Prism, CA). Student t test was used when 2 groups were compared. Data are presented as mean ± SEM.

**Supplemental References**


2. Online Figure Legends

Online Figure I. Chronic kidney disease (CKD) accelerates atherosclerosis and vascular calcification. A) En face analysis of atherosclerosis in ApoE−/− mice with CKD. 8 week-old male ApoE−/− mice with or without 5/6 nephrectomy were obtained from The Jackson Laboratory and fed ad libitum either control or a western diet for 24 weeks. B) Quantification of aortic atherosclerosis in control diet and western diet fed-mice. The aorta including ascending arch, thoracic and abdominal segments was dissected and stained with Sudan IV. The surface lesion was quantified with commercially available software (Image Quant, Zeiss). C) Aortic calcium levels in ApoE−/− mice with CKD.

Online Figure II. INT-747 reduced gene expression of osteogenic transcription factors, osteogenic markers and lipogenic enzyme in CVC treated with high-phosphate. Cells were treated with INT-747 in the presence of 2 mM phosphate for 14 days. Black bar; vehicle, gray bar; 0.3 μM INT-747, white bar; 3.0 μM INT-747. Osteogenic markers: A) ALP, B) COL1A1 and C) MGP. Osteogenic transcription factors: D) Msx2, E) Runx2 and F) Osterix. Lipogenic genes: G) SREBP-1, H) SREBP-2, I) FAS, and J) ACC1. FXR target genes: K) SHP and L) AT2R.

Online Figure III. INT-747 treatment induces SHP expression in CVC. Cells were treated with INT-747 in the presence of 2 mM phosphate for 24 hours. Black bar; vehicle, gray bar; 0.3 μM INT-747, white bar; 3.0 μM INT-747.
Online Figure IV. FXRDN blocks the alterations of gene expressions by INT-747 treatment.  A) SHP, B) ALP, C) COL1A1, D) MGP, E) Msx2 and F) osterix (Osx). Cells were treated with lentivirus expressing mock or FXRDN at 10 MOI. 24 hour after infection, cells were treated with 3μM INT-747 in the presence of 2 mM phosphate for 24 hours. **p<0.001 vs. vehicle

Online Figure V. VP16FXR reduces Msx2, osterix, ALP and COL1A1 mRNA levels. CVCs were treated with adenovirus expressing VP16FXR at 40 MOI. 24 hour after infection, cells were treated with high-phosphate for 7 days. **p<0.001 vs. Mock

Online Figure VI. Msx2 and osterix (Osx) overexpression augments mineralization of CVC: A) western blot analysis, B) Alizarin staining C) Calcium content, and D) ALP and COL1A1 mRNA. CVC were treated with adenovirus Mock (empty), Msx2 and Osx at 40 MOI in the presence of high (2.0 mM) phosphate for 7 days. Msx2 and Osx proteins were analyzed by immunoblot analysis with anti-GFP, anti-myc and anti-FLAG antibodies, respectively. GFP was used to determine the transfection efficiency. Data are representative of combined from three independent experiments. **p<0.001 vs. Mock

Online Figure VII. INT-747 did not alter gene expression of and macrophage makers (CD68 and F4/80) and pro-inflammatory cytokines (TNFα and IL-1β) in the aorta of ApoE−/− mice with CKD. Male mice were fed a western diet for 12 weeks. A) Macrophage markers: CD68 and F4/80. B) Pro-inflammatory cytokines: TNFα and IL-1β.
Online Table I. Serum parameters in 5/6 nephrectomized (5/6NX) ApoE<sup>−/−</sup> mice maintained for 12 weeks on a western diet in the presence or absence of INT-747

<table>
<thead>
<tr>
<th>Strain</th>
<th>Kidney</th>
<th>Diet</th>
<th>Treatment</th>
<th>Vehicle</th>
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<th>INT-747</th>
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<td>INT-747</td>
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<td>20 mpk</td>
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<td>10 mpk</td>
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<th>Parameter</th>
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<th>ApoE&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<th>ApoE&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tr>
<td>Total serum CHOL (mg/dl)</td>
<td>330.0 ± 21.2</td>
<td>1556.7 ± 161.9</td>
<td>1447.5 ± 205.6</td>
<td>1423.6 ± 84.2</td>
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<td>Triglycerides (mg/dl)</td>
<td>67.4 ± 8.8</td>
<td>184.0 ± 31.0</td>
<td>168.6 ± 3.2#</td>
<td>90.5 ± 0.9*</td>
<td>93.1 ± 0.7*</td>
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<td>Glucose (mg/dl)</td>
<td>189.8 ± 19.9</td>
<td>190.5 ± 22.6</td>
<td>163.2 ± 25.9</td>
<td>148.0 ± 6.7</td>
<td>144.3 ± 9.6</td>
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<td>Calcium (mg/dl)</td>
<td>8.5 ± 0.4</td>
<td>9.4 ± 0.6</td>
<td>10.0 ± 0.3</td>
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<td>9.4 ± 0.2</td>
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<tr>
<td>Phosphate (mg/dl)</td>
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<td>11.4 ± 0.3</td>
<td>14.7 ± 1.2#</td>
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<td>14.0 ± 0.4#</td>
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<td>BUN (mg/dl)</td>
<td>13.6 ± 3.7</td>
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<td>34.7 ± 2.7#</td>
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<td>Total liver CHOL (mg/g)</td>
<td>8.0 ± 0.5</td>
<td>8.4 ± 1.6</td>
<td>9.2 ± 1.9</td>
<td>8.58 ± 1.4</td>
<td>7.10 ± 0.6**</td>
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<td>Liver triglycerides (mg/g)</td>
<td>14.2 ± 2.6</td>
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<td>20.7 ± 3.5</td>
<td>13.20 ± 1.8**</td>
<td>10.72 ± 1.3**</td>
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Data expressed as mean ± SEM. *p<0.05; **p<0.01 vs. ApoE<sup>−/−</sup> mice with 5/6NX fed WTD without INT-747
#p<0.05 vs. ApoE<sup>−/−</sup> with 2 kidneys fed WTD without INT-747
Online Figure I

A. ApoE−/− mice with 2KD fed Western diet

B. ApoE−/− mice with 5/6NX fed Western diet

C. Aortic calcium (mg/tissue)

- ** ApoE−/− ApoE−/−
- Aortic calcium (mg/tissue)

- ### ApoE−/− ApoE−/−

- # ApoE−/− ApoE−/−

- ApoE−/− mice with 2KD fed Western diet
- ApoE−/− mice with 5/6NX fed Western diet

- ApoE−/− ApoE−/− ApoE−/− ApoE−/−

- Aortic calcium (mg/tissue)
- ApoE−/− ApoE−/−

- WTD

- ApoE−/− ApoE−/−

- WTD

- ApoE−/− ApoE−/−

- ApoE−/− ApoE−/−

- WTD

- ApoE−/− ApoE−/−

- ApoE−/− ApoE−/−
Online Figure II

A: ALP transcript (ratio ALP/18S)

B: COL1A1 transcript (ratio COL1A1/18S)

C: MGP transcript (ratio MGP/18S)

D: Msx2 transcript (ratio Msx2/18S)

E: Runx2 transcript (ratio Runx2/18S)

F: Osterix transcript (ratio Osterix/18S)

G: SREBP-1 transcript (Ratio SREBP-1/18S)

H: SREBP-2 transcript (Ratio SREBP-2/18S)

I: FAS transcript (Ratio FAS/18S)

J: ACC1 transcript (Ratio ACC1/18S)

K: SHP transcript (Ratio SHP/18S)

L: AT2R transcript (Ratio AT2R/18S)
Online Figure III

A

SHP transcript
(Ratio SHP/18S)

vehicle
0.3 µM INT-747
3.0 µM INT-747

**
Online Figure V

![Bar chart showing relative mRNA levels of Msx-2, Osterix, ALP, and COL1A1](#)

- **Mock**
- **VP16FXR**

Relative mRNA level (vs. Mock)

- Msx-2
- Osterix
- ALP
- COL1A1
Online Figure VI

A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No treatment</th>
<th>Mock</th>
<th>Msx2</th>
<th>OSX</th>
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<td>WB:anti-GFP</td>
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B

Mock | Msx2 | OSX

C

![Graph showing Ca content (ug/mg protein) for different treatments](image)

D

![Graph showing mRNA levels (relative to Mock) for different treatments](image)