

## Original Paper

# Fibulin-3 Attenuates Phosphate-Induced Vascular Smooth Muscle Cell Calcification by Inhibition of Oxidative Stress

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## Key Words

Fibulin-3 • Phosphate • Oxidative stress • Vascular calcification • Osteo-/chondrogenic signaling  
• Vascular smooth muscle cells

## Abstract

**Background/Aims:** Fibulin-3, an extracellular matrix glycoprotein, inhibits vascular oxidative stress and remodeling in hypertension. Oxidative stress is prevalent in chronic kidney disease (CKD) patients and is an important mediator of osteo-/chondrogenic transdifferentiation and calcification of vascular smooth muscle cells (VSMCs) during hyperphosphatemia. Therefore, the present study explored the effects of Fibulin-3 on phosphate-induced vascular calcification. **Methods:** Experiments were performed in primary human aortic smooth muscle cells (HAoSMCs) treated with control or with phosphate without or with additional treatment with recombinant human Fibulin-3 protein or with hydrogen peroxide as an exogenous source of oxidative stress. **Results:** Treatment with calcification medium significantly increased calcium deposition in HAoSMCs, an effect significantly blunted by additional treatment with Fibulin-3. Moreover, phosphate-induced alkaline phosphatase activity and mRNA expression of osteogenic and chondrogenic markers *MSX2*, *CBFA1*, *SOX9* and *ALPL* were all significantly reduced by addition of Fibulin-3. These effects were paralleled by similar regulation of oxidative stress in HAoSMCs. Phosphate treatment significantly up-regulated mRNA expression of the oxidative stress markers *NOX4* and *CYBA*, down-regulated total antioxidant capacity and increased the expression of downstream effectors of oxidative stress *PAI-1*, *MMP2* and *MMP9* as well as *BAX/BLC2* ratio in HAoSMCs, all effects blocked by additional treatment with Fibulin-3. Furthermore, the protective effects of Fibulin-3 on phosphate-induced osteogenic and chondrogenic markers expression in HAoSMCs were reversed by additional treatment

with hydrogen peroxide. **Conclusions:** Fibulin-3 attenuates phosphate-induced osteo-/chondrogenic transdifferentiation and calcification of VSMCs, effects involving inhibition of oxidative stress. Up-regulation or supplementation of Fibulin-3 may be beneficial in reducing the progression of vascular calcification during hyperphosphatemic conditions such as CKD.

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## Introduction

Fibulin-3 is an extracellular matrix protein [1, 2] widely expressed in various tissues [2] including the vasculature [1, 3]. The functions of Fibulin-3 include basement membrane organization and elastic fiber formation [4]. It also interacts with other matrix proteins, forming anchoring structures to regulate cell proliferation and migration [4, 5]. Furthermore, Fibulin-3 promotes angiogenesis [6]. In addition to its role in extracellular matrix organization, Fibulin-3 participates in various intracellular signaling pathways [4, 7-9].

In the media of arterial wall, Fibulin-3 expression is up-regulated at hypertensive vascular remodeling [3, 10]. Fibulin-3 has previously been suggested as an arterial growth factor [3, 6] as injection of Fibulin-3 protein increases aortic wall thickness in spontaneously hypertensive rats [10]. However, Fibulin-3 reduces systolic blood pressure and improves vascular health [10], at least in part, by reducing aortic oxidative stress [10-12] and expression of matrix gelatinases [10, 13]. In vascular smooth muscle cells (VSMCs), gelatinase expression and activity are stimulated by excessive oxidative stress [14-17].

Oxidative stress is commonly associated with cardiovascular disease [18, 19] and all-cause mortality in patients with chronic kidney disease (CKD) [20]. The high cardiovascular morbidity and mortality of CKD patients is associated with medial vascular calcification [21]. Vascular calcification is an active process promoted by osteo-/chondrogenic reprogramming of VSMCs [22, 23]. Various pathological factors [22, 24-26], most importantly phosphate [27], induce the phenotypical transdifferentiation of contractile VSMCs into osteoblast and chondroblast-like cells via complex signaling pathways. Oxidative stress is an important mediator of osteo-/chondrogenic transdifferentiation of VSMCs [28-30]. These transdifferentiated VSMCs express osteogenic and chondrogenic transcription factors, such as msh homeobox 2 (MSX2), core-binding factor  $\alpha$ -1 (CBFA1) and SRY-Box 9 (SOX9) [24, 31, 32], as well as osteogenic enzymes, such as tissue non-specific alkaline phosphatase (ALPL) [33] to cause an active mineralization of vascular tissue.

The present study explored the effects of Fibulin-3 protein on phosphate-induced osteo-/chondrogenic transdifferentiation and calcification of VSMCs in-vitro as well as the possible involvement of oxidative stress suppression in mediating these effects.

## Materials and Methods

### *Cell culture of primary human aortic smooth muscle cells*

Primary human aortic smooth muscle cells (HAoSMCs) commercially obtained from Thermo Fisher Scientific were cultured in medium containing a 1:1 ratio of Waymouth's MB 752/1 medium and Ham's F-12 nutrient mixture (Thermo Fisher Scientific) supplemented with 10% FBS (Thermo Fisher Scientific), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (Thermo Fisher Scientific) [34, 35]. At least 4 different batches of HAoSMCs were used during the course of this study and each experiment was performed in at least 2 different batches of HAoSMCs at different passages, depending on the availability of the cells. HAoSMCs were grown to confluence and used in all experiments from passages 4 to 10. HAoSMCs were treated for the indicated periods with 2 mM  $\beta$ -glycerophosphate (Sigma Aldrich) [23, 28, 34, 36-38], 200 ng/ml recombinant human Fibulin-3 protein (R&D Systems) [9, 39] or 10  $\mu$ M  $H_2O_2$  (Sigma Aldrich) [40-42]. Treatment with 10 mM  $\beta$ -glycerophosphate and 1.5 mM

CaCl<sub>2</sub> (Sigma-Aldrich) for 11 days was used as calcification media for the calcium deposition analysis and Alizarin Red staining [43]. Fresh media with agents were added every 2-3 days.

### *Analysis of calcification*

To quantify the calcium deposition, HAoSMCs were decalcified in 0.6 M HCl for 24 hours at 4°C. Calcium content in the supernatant was determined by using QuantiChrom Calcium assay kit (BioAssay Systems) according to the manufacturer's protocol. HAoSMCs were lysed with 0.1 M NaOH/ 0.1% SDS and protein concentration was measured by the Bradford assay (Bio-Rad Laboratories). The results are shown normalized to total protein concentration [44]. To visualize calcification, HAoSMCs were fixed with 4% paraformaldehyde and stained with 2% Alizarin Red (pH 4.5). The calcified areas are shown as red staining [28].

### *Alkaline phosphatase (ALPL) activity assay*

ALPL activity in HAoSMCs was determined by using the ALP colorimetric assay kit (Abcam) according to the manufacturer's protocol [35]. The results are shown normalized to total protein concentration measured by the Bradford assay (Bio-Rad Laboratories).

### *Antioxidant capacity assay*

Total antioxidant capacity of HAoSMCs was measured in the cell lysate by using the colorimetric antioxidant assay kit (Cayman Chemical) according to the manufacturer's protocols. The capacity of the antioxidants in the samples was compared to that of Trolox as standard [28]. The results were normalized to total protein concentration as assessed by the Bradford assay (Bio-Rad Laboratories) and to the control group.

### *Quantitative RT-PCR*

Total RNA was isolated from HAoSMCs by using Trizol Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions [45-47]. Reverse transcription of 2 µg RNA was performed using oligo(dT)12-18 primers (Thermo Fisher Scientific) and SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). Quantitative RT-PCR was performed with the iCycler iQTM Real-Time PCR Detection System (Bio-Rad Laboratories) and iQTM Sybr Green Supermix (Bio-Rad Laboratories) according to the manufacturer's instructions. The following human primers were used (5'→3' orientation):

*ALPL* fw: GGGACTGGTACTCAGACAACG;  
*ALPL* rev: GTAGGCGATGTCCTTACAGCC;  
*BAX* fw: CCCGAGAGGTCTTTTTCCGAG;  
*BAX* rev: CCAGCCCATGATGGTTCTGAT;  
*BCL2* fw: GGTGGGGTCATGTGTGTGG;  
*BCL2* rev: CGGTTCAAGTACTCAGTCATCC;  
*CBFA1* fw: GCCTTCCACTCTCAGTAAGAAGA;  
*CBFA1* rev: GCCTGGGGTCTGAAAAAGGG;  
*CYBA* fw: CCCAGTGGTACTTTGGTGCC;  
*CYBA* rev: GCGGTCATGTACTTCTGTCCC;  
*EFEMP1* fw: TGCAGACTGGCCGAAATAACT;  
*EFEMP1* rev: CACACTGGATACGGTGGGAA;  
*GAPDH* fw: GAGTCAACGGATTGGTTCGT;  
*GAPDH* rev: GACAAGCTTCCCGTTCTCAG;  
*MMP2* fw: TACAGGATCATTTGGCTACACACC;  
*MMP2* rev: GGTACATCGCTCCAGACT;  
*MMP9* fw: AGACCTGGGCAGATTCCAAAC;  
*MMP9* rev: CGGCAAGTCTTCCGAGTAGT;  
*MSX2* fw: TGCAGAGCGTGCAGAGTTC;  
*MSX2* rev: GGCAGCATAGTTTTGCAGC;  
*NOX4* fw: TGACGTTGCATGTTTCAGGAG;  
*NOX4* rev: AGCTGGTTCGGTTAAGACTGAT;

*PAI-1* fw: ACCGCAACGTGGTTTTCTCA;  
*PAI-1* rev: TTGAATCCCATAGCTGCTTGAAT;  
*SOX9* fw: AGCGAACGCACATCAAGAC;  
*SOX9* rev: CTGTAGGCGATCTGTTGGGG.

The specificity of the PCR products was confirmed by analysis of the melting curves. All PCRs were performed in duplicate and relative mRNA expression was calculated by the  $2^{-\Delta\Delta Ct}$  method using GAPDH as internal reference normalized to the control group.

#### Western blot analysis

HAoSMCs were lysed with ice-cold IP lysis buffer (Thermo Fisher Scientific) supplemented with complete protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific) [44]. After centrifugation at 10000 rpm for 5 minutes, the proteins were boiled in Roti-Load1 Buffer (Carl Roth GmbH) at 100°C for 10 min. Equal amounts of proteins were separated on SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were incubated overnight at 4°C with primary mouse anti-EFEMP1 antibody (diluted 1:1000, sc-33722, Santa Cruz Biotechnology) or rabbit anti-GAPDH antibody (diluted 1:5000, #2118, Cell Signaling) and then with secondary anti-mouse HRP-conjugated (diluted 1:1000, Cell Signaling) or anti-rabbit HRP-conjugated antibody (diluted 1:1000, Cell Signaling) for 1 hour at RT. For loading controls, the membranes were stripped in stripping buffer (Thermo Fisher Scientific) at RT for 10 minutes. Antibody binding was detected with ECL detection reagent (Thermo Fisher Scientific). Bands were quantified by using ImageJ software and the results are shown as the ratio of total protein to GAPDH normalized to the control group.

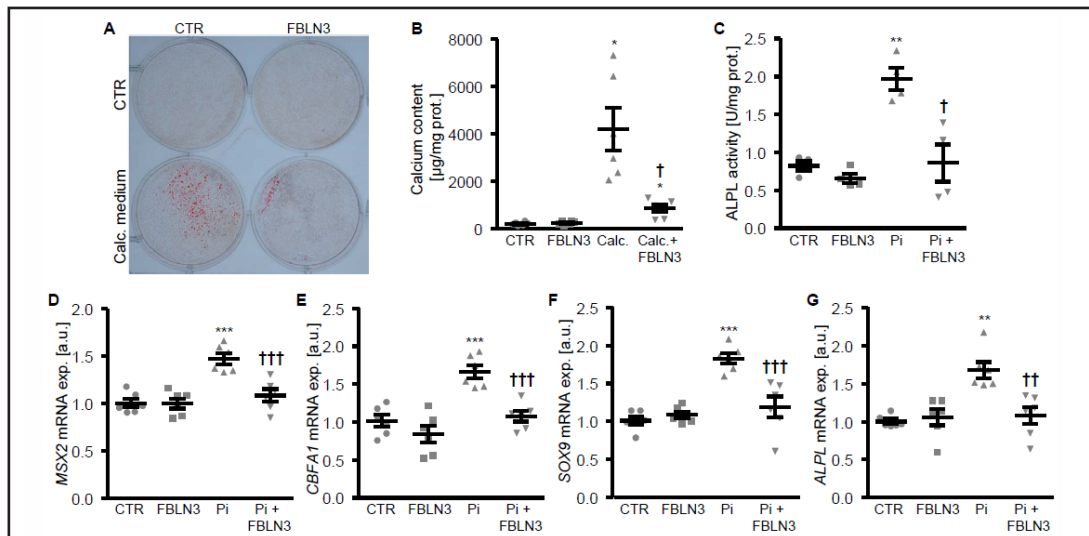
#### Statistics

Data are shown as scatterdot plots and arithmetic means  $\pm$  SEM. N indicates the number of independent experiments performed at different passages of the cells. Normality was tested with Shapiro-Wilk test. Non-normal datasets were transformed (log, reciprocal or sqrt) prior to statistical testing to provide normality according to Shapiro-Wilk test. Statistical testing was performed by one-way Anova followed by Tukey-test for homoscedastic data or Games-Howell test for heteroscedastic data. Non-normal data were tested by the Steel-Dwass method. Two groups were compared by unpaired two-tailed t-test.  $P < 0.05$  was considered statistically significant.

## Results

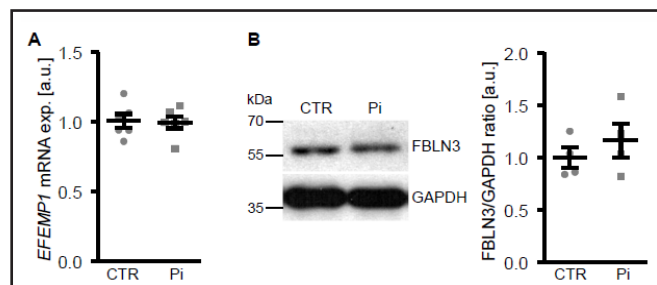
To investigate the effects of Fibulin-3 on phosphate-induced vascular calcification, a first series of experiments was performed in primary human aortic smooth muscle cells (HAoSMCs) following treatment with calcification media without or with additional treatment with recombinant human Fibulin-3 protein. As a result, Alizarin Red staining (Fig. 1A) and quantification of calcium deposition (Fig. 1B) in HAoSMCs revealed extensive calcification following treatment with calcification media, an effect significantly reduced by additional treatment with Fibulin-3. As shown in Fig. 1C-G, phosphate treatment significantly up-regulated ALPL activity and the mRNA expression of osteogenic markers *MSX2*, *CBFA1* and *ALPL* and of chondrogenic marker *SOX9* in HAoSMCs, all effects again significantly blunted by additional treatment with Fibulin-3. Treatment with Fibulin-3 alone did not significantly modify the calcium deposition and osteo-/chondrogenic markers expression in HAoSMCs (Fig. 1). Taken together, Fibulin-3 inhibits phosphate-induced osteo-/chondrogenic transdifferentiation and calcification of VSMCs in-vitro.

Additional experiments were performed to elucidate the underlying mechanisms of the protective effects of Fibulin-3 on VSMC calcification. Phosphate treatment did not significantly affect Fibulin-3 (encoded by the *EFEMP1* gene) mRNA and protein expression in HAoSMCs (Fig. 2). Nonetheless, additional treatment with Fibulin-3 blunted the increased mRNA expression of *NOX4* and p22phox (encoded by the *CYBA* gene) induced by high phosphate



**Fig. 1.** Fibulin-3 inhibits phosphate-induced osteoinductive signaling and calcification in HAoSMCs. **A.** Representative original images showing Alizarin red staining in HAoSMCs following treatment for 11 days with control or with calcification medium (Calc.) without or with additional treatment with 200 ng/ml recombinant human Fibulin-3 protein. The calcified areas are shown as red staining. Images are representative for three independent experiments. **B.** Scatterdot plots and arithmetic means  $\pm$  SEM ( $n=6$ ,  $\mu\text{g}/\text{mg}$  protein) of calcium content in HAoSMCs following treatment for 11 days with control or with calcification medium (Calc.) without or with additional treatment with 200 ng/ml recombinant human Fibulin-3 protein. **C.** Scatterdot plots and arithmetic means  $\pm$  SEM ( $n=4$ , U/mg protein) of ALPL activity in HAoSMCs following treatment for 7 days with control or with 2 mM  $\beta$ -glycerophosphate (Pi) without or with additional treatment with 200 ng/ml recombinant human Fibulin-3 protein. **D-G.** Scatterdot plots and arithmetic means  $\pm$  SEM ( $n=6$ ; arbitrary units, a.u.) of *MSX2* (**D**), *CBFA1* (**E**), *SOX9* (**F**) and *ALPL* (**G**) relative mRNA expression in HAoSMCs following treatment for 24 hours with control or with 2 mM  $\beta$ -glycerophosphate (Pi) without or with additional treatment with 200 ng/ml recombinant human Fibulin-3 protein. \*( $p<0.05$ ), \*\*( $p<0.01$ ), \*\*\*( $p<0.001$ ) statistically significant vs. control treated HAoSMCs; †( $p<0.05$ ), ††( $p<0.01$ ), †††( $p<0.001$ ) statistically significant vs. HAoSMCs treated with Calc./Pi alone.

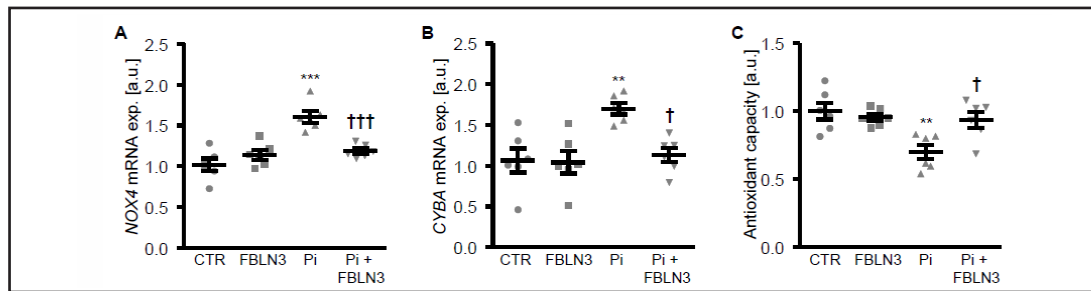
**Fig. 2.** Fibulin-3 expression is not modified by phosphate in HAoSMCs. **A.** Scatterdot plots and arithmetic means  $\pm$  SEM ( $n=6$ ; arbitrary units, a.u.) of *EFEMP1* relative mRNA expression in HAoSMCs following treatment for 24 hours with control or with 2 mM  $\beta$ -glycerophosphate (Pi). **B.** Representative original Western blots and scatterdot plots and arithmetic means  $\pm$  SEM ( $n=4$ ; a.u.) of normalized Fibulin-3/GAPDH protein ratio in HAoSMCs following treatment for 24 hours with control or with 2 mM  $\beta$ -glycerophosphate (Pi).



**Fig. 2.** Fibulin-3 expression is not modified by phosphate in HAoSMCs. **A.** Scatterdot plots and arithmetic means  $\pm$  SEM ( $n=6$ ; arbitrary units, a.u.) of *EFEMP1* relative mRNA expression in HAoSMCs following treatment for 24 hours with control or with 2 mM  $\beta$ -glycerophosphate (Pi). **B.** Representative original Western blots and scatterdot plots and arithmetic means  $\pm$  SEM ( $n=4$ ; a.u.) of normalized Fibulin-3/GAPDH protein ratio in HAoSMCs following treatment for 24 hours with control or with 2 mM  $\beta$ -glycerophosphate (Pi).

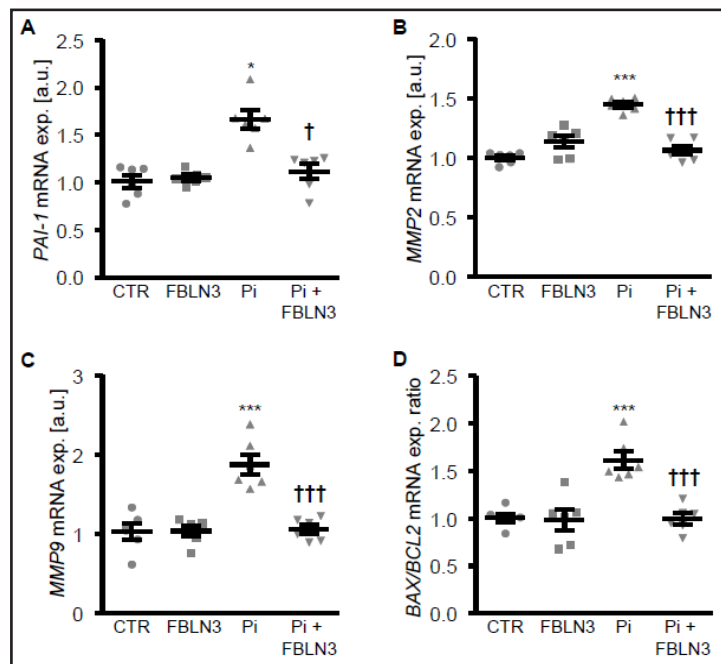
conditions in HAoSMCs, two critical components of the superoxide-generating NADH/NADPH oxidase system and markers of oxidative stress (Fig. 3A,B). Moreover, Fibulin-3 ameliorated the decrease of total antioxidant capacity in phosphate treated HAoSMCs (Fig. 3C). Thus, Fibulin-3 inhibits phosphate-induced oxidative stress in HAoSMCs.

These effects were paralleled by similar alteration of the oxidative stress downstream effectors in HAoSMCs. Phosphate treatment up-regulated the mRNA expression of plasminogen activator inhibitor *PAI-1* (Fig. 4A) and of matrix gelatinases *MMP2* and *MMP9* (Fig. 4B,C) in HAoSMCs, all effects significantly reduced following additional treatment with Fibulin-3 protein. Furthermore, phosphate treatment induced indicators of apoptosis in



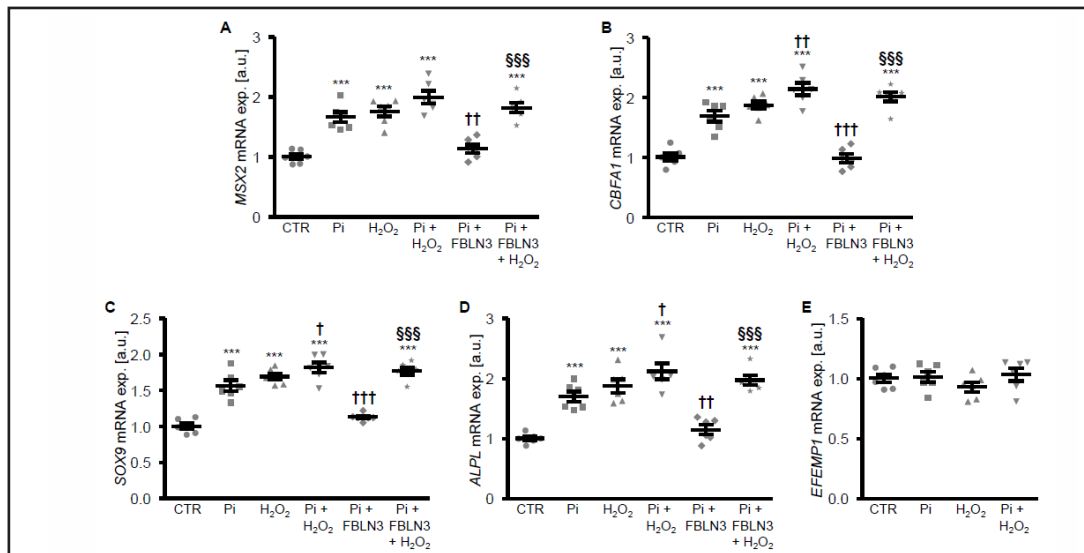
**Fig. 3.** Fibulin-3 attenuates phosphate-induced oxidative stress in HAoSMCs. A,B. Scatterdot plots and arithmetic means  $\pm$  SEM (n=6; arbitrary units, a.u.) of *NOX4* (A) and *CYBA* (B) relative mRNA expression in HAoSMCs following treatment for 24 hours with control or with 2 mM  $\beta$ -glycerophosphate (Pi) without or with additional treatment with 200 ng/ml recombinant human Fibulin-3 protein. C. Scatter dot plots and arithmetic means  $\pm$  SEM (n=6; a.u.) of normalized total antioxidant capacity of HAoSMCs following treatment for 24 hours with control or with 2 mM  $\beta$ -glycerophosphate (Pi) without or with additional treatment with 200 ng/ml recombinant human Fibulin-3 protein. \*\* (p<0.01), \*\*\* (p<0.001) statistically significant vs. control treated HAoSMCs; † (p<0.05), ††† (p<0.001) statistically significant vs. HAoSMCs treated with Pi alone.

**Fig. 4.** Fibulin-3 inhibits the expression of oxidative stress downstream effectors during high phosphate conditions in HAoSMCs. A-D. Scatterdot plots and arithmetic means  $\pm$  SEM (n=6; arbitrary units, a.u.) of *PAI-1* (A), *MMP2* (B) and *MMP9* (C) relative mRNA expression as well as *BAX/BCL2* relative mRNA expression ratio (D) in HAoSMCs following treatment for 24 hours with control or with 2 mM  $\beta$ -glycerophosphate (Pi) without or with additional treatment with 200 ng/ml recombinant human Fibulin-3 protein. \* (p<0.05), \*\*\* (p<0.001) statistically significant vs. control treated HAoSMCs; † (p<0.05), ††† (p<0.001) statistically significant vs. HAoSMCs treated with Pi alone.



HAoSMCs, as shown by an increase in *BAX/BCL2* mRNA expression ratio (Fig. 4D). These effects were again significantly ameliorated by Fibulin-3 treatment.

To further investigate whether inhibition of oxidative stress is responsible for the anti-calcific effects of Fibulin-3, low concentrations of  $H_2O_2$  were added to HAoSMCs as an exogenous source of oxidative stress. As shown in Fig. 5, treatment with  $H_2O_2$  alone significantly up-regulated *MSX2*, *CBFA1*, *SOX9* and *ALPL* mRNA expression in HAoSMCs to similarly high levels as phosphate treatment and aggravated osteo-/chondrogenic markers expression in phosphate treated HAoSMCs (Fig. 5A-D) without significantly affecting *EFEMP1* mRNA expression (Fig. 5E). More importantly, addition of  $H_2O_2$  blocked the protective effects of Fibulin-3 on phosphate-induced osteo-/chondrogenic transdifferentiation of HAoSMCs (Fig. 5). Thus, the protective effects of Fibulin-3 on phosphate-induced osteo-/chondrogenic transdifferentiation of VSMCs are due, at least in part, to inhibition of endogenous oxidative stress.



**Fig. 5.** The protective effects of Fibulin-3 on phosphate-induced osteoinduction are blunted by addition of H<sub>2</sub>O<sub>2</sub> as an exogenous source of oxidative stress in HAoSMCs. A-D. Scatterdot plots and arithmetic means  $\pm$  SEM (n=6; arbitrary units, a.u.) of *MSX2* (A), *CBFA1* (B), *SOX9* (C) and *ALPL* (D) relative mRNA expression in HAoSMCs following treatment for 24 hours with control, 2 mM  $\beta$ -glycerophosphate alone (Pi), 10  $\mu$ M H<sub>2</sub>O<sub>2</sub> alone or 2 mM  $\beta$ -glycerophosphate together with 10  $\mu$ M H<sub>2</sub>O<sub>2</sub> (Pi+H<sub>2</sub>O<sub>2</sub>), 200 ng/ml recombinant human Fibulin-3 protein (Pi+FBLN3) or 200 ng/ml recombinant human Fibulin-3 protein and 10  $\mu$ M H<sub>2</sub>O<sub>2</sub> (Pi+FBLN3+H<sub>2</sub>O<sub>2</sub>). \*\*\* (p<0.001) statistically significant vs. control treated HAoSMCs; † (p<0.05), †† (p<0.01), ††† (p<0.001) statistically significant vs. HAoSMCs treated with Pi alone; §§§ (p<0.001) statistically significant between Pi+FBLN3 and Pi+FBLN3+H<sub>2</sub>O<sub>2</sub> HAoSMCs. E. Scatterdot plots and arithmetic means  $\pm$  SEM (n=6; a.u.) of *EFEMP1* relative mRNA expression in HAoSMCs following treatment for 24 hours with control, 2 mM  $\beta$ -glycerophosphate alone (Pi), 10  $\mu$ M H<sub>2</sub>O<sub>2</sub> alone or 2 mM  $\beta$ -glycerophosphate together with 10  $\mu$ M H<sub>2</sub>O<sub>2</sub> (Pi+H<sub>2</sub>O<sub>2</sub>).

## Discussion

The present study reveals a novel role of Fibulin-3 in the inhibition of osteo-/chondrogenic transdifferentiation and calcification of VSMCs during hyperphosphatemia in-vitro. Fibulin-3 treatment blunts phosphate-induced oxidative stress, while addition of an exogenous source of oxidative stress suppresses the anti-calcific effects of Fibulin-3 in VSMCs.

Oxidative stress represents the imbalance between antioxidant systems and oxidative products [20]. Phosphate up-regulates the expression of *NOX4* and *CYBA*, two critical components of the superoxide-generating NADH/NADPH oxidase system [48, 49] and reduces total antioxidant capacity of VSMCs. Fibulin-3 supplementation is able to suppress the expression of NADH/NADPH oxidase system components and may, thus, interfere with reactive oxygen species production in VSMCs. In accordance with previous observations [10], Fibulin-3 treatment is able to block the accumulation of oxidative stress in phosphate treated VSMCs.

Excessive oxidative stress is involved in the pathogenesis of vascular calcification [20, 28-30]. Reactive oxygen species are mediators of CKD-related vascular calcification [48, 50]. Similarly, increased NADPH oxidase activity is associated with coronary artery calcification in asymptomatic men [49]. Oxidative stress contributes to the progression of vascular calcification via active cellular mechanisms by stimulating osteo-/chondrogenic transdifferentiation of VSMCs [29, 50]. Accordingly, hydrogen peroxide induces the phenotypical transformation of VSMCs into osteoblast-like cells, including up-regulation of CBFA1 expression [29] and ALPL activity [30] to facilitate mineral deposition. Conversely,

antioxidants are able to reduce osteo-/chondrogenic transdifferentiation and calcification of VSMCs [51]. The present data shows that Fibulin-3 suppresses the increased expression of osteogenic transcription factors *MSX2* and *CBFA1* and of chondrogenic transcription factor *SOX9* as well as *ALPL* expression and activity in VSMCs following high phosphate treatment. Previous studies similarly show that Fibulin-3 is able to reduce *SOX9* expression and to inhibit chondrogenesis in bone marrow-derived mesenchymal stem cells as well as differentiation of chondrocytes [52, 53].

Downstream effectors of oxidative stress in VSMCs include plasminogen activator inhibitor PAI-1 [16, 54] and the matrix metalloproteinases MMP2 and MMP9, also known as gelatinases [14-17, 55]. The increased *PAI-1* expression, a regulator of VSMCs calcification [28, 56], is attenuated by Fibulin-3 during high phosphate conditions. Similarly, Fibulin-3 reduces phosphate-induced *MMP2* and *MMP9* expression in VSMCs. Gelatinases are essential for phenotypical transformation of VSMCs, matrix remodeling and initiation of vascular calcification [57, 58] by up-regulation of bone morphogenetic protein-2 (BMP-2) which induces the expression of *CBFA1* and *MSX2* [59]. Furthermore, oxidative stress is able to induce apoptosis of VSMCs [60] and apoptosis may promote the initiation and progression of vascular calcification [61]. Accordingly, Fibulin-3 is able to inhibit phosphate-induced increase in *BAX/BCL2* ratio in VSMCs, a maker of increased apoptosis.

The inhibitory effect of Fibulin-3 on phosphate-induced osteogenic and chondrogenic markers expression in VSMCs is blunted by additional treatment with low concentrations of hydrogen peroxide. Thus, in the presence of an exogenous source of oxidative stress, Fibulin-3 fails to inhibit the osteo-/chondrogenic signaling in VSMCs. Taken together, the present observations suggest that Fibulin-3 may be effective not by interfering with oxidative stress-downstream osteoinductive signaling in VSMCs, but rather by interfering with reactive oxygen species production and accumulation of oxidative stress in VSMCs. Excessive oxidative stress does not modify Fibulin-3 expression in VSMCs. However, oxidation may render Fibulin-3 biologically inactive [1, 2, 62-64]. Further studies are clearly required to elucidate whether Fibulin-3 function is affected by calcific conditions in VSMCs. Nonetheless, the current observations suggest that Fibulin-3 supplementation reduces osteo-/chondrogenic signaling in VSMCs and, thus, vascular calcification, effects involving inhibition of endogenous oxidative stress.

In addition, other mechanisms may contribute to the anti-calcific effects of Fibulin-3 in the vascular tissue. Fibulin-3 may hinder extracellular matrix remodeling and elastin degradation [4, 5, 61, 65, 66]. Furthermore, Fibulin-3 may interfere with various intracellular signaling pathways [7-9] in vascular calcification. Fibulin-3 was previously shown to be an inhibitor of TGF- $\beta$ -dependent signaling [9], a key intracellular pathway in osteo-/chondrogenic transdifferentiation of VSMCs [67, 68]. However, addition of Fibulin-3 failed to suppress the TGF- $\beta$ -dependent osteoinductive signaling in VSMCs (data not shown).

Despite the finding that phosphate did not directly affect Fibulin-3 expression in VSMCs, the present observations suggest that Fibulin-3 may reduce the progression of vascular calcification in conditions of hyperphosphatemia such as CKD [21, 27]. In these patients, oxidative stress is further associated with increased cardiovascular and all-cause mortality [18-20] and thus, Fibulin-3 may have overall protective effects in systemic changes during disease progression. However, Fibulin-3 may promote neoplastic growth in some tumors [69, 70]. Also, tumor suppressive properties of Fibulin-3 were described [71]. These observations warrant caution and further investigations of a possible safe further translation of Fibulin-3 treatment in CKD patients [72].

In theory, Fibulin-3 may be effective in reducing vascular calcification in other pathologies associated with oxidative stress such as diabetes mellitus [73, 74] or aging [28, 75]. Fibulin-3 expression is decreased with aging [52] and Fibulin-3 deficiency in mice leads to early onset of aging-associated phenotypes [76]. Further studies are necessary to confirm the potential benefits of Fibulin-3 in these conditions.

In conclusion, Fibulin-3 reduces phosphate-induced osteo-/chondrogenic transdifferentiation of VSMCs and, thus, vascular calcification by inhibiting endogenous



oxidative stress. The present observations reveal that up-regulation or supplementation of Fibulin-3 may be beneficial in reducing the progression of vascular calcification during high phosphate conditions such as CKD.

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## Disclosure Statement

All authors disclose that they have no potential conflict of interest.

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