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Inhibition of vascular smooth muscle cell calcification by vasorin through interference with TGFβ1 signaling

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ABSTRACT

Elevated transforming growth factor β1 (TGFβ1) levels are frequently observed in chronic kidney disease (CKD) patients. TGFβ1 contributes to development of medial vascular calcification during hyperphosphatemia, a pathological process promoted by osteo−/chondrogenic transdifferentiation of vascular smooth muscle cells (VSMCs). Vasorin is a transmembrane glycoprotein highly expressed in VSMCs, which is able to bind TGFβ to inhibit TGFβ signaling. Thus, the present study explored the effects of vasorin on osteo−/chondrogenic transdifferentiation and calcification of VSMCs. Primary human aortic smooth muscle cells (HAoSMCs) were treated with recombinant human TGFβ1 or β-glycerophosphate without or with recombinant human vasorin or vasorin gene silencing by siRNA. As a result, TGFβ1 down-regulated vasorin mRNA expression in HAoSMCs. Vasorin supplementation inhibited TGFβ1-induced pathway activation, SMAD2 phosphorylation and downstream target genes expression in HAoSMCs. Furthermore, treatment with exogenous vasorin blunted, while vasorin knockdown augmented TGFβ1-induced osteo−/chondrogenic transdifferentiation of HAoSMCs. In addition, phosphate down-regulated vasorin mRNA expression in HAoSMCs. Phosphate-induced *TGFβ1* expression was not affected by addition of exogenous vasorin. Nonetheless, the phosphate-induced TGFβ1 signaling, osteo−/chondrogenic transdifferentiation and calcification of HAoSMCs were all blunted by vasorin. Conversely, silencing of vasorin aggravated osteoinduction in HAoSMCs during high phosphate conditions. Aortic vasorin expression was reduced in the hyperphosphatemic klotho-hypomorphic mouse model of CKD-related vascular calcification. In conclusion, vasorin, which suppresses TGFβ1 signaling and protects against osteo−/chondrogenic transdifferentiation and calcification of VSMCs, is reduced by pro-calcifying conditions. Thus, vasorin is a novel key regulator of VSMC calcification and may represent a potential therapeutic target for vascular calcification during CKD.

1. Introduction

Vasorin is a transmembrane glycoprotein [1,2], expressed in a multitude of tissues [2–5] and at particularly high levels in the arteries [2,6]. In vascular tissue, vasorin is predominantly expressed by vascular smooth muscle cells (VSMCs) [2]. Vasorin is mainly localized at the cell membrane [1,2,7], but may also be cleaved and secreted into the extracellular space [4,7]. The functions of vasorin are not yet completely understood [1], but it is believed to play a key role in the transforming growth factor β (TGFβ) signaling pathway [1,2,6]. At the cell

Abbreviations: ALK5, activin receptor-like kinase 5; ALPL, tissue non-specific alkaline phosphatase; BMP, bone morphogenetic protein; CBFA1, core-binding factor α-1; CKD, chronic kidney disease; COL2A1, collagen type II alpha 1 chain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HAoSMCs, human aortic smooth muscle cells; INHBA, inhibin, beta A; MMP13, matrix metallopeptidase 13; PAI-1, plasminogen activator inhibitor 1; Pi, phosphate; SMAD, small mothers against decapentaplegic; SOX9, SRY-Box 9; TGFβ, transforming growth factor β; VASN, vasorin; VSMCs, vascular smooth muscle cells

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membrane and in the extracellular space, vasorin can directly bind to TGFβ1, TGFβ2 or TGFβ3 [2] and thereby inhibits TGFβ-induced intracellular signaling [1,2,4,6].

Elevated TGFβ1 levels are frequently observed in chronic kidney disease (CKD) [8,9] and presumably contribute to the progression of CKD-related complications including medial vascular calcification [10–16]. In CKD, vascular calcification is considered a major cardiovascular risk factor and is associated with cardiovascular and all-cause morbidity and mortality [15,17–19]. Vascular calcification is an active process with some parallels to physiological bone formation [20,21]. A critical role is attributed to VSMCs, which are able to change their contractile phenotype into an osteoblast- and chondroblast-like phenotype [12,22–24]. Osteo−/chondrogenic VSMCs actively promote vascular tissue mineralization [14,25]. Complex intracellular signaling pathways control this osteo−/chondrogenic transdifferentiation of VSMCs [14,21,22,26–30].

TGFβ1 is a decisive stimulator of VSMC osteo−/chondrogenic transdifferentiation, at least partly, by triggering cellular senescence [10–13]. The osteoinductive signaling pathways activated by TGFβ1 include up-regulation of plasminogen activator inhibitor (PAI-1) [10–12,14,31], a marker and mediator of senescence that has osteoinductive effects [10,11,32]. Moreover, TGFβ1-dependent osteoinductive signaling involves increased expression of the chondrogenic transcription factor SRY-Box 9 (SOX9) [11,31]. The osteoinductive pathways eventually lead to up-regulation of osteogenic transcription factors such as core-binding factor alpha 1 (CBFA1) and of the osteogenic enzymes such as tissue-nonspecific alkaline phosphatase (ALPL), key mediators of vascular tissue calcification [17,23,33]. More importantly, inhibition of TGFβ1-dependent signaling is able to reduce osteo−/chondrogenic transdifferentiation of VSMCs and vascular calcification [10,11,31].

TGFβ1 expression in VSMCs is promoted by phosphate [11,31], one of the strongest promoters of vascular calcification in CKD [17,34]. In turn, TGFβ1 participates in the complex osteoinductive signaling regulating phosphate-induced vascular calcification [11,31].

The present study explored the effects of vasorin on TGFβ1 signaling and TGFβ1-mediated osteo−/chondrogenic transdifferentiation of VSMCs and vascular calcification *in vitro*.

2. Materials and methods

2.1. Cell culture of HAoSMCs

Primary human aortic smooth muscle cells (HAoSMCs) commercially obtained from Thermo Fisher Scientific (Cat. C0075C) [22,26,27,35–37] and Sigma Aldrich (Cat. 354-05A) were routinely cultured as previously described. The culture medium contained Waymouth's MB 752/1 medium and Ham's F-12 nutrient mixture (1:1 ratio, Thermo Fisher Scientific) supplemented with 10% FBS (Thermo Fisher Scientific), 100 U/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific). Each experiment was performed in at least two different batches of HAoSMCs at different passages (from passages 4 to 11), depending on the availability of the cells.

Where indicated, HAoSMCs were pre-treated for 30 min with 100 ng/ml recombinant human vasorin protein (Biorbyt, Cat. orb49733, stock in PBS) [6] followed by additional treatment for 30 min (phosphorylated proteins, luciferase assay), 24 h (total protein abundance, mRNA expression) or 7 days (ALPL activity) with 10 ng/ml recombinant human TGFβ1 protein (R&D Systems, stock in 4 mM HCl, 1 mg/ml BSA) [11,31] or with 2 mM β-glycerophosphate (Sigma Aldrich) [22,26,28,29]. Equal amounts of vehicle were used as control. Treatment for 11 days with calcification media containing 10 mM β-glycerophosphate and 1.5 mM $CaCl_2$ (Sigma-Aldrich) was used for the analysis of mineral deposition [26,35,37,38]. Fresh media with agents were added every 2–3 days.

HAoSMCs were transfected with 20 nM VASN siRNA (ID no. s41740, Thermo Fisher Scientific) or 20 nM negative control siRNA (ID no. 4390843, Thermo Fisher Scientific) using siPORT amine transfection agent (Thermo Fisher Scientific), according to the manufacturer's protocol [37,39]. The cells were used 48 h after transfection and silencing efficiency was verified by quantitative RT-PCR.

2.2. Animal experiments

All animal experiments were conducted according to the recommendations of the Guide for Care and Use of Laboratory Animals of the National Institutes of Health as well as the German law for the welfare of animals, and reviewed and approved by the local government authority. Male and female klotho-hypomorphic (*kl/kl*) mice and corresponding wild-type (WT) mice [11,40] were sacrificed in isoflurane anesthesia and aortic tissues were snap frozen in liquid nitrogen.

2.3. RNA isolation and quantitative RT-PCR

Total RNA was isolated from HAoSMCs and aortic tissues by using Trizol Reagent (Thermo Fisher Scientific) [22,27]. Reverse transcription was performed with SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) and oligo$(dT)_{12–18}$ primers (Thermo Fisher Scientific). Quantitative RT-PCR was performed in duplicate by using iQ™ Sybr Green Supermix (Bio-Rad Laboratories) and CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories). The specificity of the PCR products was confirmed by analysis of the melting curves. Relative mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method using GAPDH as internal control, normalized to the control group. The following human primers were used (5′ → 3′ orientation, Thermo Fisher Scientific) [22,31,35,41]:

ALK5 fw: GCTGTATTGCAGACTTAGGACTG;
ALK5 rev: TTTTTGTTCCCACTCTGTGGTT;
ALPL fw: GGGACTGGTACTCAGACAACG;
ALPL rev: GTAGGCGATGTCCTTACAGCC;
BMP2 fw: ACTACCAGAAACGAGTGGGAA;
BMP2 rev: GCATCTGTTCTCGGAAAACCT;
BMP4 fw: TAGCAAGAGTGCCGTCATTCC;
BMP4 rev: GCGCTCAGGATACTCAAGACC;
BMP7 fw: GGAACGCTTCGACAATGAGAC;
BMP7 rev: GCAGGAAGAGATCCGATTCCC;
CBFA1 fw: GCCTTCCACTCTCAGTAAGAAGA;
CBFA1 rev: GCCTGGGGTCTGAAAAAGGG;
COL2A1 fw: TGGACGCCATGAAGGTTTTCT;
COL2A1 rev: TGGGAGCCAGATTGTCATCTC;
GAPDH fw: GAGTCAACGGATTTGGTCGT;
GAPDH rev: GACAAGCTTCCCGTTCTCAG;
INHBA fw: CAACAGGACCAGGACCAAAGT;
INHBA rev: GAGAGCAACAGTTCACTCCTC;
MMP13 fw: CCAGACTTCACGATGGCATTG;
MMP13 rev: GGCATCTCCTCCATAATTTGGC;
PAI-1 fw: ACCGCAACGTGGTTTTCTCA;
PAI-1 rev: TTGAATCCCATAGCTGCTTGAAT;
SOX9 fw: AGCGAACGCACATCAAGAC;
SOX9 rev: CTGTAGGCGATCTGTTGGGG;
TGFB1 fw: CAATTCCTGGCGATACCTCAG;
TGFB1 rev: GCACAACTCCGGTGACATCAA;
TGFB2 fw: CCATCCCGCCCACTTTCTAC;
TGFB2 rev: AGCTCAATCCGTTGTTCAGGC;
TGFB3 fw: GGAAAACACCGAGTCGGAATAC;
TGFB3 rev: GCGGAAAACCTTGGAGGTAAT;
VASN fw: GAGAGCCACGTCACACTGG;
VASN rev: CAAAGTCGGCGTAGTCAAGC.

The following mouse primers were used (5′ → 3′ orientation,

Thermo Fisher Scientific):

Gapdh fw: AGGTCGGTGTGAACGGATTTG;
Gapdh rev: TGTAGACCATGTAGTTGAGGTCA;
Vasn fw: TTGAGAACGGCATCACGACAC;
Vasn rev: ACGGAAGGTCTCGTTGGAGAT.

2.4. Protein isolation and Western blotting

HAoSMCs were lysed in ice-cold IP lysis buffer (Thermo Fisher Scientific) supplemented with complete protease and phosphatase inhibitors cocktail (Thermo Fisher Scientific) [22,26,35]. Equal amounts of proteins were boiled in Roti-Load1 Buffer (Carl Roth) at 100 °C for 10 min. Proteins were separated on SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were incubated with primary rabbit anti-RUNX2 (1:1000, #8486, Cell Signaling), rabbit anti-phospho-SMAD2 ($Ser^{465/467}$)/ SMAD3 ($Ser^{423/425}$) (1:1000, #8828, Cell Signaling), rabbit anti-SMAD2/3 (1:1000, #8685, Cell Signaling), rabbit anti-phospho- SMAD1 ($Ser^{463/465}$)/ SMAD5 ($Ser^{463/465}$)/ SMAD9 ($Ser^{465/467}$) (1:1000, #13820, Cell Signaling), rabbit anti-SMAD1 (1:1000, #6944, Cell Signaling) or rabbit anti-GAPDH (1:1000, #2118, Cell Signaling) antibodies overnight at 4 °C and then with secondary anti-rabbit HRP-conjugated antibody (1:1000, Cell Signaling) for 1 h at room temperature. For loading controls, the membranes were stripped in Restore PLUS Western Blot stripping buffer (Thermo Fisher Scientific) for 10 min at room temperature. 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 phosphorylated to total protein to GAPDH and of total protein to GAPDH, normalized to the control group [22,26]. Due to the short exposure needed to visualize the phosphorylation of SMAD2 following TGFβ1 treatment, the data was normalized to the TGFβ1 alone treated group.

2.5. Luciferase assay

HAoSMCs were transfected for 24 h with 0.7 μg DNA mixture of a 40:1 ratio of SMAD-responsive luciferase construct and constitutively expressing *Renilla* construct (Qiagen) using X-tremeGENE HP DNA transfection reagent (Roche Applied Science) according to the manufacturer's protocol. HAoSMCs were lysed with Passive Lysis Buffer (Promega) and assayed for transcriptional activity using Dual-Luciferase Reporter Assay (Promega) and a microplate luminometer (GloMax Navigator System, Promega) according to the manufacturer's protocol. Results are shown as the ratio of SMAD Firefly-Luciferase to *Renilla*-Luciferase, normalized to control treated HAoSMCs.

2.6. ALPL activity assay

ALPL activity in the cell extracts from HAoSMCs was determined by using the ALP colorimetric assay kit (Abcam) [22,37]. The results are shown normalized to total protein concentration measured by the Bradford assay (Bio-Rad Laboratories).

2.7. Alizarin red staining

To visualize calcification, HAoSMCs were fixed with 4% paraformaldehyde and stained with 2% Alizarin Red (pH 4.5) at room temperature. The calcified areas are shown as red staining [22,26].

2.8. Quantification of calcification

HAoSMCs were decalcified in 0.6 M HCl for 24 h at 4 °C and the calcium content in the supernatant was determined by using the QuantiChrom Calcium assay kit (BioAssay Systems) [28,38]. HAoSMCs were lysed with 0.1 M NaOH/ 0.1% SDS. The results are shown normalized to total protein concentration measured by the Bradford assay (Bio-Rad Laboratories).

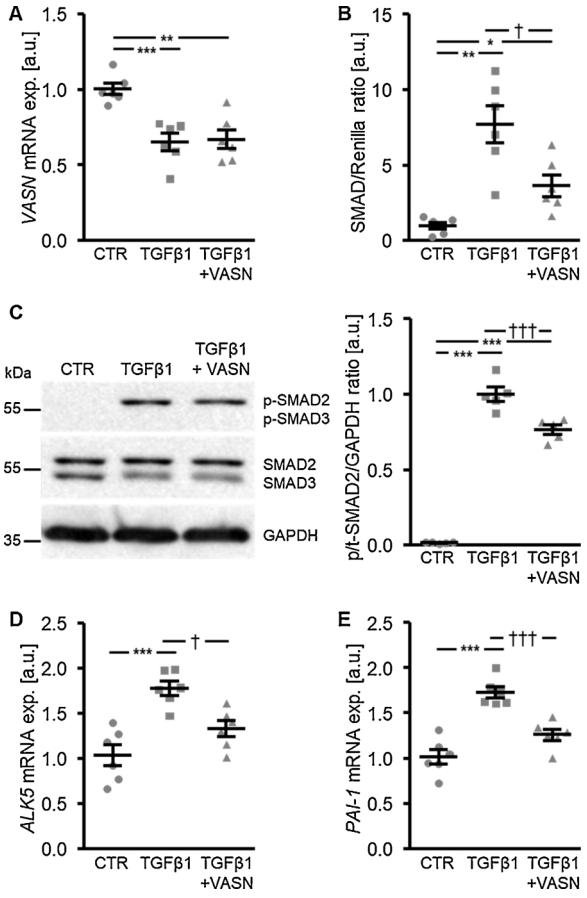

Fig. 1. Vasorin inhibits TGFβ1-dependent signaling in primary human aortic smooth muscle cells. **A.** Scatter dot plots and arithmetic means ± SEM ($n = 6$; arbitrary units, a.u.) of *VASN* relative mRNA expression in HAoSMCs treated with control (CTR) or with 10 ng/ml recombinant human TGFβ1 without or with additional treatment with 100 ng/ml recombinant human vasorin (VASN). **B.** Scatter dot plots and arithmetic means ± SEM ($n = 6$; a.u.) of TGFβ pathway activity measured by luciferase reporter assay in HAoSMCs following transfection with SMAD-responsive luciferase/*Renilla* constructs and treatment with control (CTR) or with 10 ng/ml recombinant human TGFβ1 without or with additional treatment with 100 ng/ml recombinant human vasorin (VASN). **C.** Representative original Western blots and scatter dot plots and arithmetic means ± SEM ($n = 5$; a.u.) of normalized phospho-SMAD2 ($Ser^{465/467}$)/ SMAD2/ GAPDH protein ratio in HAoSMCs treated with control (CTR) or with 10 ng/ml recombinant human TGFβ1 without or with additional treatment with 100 ng/ml recombinant human vasorin (VASN). **D, E.** Scatter dot plots and arithmetic means ± SEM ($n = 6$; a.u.) of *ALK5* (**D**) and *PAI-1* (**E**) relative mRNA expression in HAoSMCs treated with control (CTR) or with 10 ng/ml recombinant human TGFβ1 without or with additional treatment with 100 ng/ml recombinant human vasorin (VASN). *($p < .05$), **($p < .01$), ***($p < .001$) significant compared to control HAoSMCs; †(p < .05), †††(p < .001) significant compared to HAoSMCs treated with TGFβ1 alone.

2.9. Statistics

Data are shown as scatter dot plots and arithmetic means ± SEM and n indicate the number of independent experiments performed at different passages of the cells or the number of mice, respectively. 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 (homoscedastic

data) or Games-Howell test (heteroscedastic data). Non-normal data were tested by the Steel-Dwass method. $P < .05$ was considered statistically significant.

3. Results

A first series of experiments was performed to elucidate the effects of vasorin on TGFβ1 downstream signaling in VSMCs. To this end, HAoSMCs were treated with recombinant human TGFβ1 without or with additional treatment with recombinant human vasorin. As shown in Fig. 1A, TGFβ1 significantly down-regulated vasorin (*VASN*) mRNA expression in HAoSMCs. Addition of exogenous vasorin to the cell culture medium did not modify this effect. TGFβ1 treatment triggered the activation of TGFβ pathway in HAoSMCs, as shown by a SMAD-reporter assay (Fig. 1B). These effects were significantly blunted by additional treatment with vasorin. Furthermore, vasorin significantly reduced TGFβ1-induced phosphorylation of SMAD2 ($Ser^{465/467}$), but not SMAD3 ($Ser^{423/425}$)(Fig. 1C) and up-regulation of TGFβ type I receptor (*ALK5*) and plasminogen activator inhibitor (*PAI-1*) mRNA expression, downstream target genes of TGFβ1 signaling in VSMCs (Fig. 1D,E). Thus, vasorin interfered with TGFβ1-induced signaling in VSMCs.

Further experiments investigated whether the inhibitory effects of vasorin on TGFβ1-dependent signaling influences TGFβ1-induced osteo−/chondrogenic transdifferentiation of VSMCs. As illustrated in Fig. 2A-C, TGFβ1 up-regulated chondrogenic markers *SOX9*, matrix metallopeptidase 13 (*MMP13*) and collagen type II alpha 1 chain (*COL2A1*) mRNA expression in HAoSMCs. These effects were significantly inhibited in the presence of vasorin. Moreover, the TGFβ1-induced up-regulation of *CBFA1*, a critical osteogenic transcription factor and marker of osteo−/chondrogenic transdifferentiation, was significantly blunted by vasorin at the mRNA and protein level in HAoSMCs (Fig. 2D,E). Similarly, TGFβ1 increased *ALPL* mRNA expression as well as activity in HAoSMCs. These effects were again significantly inhibited by additional treatment with vasorin (Fig. 2F,G). Taken together, vasorin inhibited osteoinductive signaling and osteo−/chondrogenic transdifferentiation of VSMCs promoted by TGFβ1.

Next, the impact of endogenous vasorin on TGFβ1-induced osteo−/chondrogenic signaling in VSMCs was determined by silencing of the *VASN* gene in HAoSMCs using small interfering RNA (siRNA). As a result, transfection with vasorin siRNA significantly down-regulated *VASN* mRNA expression in HAoSMCs as compared to negative control siRNA transfected cells (Suppl. Fig. 1A). Silencing of vasorin alone significantly up-regulated osteo−/chondrogenic markers *SOX9*, *CBFA1* and *ALPL* mRNA expression in HAoSMCs (Fig. 3A-C). Moreover, TGFβ1-induced *SOX9* and *ALPL* mRNA expression was significantly augmented by silencing of vasorin in HAoSMCs (Fig. 3A,C). Vasorin knockdown tended to augment TGFβ1-induced *CBFA1* mRNA expression (Fig. 3B), a difference, however, not reaching statistical significance ($p = .0975$). Thus, vasorin knockdown promoted osteoinduction and aggravated TGFβ1-induced osteo−/chondrogenic transdifferentiation of VSMCs.

To further explore whether vasorin supplementation exerts similar protective effects on osteo−/chondrogenic transdifferentiation of VSMCs during high phosphate conditions, HAoSMCs were treated with β-glycerophosphate and/or with recombinant human vasorin. As

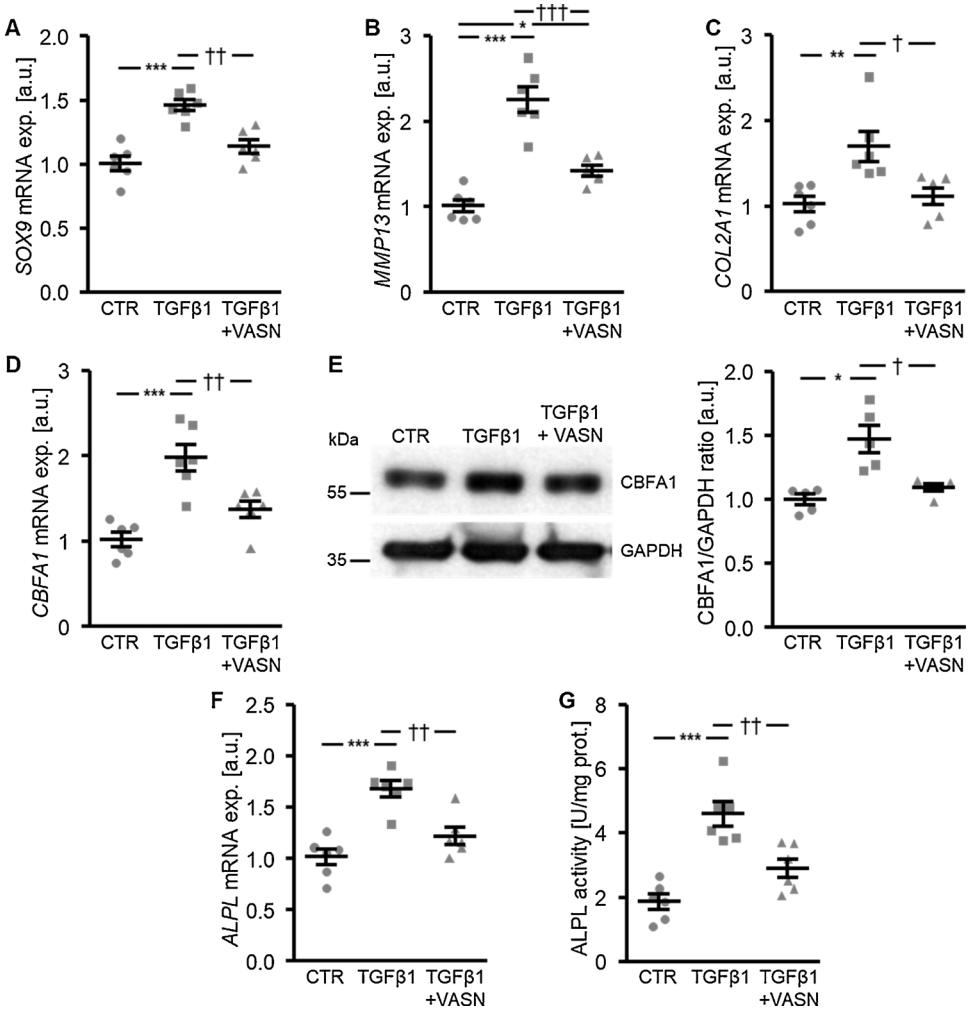

Fig. 2. Vasorin blunts TGFβ1-induced osteo−/chondrogenic transdifferentiation of primary human aortic smooth muscle cells. **A-D.** Scatter dot plots and arithmetic means ± SEM ($n = 6$; arbitrary units, a.u.) of *SOX9* (**A**), *MMP13* (**B**), *COL2A1* (**C**) and *CBFA1* (**D**) relative mRNA expression in HAoSMCs treated with control (CTR) or with 10 ng/ml recombinant human TGFβ1 without or with additional treatment with 100 ng/ml recombinant human vasorin (VASN). **E.** Representative original Western blots and scatter dot plots and arithmetic means ± SEM (n = 5; a.u.) of normalized CBFA1/ GAPDH protein ratio in HAoSMCs treated with control (CTR) or with 10 ng/ml recombinant human TGFβ1 without or with additional treatment with 100 ng/ml recombinant human vasorin (VASN). **F.** Scatter dot plots and arithmetic means ± SEM (n = 6; a.u.) of *ALPL* relative mRNA expression in HAoSMCs treated with control (CTR) or with 10 ng/ml recombinant human TGFβ1 without or with additional treatment with 100 ng/ml recombinant human vasorin (VASN). **G.** Scatter dot plots and arithmetic means ± SEM (n = 6; U/mg protein) of ALPL activity in HAoSMCs treated with control (CTR) or with 10 ng/ml recombinant human TGFβ1 without or with additional treatment with 100 ng/ml recombinant human vasorin (VASN). *($p < .05$), **($p < .01$), ***($p < .001$) significant compared to control HAoSMCs; †($p < .05$), ††($p < .01$), †††($p < .001$) significant compared to HAoSMCs treated with TGFβ1 alone.

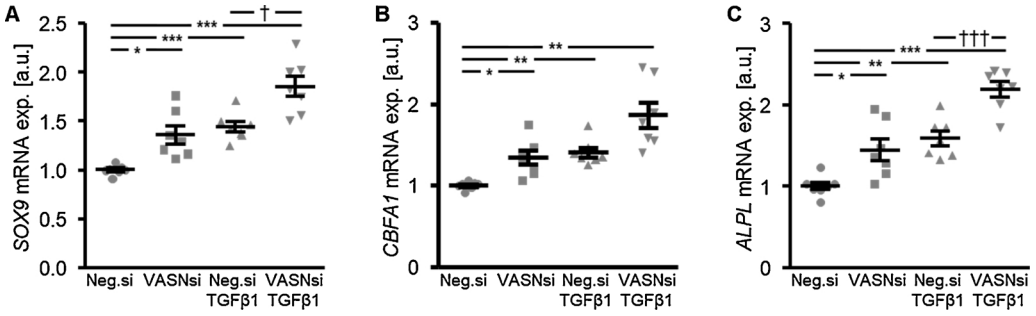

Fig. 3. Silencing of vasorin aggravates TGFβ1-induced osteo−/chondrogenic transdifferentiation of primary human aortic smooth muscle cells. **A-C.** Scatter dot plots and arithmetic means ± SEM (n = 7; arbitrary units, a.u.) of *SOX9* (**A**), *CBFA1* (**B**) and *ALPL* (**C**) relative mRNA expression in HAoSMCs following silencing with negative control siRNA (Neg.si) or vasorin siRNA (VASNsi) and additional treatment with control or with 10 ng/ml recombinant human TGFβ1. *(p < .05), **(p < .01), ***(p < .001) significant compared to Neg.si transfected HAoSMCs; †(p < .05), †††(p < .001) significant compared to Neg.si transfected and TGFβ1 treated HAoSMCs.

illustrated in Fig. 4A, phosphate down-regulated *VASN* mRNA expression in HAoSMCs as compared to control treated HAoSMCs. Addition of exogenous vasorin did not modify this effect. Phosphate treatment increased *TGFB1* mRNA expression in HAoSMCs, an effect not significantly modified by addition of exogenous vasorin (Fig. 4B). Surprisingly, treatment with vasorin alone significantly up-regulated *TGFB1* mRNA expression in HAoSMCs (Fig. 4B), but did not affect the expression of other TGFβ family and BMP ligands such as *TGFB2*, *TGFB3*, *INHBA*, *BMP2*, *BMP4* or *BMP7* (Suppl. Fig. 2). Nonetheless, vasorin decreased phosphate-induced TGFβ pathway activity (Fig. 4C), SMAD2 phosphorylation ($Ser^{465/467}$) (Fig. 4D) as well as *ALK5* and *PAI-1* mRNA expression (Fig. 4E,F). SMAD1 phosphorylation was not significantly modified by vasorin in phosphate treated HAoSMCs (Suppl. Fig. 3). Taken together, these observations suggest that vasorin interfered with TGFβ1-downstream signaling in HAoSMCs during conditions of elevated phosphate levels.

Furthermore, vasorin inhibited phosphate-induced *SOX9*, *CBFA1* and *ALPL* mRNA expression (Fig. 5A-C) and, thus, osteo−/chondrogenic transdifferentiation of HAoSMCs. In accordance, the calcification of HAoSMCs triggered in the presence of calcification medium was significantly blunted by vasorin (Fig. 5D,E). Thus, vasorin supplementation inhibited TGFβ1 signaling and osteo−/chondrogenic transdifferentiation and calcification of HAoSMCs during high phosphate conditions.

Conversely, silencing of vasorin in HAoSMCs significantly augmented phosphate-induced osteo−/chondrogenic markers mRNA

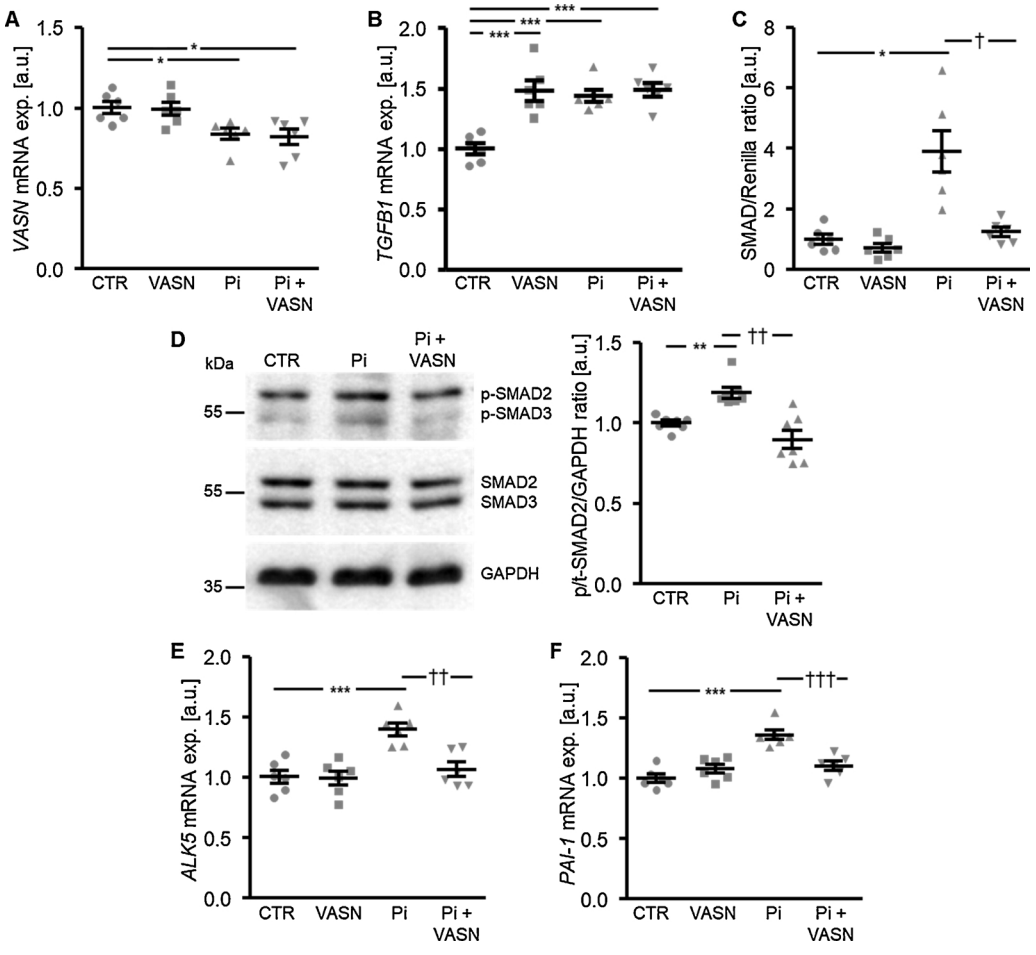

Fig. 4. Vasorin suppresses phosphate-induced TGFβ1-dependent signaling in primary human aortic smooth muscle cells. **A,B.** Scatter dot plots and arithmetic means ± SEM (n = 6; arbitrary units, a.u.) of *VASN* (**A**) and *TGFB1* (**B**) relative mRNA expression in HAoSMCs treated with control (CTR) or 100 ng/ml recombinant human vasorin (VASN) without or with additional treatment with 2 mM β-glycerophosphate (Pi). **C.** Scatter dot plots and arithmetic means ± SEM (n = 6; a.u.) of TGFβ pathway activity measured by luciferase reporter assay in HAoSMCs following transfection with SMAD-responsive luciferase/*Renilla* constructs and treatment with control (CTR) or 100 ng/ml recombinant human vasorin (VASN) without or with additional treatment with 2 mM β-glycerophosphate (Pi). **D.** Representative original Western blots and scatter dot plots and arithmetic means ± SEM (n = 7; a.u.) of normalized phospho-SMAD2 ($Ser^{465/467}$)/ SMAD2/ GAPDH protein ratio in HAoSMCs treated with control (CTR) or with 2 mM β-glycerophosphate (Pi) without or with additional treatment with 100 ng/ml recombinant human vasorin (VASN). **E,F.** Scatter dot plots and arithmetic means ± SEM (n = 6; a.u.) of *ALK5* (**E**) and *PAI-1* (**F**) relative mRNA expression in HAoSMCs treated with control (CTR) or 100 ng/ml recombinant human vasorin (VASN) without or with additional treatment with 2 mM β-glycerophosphate (Pi). *(p < .05), **(p < .01), ***(p < .001) significant compared to control HAoSMCs; †(p < .05), ††(p < .01), †††(p < .001) significant compared to HAoSMCs treated with Pi alone.

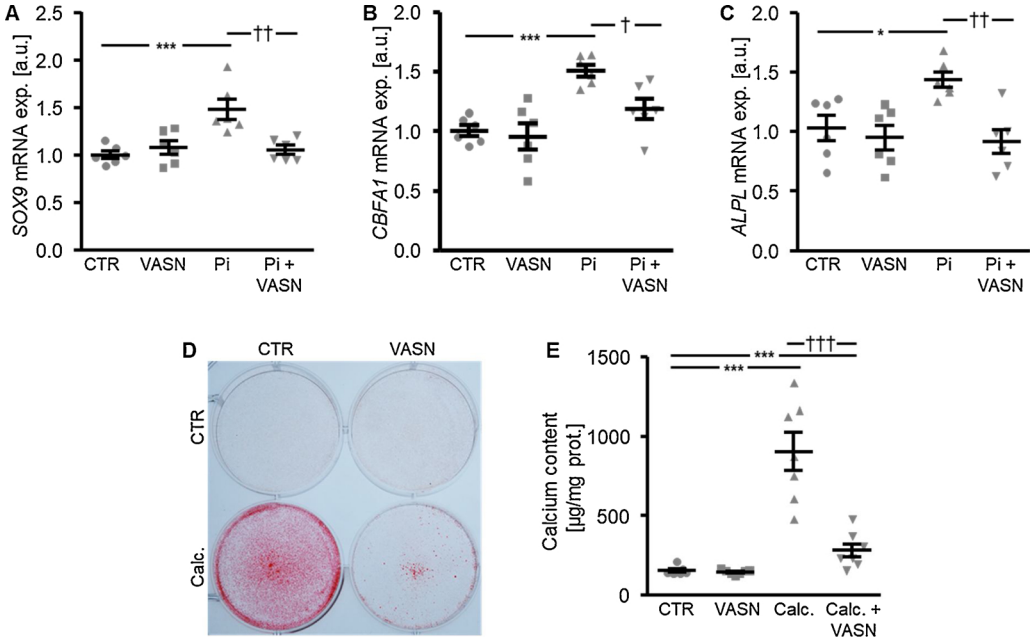

Fig. 5. Vasorin ameliorates osteo−/chondrogenic transdifferentiation and calcification of primary human aortic smooth muscle cells during high phosphate conditions. **A-C.** Scatter dot plots and arithmetic means ± SEM (n = 6; arbitrary units, a.u.) of *SOX9* (**A**), *CBFA1* (**B**) and *ALPL* (**C**) relative mRNA expression in HAoSMCs treated with control (CTR) or 100 ng/ml recombinant human vasorin (VASN) without or with additional treatment with 2 mM β-glycerophosphate (Pi). **D.** Representative original images ($n = 4$) showing Alizarin red staining in HAoSMCs treated with control (CTR) or 100 ng/ml recombinant human vasorin (VASN) without or with additional treatment with calcification medium (Calc.). The calcified areas are shown as red staining. **E.** Scatter dot plots and arithmetic means ± SEM (n = 7; µg/mg protein) of calcium content in HAoSMCs treated with control (CTR) or 100 ng/ml recombinant human vasorin (VASN) without or with additional treatment with calcification medium (Calc.). *(p < .05), ***(p < .001) significant compared to control HAoSMCs; †(p < .05), ††(p < .01), †††(p < .001) significant compared to HAoSMCs treated with Pi/Calc. alone. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expression (Fig. 6A–C, Suppl. Fig. 1B). Thus, vasorin produced by VSMCs participated in the regulation of osteo−/chondrogenic transdifferentiation of VSMCs during hyperphosphatemic conditions.

In an additional experiment, vascular vasorin expression was determined in the hyperphosphatemic klotho-hypomorphic (*kl/kl*) mouse model of CKD-related vascular calcification. As shown in Fig. 7, *Vasn* mRNA expression was significantly lower in the aortic tissue of *kl/kl* mice as compared to wild-type mice.

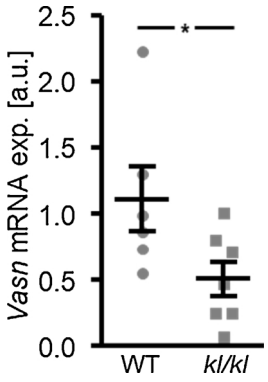

Fig. 7. Aortic vasorin expression is reduced in the hyperphosphatemic klotho-hypomorphic mouse model. Scatter dot plots and arithmetic means ± SEM (n = 6–7; arbitrary units, a.u.) of *Vasn* relative mRNA expression in aortic tissue of klotho-hypomorphic (*kl/kl*) mice and corresponding wild-type (WT) mice. *(p < .05) significant compared to WT mice.

4. Discussion

The present findings identified a novel role of vasorin in controlling osteo−/chondrogenic transdifferentiation of VSMCs and, thus, vascular calcification by regulation of TGFβ1-mediated osteoinductive signaling. Vasorin expression is reduced in VSMCs during pro-calcific conditions with high TGFβ1 or phosphate levels and in vascular tissue of a mouse model of CKD-related vascular calcification. Endogenous vasorin knockdown augments osteo−/chondrogenic transdifferentiation of VSMCs. Conversely, exogenous vasorin supplementation is able to interfere with TGFβ1-induced osteo−/chondrogenic phenotype switch of VSMCs, attenuating VSMC calcification *in vitro*.

Excessive TGFβ1 levels promote osteo−/chondrogenic transdifferentiation of VSMCs [10,12,13,31], a prerequisite for medial vascular tissue mineralization [12,14,18,23,34]. Inhibition of TGFβ1-dependent signaling reduces vascular calcification [10,11,31]. The osteoinductive intracellular pathways triggered by TGFβ1 in VSMCs involve signaling through the TGFβ type I receptor (ALK5) [10,42] and the increase of

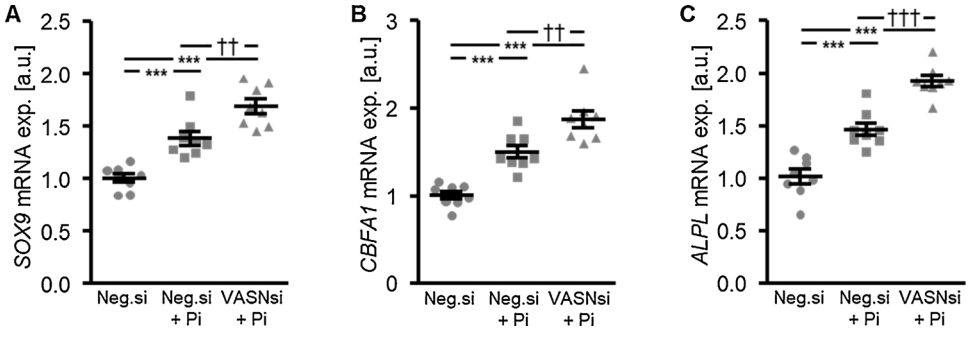

Fig. 6. Vasorin knockdown augments phosphate-induced osteo−/chondrogenic transdifferentiation of primary human aortic smooth muscle cells. **A-C.** Scatter dot plots and arithmetic means ± SEM ($n = 8$; arbitrary units, a.u.) of *SOX9* (**A**), *CBFA1* (**B**) and *ALPL* (**C**) relative mRNA expression in HAoSMCs following silencing with negative control siRNA (Neg.si) or vasorin siRNA (VASNsi) and additional treatment with control or with 2 mM β-glycerophosphate (Pi). ***(p < .001) significant compared to Neg.si transfected HAoSMCs; ††(p < .01), †††(p < .001) significant compared to Neg.si transfected and Pi treated HAoSMCs.

SMAD2 phosphorylation [10]. Others have previously found that vasorin is able to inhibit TGFβ signaling in various cell types [1], including but not limited to VSMCs [1,2,4,6]. Vasorin modulates TGFβ downstream signaling by reducing SMAD2 phosphorylation [2,6]. Moreover, recent studies describe that only the soluble form of vasorin may be able to interfere with TGFβ signaling pathways and to reduce the phosphorylated SMAD2 abundance in response to TGFβ [4,7]. The functions of soluble vasorin are not yet completely elucidated [1].

In accordance with the previous studies, vasorin supplementation of the cell culture medium is able to decrease TGFβ pathway activation and the phosphorylation of SMAD2 in VSMCs following TGFβ1 treatment. Consequently, the expression of TGFβ1 pathway downstream targets is also reduced by vasorin supplementation. In VSMCs, vasorin suppresses the expression of *PAI-1*, a direct TGFβ1 signaling target gene [43–45] and a critical mediator of vascular calcification [10,11,28,31,35]. TGFβ1 - PAI-1 is an important osteoinductive pathway in VSMCs and its inhibition is able to reduce vascular calcification [10,11,31]. Furthermore, the increased expression of chondrogenic and osteogenic transcription factors [12,33,46] in response to high TGFβ1 levels is blunted by vasorin. Similarly, vasorin inhibits the expression and activity of ALPL, a key enzyme regulating vascular calcification [18,33] by promoting the degradation of the calcification inhibitor pyrophosphate, which further allows for calcium phosphate precipitation and deposition in the vascular tissue [18,33,46]. Vasorin supplementation does not affect the increased expression of *TGFβ1* in VSMCs following phosphate treatment [11,31], but inhibits phosphate-induced TGFβ1-dependent osteo−/chondrogenic signaling, findings suggesting that vasorin acts downstream of TGFβ1 expression to reduce osteo−/chondrogenic transdifferentiation of VSMCs during elevated phosphate conditions. Accordingly, vasorin treatment is able to suppress VSMC mineralization *in vitro*. Surprisingly, treatment with vasorin alone up-regulates the mRNA expression of *TGFB1*, but not of other TGFβ family or BMP ligands, in VSMCs. Hypothetically, this increase may be a counterregulation due to inhibition of the TGFβ pathway activity observed in the presence of vasorin. The functional significance and the underlying mechanisms of this regulation require further study. Taken together, the current observations prove for the first time that vasorin interferes with TGFβ1-dependent osteoinduction in VSMCs and, thus, attenuates VSMC calcification during pro-calcific conditions *in vitro*. Additional studies are necessary to confirm the anti-calcific effects of vasorin supplementation in relevant *ex vivo* and animal models of vascular calcification.

The anti-calcific effects of vasorin in VSMCs may potentially involve mechanisms beyond and independent of the regulation of TGFβ1 signaling. A previous study describes that in embryonic fibroblasts, vasorin may translocate into the mitochondrial membrane [3] and protect cells from hypoxia and TNFα-induced mitochondrial dysfunction, oxidative stress and apoptosis [3], mechanisms which are all contributing to initiation and progression of vascular calcification during hyperphosphatemia [35,37,47–49]. In addition, vasorin may modulate VSMC osteo−/chondrogenic transdifferentiation [50] by regulating Notch signaling [1,3].

Vascular vasorin may also play an important role in the cellular response to various pathological stimuli, such as vascular injury, aging and angiotensin II exposure [2,6]. Reduced expression of vasorin may allow for unrestrained pathophysiological vascular remodeling as well as vascular calcification, while vasorin supplementation or up-regulation may limit their development. Further research is required to elucidate the regulation and potential benefits of vasorin.

Excessive TGFβ1 activation appears to be critically involved in the progression of vascular calcification during CKD [10,12,38,51,52] and may contribute to the high mortality of these patients [16–19]. By inhibiting TGFβ1-dependent osteoinductive signaling, vasorin may, thus, have beneficial effects in reducing the progression of vascular calcification in CKD patients, which could potentially translate into cardiovascular and survival benefits. In addition, TGFβ is involved in kidney fibrosis, as hallmark of progressive renal injury [53], abnormal bone remodeling [54], immune dysfunction [55] or uraemic cardiomyopathy [56]. Thus, beyond its effects on vascular calcification, vasorin may play a decisive role as TGFβ1 inhibitor and its supplementation may have an overall protective effect during renal disease progression. Vasorin has already been suggested as a potential biomarker for various nephropathies [1].

5. Conclusions

Vascular vasorin expression is reduced during pro-calcifying conditions. Vasorin suppresses osteo−/chondrogenic transdifferentiation and calcification of VSMCs, effects involving, at least in part, interference with TGFβ1-dependent osteoinductive signaling. Thus, up-regulation or supplementation of vasorin may be beneficial in reducing the progression of vascular calcification during CKD.

Declarations of Competing Interest

None.

Author contributions

J.V. and I.A. designed research; T.T.D.L., M.E., B.B. and I.A. performed experiments; B.P., F.L., K.U.E, J.V. and I.A. analyzed and interpreted data; J.V. and I.A. wrote the manuscript with comments and edits from all authors. All authors have approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.109414.

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