Secreted Frizzled-Related Protein 5 Ameliorates Vascular Calcification in a Rat Model of Chronic Kidney Disease through the Wnt/ β -Catenin Pathway

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Keywords

Vascular calcification · Secreted frizzled-related protein 5 · Wnt/ β -catenin pathway · Chronic kidney disease

Abstract

Introduction: Vascular calcification (VC) is highly prevalent and a major cardiovascular risk factor in chronic kidney disease (CKD) patients. Secreted frizzled-related protein 5 (SFRP5), an inhibitor of the Wnt pathway, is an adipokine with a positive effect on metabolic and cardiovascular diseases. Our previous in vitro study showed that SFRP5 attenuates high phosphate-induced calcification in vascular smooth muscle cells by inhibiting the Wnt/ β -catenin pathway. Therefore, we hypothesized that SFRP5 may protect against CKD-associated VC (CKD-VC) through the same signalling. **Methods:** The rat model of CKD with VC was induced by 0.75% adenine combined with 1.8% high phosphate diet, which were administered with adenovirus vectors of SFRP5. We evaluated the SFRP5 effect on VC by von Kossa staining and calcium content analysis and osteogenic markers by immunohistochemistry and Western blot. The components of Wnt/ β -catenin signalling were also evaluated. **Results:** SFRP5 local and serum levels were significantly decreased in

the CKD-VC rat model compared with the control group. Adenovirus-mediated overexpression of SFRP5 significantly inhibited VC, which was due to suppression of CKD-induced expression of calcification and osteoblastic markers. Additionally, SFRP5 abrogated activation of the Wnt/ β -catenin pathway that plays a major role in the pathogenesis of VC. The specificity of SFRP5 for inhibition of VC was confirmed using an empty adenovirus as a control. **Conclusion:** Our results suggest that SFRP5 ameliorates VC of CKD rats by inhibiting the expression of calcification and osteoblastic markers as well as the Wnt/ β -catenin pathway. Collectively, this study suggests that SFRP5 is a potential therapeutic target in CKD-VC.

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Introduction

Chronic kidney disease (CKD) has become a public health problem worldwide and poses a serious threat to human health. CKD mineral and bone disorder is a common complication that contributes to the progression of vascular calcification (VC) [1, 2]. VC is an independent

risk factor that substantially contributes to cardiovascular events and increases the mortality of CKD patients [3, 4]. In addition to kidney damage, vascular stiffness caused by CKD-associated VC (CKD-VC) also leads to escalated cardiovascular mortality. In these patients, both intimal and medial calcification occurs, but medial arterial calcification is the most common. Although passive deposition of hydroxyapatites is considered the main pathological process of VC, recent studies have reported VC to be a complex process with involvement of various molecular pathways, which includes active transformation of vascular smooth muscle cells (VSMCs) into osteoblast-like cells [4, 5]. However, the molecular mechanism of CKD-VC has not been fully elucidated.

Secreted frizzled-related protein 5 (SFRP5) is an anti-inflammatory secreted adipokine that belongs to the SFRP family [6]. SFRPs are inhibitors of the Wnt pathway, and SFRP5 inhibits either canonical or non-canonical signalling, which depends on the cell type. SFRP5 is a beneficial adipokine with protective effects on some cardiovascular diseases including VC [7–9]. Expression of SFRP5 in VSMCs and cardiomyocytes has also been demonstrated [10, 11]. Our previous *in vitro* study showed that SFRP5 interferes with the canonical Wnt pathway and mitigates high phosphate-induced VC [12]. A recent study found that serum SFRP5 levels were low in CKD patients with VC [13]. Therefore, SFRP5 might have a protective effect against CKD-VC. Because SFRP5 is an inhibitor of the Wnt/ β -catenin pathway in VSMCs, we hypothesized that SFRP5 may protect against CKD-VC through the Wnt/ β -catenin axis. To test our hypothesis, we established a CKD rat model to mimic clinical CKD-VC *in vivo*.

Materials and Methods

Animal Model

Eight-week-old male Sprague Dawley rats (250–300 g) were obtained from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (Beijing, China). Animals were housed in a climate-controlled room with a 12-h light/dark cycle and allowed free access to food and water. The animals were randomly assigned to 4 groups: (1) adenine– SFRP5–: normal diet + saline, $n = 10$; (2) adenine+ SFRP5–: adenine diet + saline, $n = 10$; (3) adenine+ Ad-SFRP5+: adenine diet + adenovirus-SFRP5, $n = 10$; (4) adenine+ Ad-GFP+: adenine diet + control adenovirus, $n = 10$. Standard chow (1.0% calcium [Ca] and 1.2% phosphorus [P]) was fed to control rats, and adenine-rich chow (0.75% adenine, 1.0% Ca, and 1.8% P; KEAOXIELI FEED Co., LTD., Beijing, China) was fed to rats for 6 weeks to induce CKD and VC. Then, rats were switched to a normal diet until 8 weeks. At the beginning of the experiment, the rats were administered the following treat-

Table 1. Primers used for quantitative RT-PCR analyses

Primer	Sequence
SFRP5	F 5'-ACCTTGAATGCCGTGAA-3' R 5'-AATCCAGTTGGTGAGCC-3'
c-Myc	F 5'-CTACTTGGAGGAGACATGGTG-3' R 5'-TGGAGGTGGAGCAGACG-3'
Cyclin D1	F 5'-AGTAGCAGCGAGCAGCAGAGT-3' R 5'-TTCATCTTAGAGGCCACGAA-3'
GAPDH	R 5'-TGGAGGTGGAGCAGACG-3' F 5'-GAGGCTCTCTCCAGCCTTC-3'

ments. Groups 1 and 2 were treated by tail vein injection of saline. Rats in group 3 received a tail vein injection of 2×10^{11} plaque-forming units adenovirus-SFRP5 (National Human Genome Northern Research Center, Beijing, China). Group 4 received a tail vein injection of 2×10^{11} plaque-forming units control adenovirus. One week later, the injections were repeated. The rats were sacrificed at 8 weeks, and blood samples and aortic tissues were collected for various analyses. All animal procedures were carried out in accordance with local institutional and governmental regulations on the use of experimental animals.

Biochemical Parameters

Venous blood samples were collected in EDTA/acetic acid-containing tubes and centrifuged at 3,000 g for 10 min at 4°C. Serum levels of urea nitrogen (BUN), creatinine (Cr), Ca, P, and albumin were analysed using a Hitachi 7170 Autoanalyzer (Hitachi, Tokyo, Japan). The serum level of SFRP5 in rats was evaluated by using an SFRP5 micro-plate ELISA kit (Cloud Clone Corp, Wuhan, China) in accordance with the manufacturer's instructions.

Quantification of Calcification

To quantitatively evaluate the degree of VC, frozen aortic tissue was weighed and hydrolysed in 1 mL of 6 mol/L hydrochloric acid for 24 h. The Ca content of the supernatant was determined using a commercially available kit (QuantiChrom™ Calcium Assay Kit; Bioassay System, Hayward, CA, USA) and normalized to the wet tissue weight ($\mu\text{g}/\text{mg}$ wet tissue weight). Additionally, aortic tissues were fixed with 4% paraformaldehyde, embedded in paraffin, cut into 3- μm -thick sections, and stained for calcification using a von Kossa staining kit (GenMed, Shanghai, China) in accordance with the manufacturer's instructions. Dark brown staining indicated areas of calcification in vessel walls. Calcification was evaluated semiquantitatively by the calcification score (CS) using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The percentage of the calcified area was calculated as the von Kossa-positive area normalized to the total tissue area, which was measured in 10 representative areas and expressed as the mean value.

Immunohistochemistry

Paraffin-embedded sections of aortic tissues were incubated with primary antibodies against SFRP5 (1:100, ab230425; Abcam) and Runx2 (1:100, ab76956; Abcam) overnight at 4°C. Staining was performed using an ABC ELITE kit (Vector Laboratories, Burlingame, CA, USA) including biotinylated secondary antibodies in accordance with the manufacturer's instructions. Sections stained

Table 2. Physiological and biochemical parameters of rats

	Adenine– Ad-SFRP5– (n = 10)		Adenine+ Ad-SFRP5– (n = 9)		Adenine+ Ad-SFRP5+ (n = 9)		Adenine+ Ad-GFP+ (n = 9)	
	baseline	week 8	baseline	week 8	baseline	week 8	baseline	week 8
Body weight, g	407.5±23.8	491.9±33.1	404.9±20.4	252.0±23.6*	405.8±19.7	237.7±17.5	404.6±18.9	232.2±10.6
Food intake, g/kg/d	6.1±0.6	5.8±0.6	6.4±1.0	2.6±0.5*	5.5±0.4	2.6±0.4	5.9±0.8	2.5±0.5
Blood urea nitrogen, mmol/L	4.68±0.56	5.47±0.92	5.23±0.93	73.33±12.92*	5.52±0.92	72.84±20.20*	5.53±1.26	72.75±9.84*
Serum creatinine, µmol/L	35.3±1.6	43.2±4.8	36.1±2.5	339.4±40.4*	33.6±4.6	311.9±35.7*	33.5±3.9	305.2±23.0*
Serum calcium, mmol/L	2.50±0.09	2.29±0.13	2.50±0.04	1.96±0.39*	2.46±0.16	2.10±0.34	2.36±0.07	2.07±0.25
Serum phosphate, mmol/L	3.22±0.13	2.83±0.35	3.21±0.16	4.91±1.10*	2.97±0.29	4.93±0.81*	2.97±0.14	4.96±0.77*
Serum albumin, g/L	42.5±1.1	39.0±2.7	43.6±2.4	30.4±1.0*	44.4±2.2	28.6±2.5*	43.4±1.1	28.4±2.9*

Data are mean ± SD. * $p < 0.05$ (vs. baseline).

with secondary antibodies alone were confirmed to be negative. Image acquisition was performed using the analysis system described previously [14].

Western Blot Analysis

Preparation of thoracic aorta tissue homogenates and immunoblotting were performed as described previously [12, 14]. Primary antibodies and dilutions were as follows: anti-GAPDH (1:5000, ab181602; Abcam), anti-BMP-2 (1:1000, ab214821; Abcam), and anti-β-catenin (1:1000, ab6301; Abcam).

Quantitative Real-Time PCR

RNA isolation and quantitative real-time PCR (RT-PCR) were performed as described previously [14]. The primers used for PCR amplification are shown in Table 1. Quantitative RT-PCR was performed in duplicate with Power SYBR Green PCR Master Mix (Applied Biosystems) on an ABI 7500 sequence detection system in accordance with the manufacturer's protocol. The mRNA levels of target genes were calculated after normalization to glyceraldehyde-3-phosphate dehydrogenase mRNA.

Statistical Analysis

Results are expressed as mean ± standard error of the mean. Differences between control and experimental groups were determined by one-way ANOVA and the Student-Newman-Keuls test for post hoc comparisons. All statistical analyses were performed using SPSS software (version 20.0). $p < 0.05$ was considered statistically significant.

Results

Changes in Body Weight, Biochemical Analysis, and Serum SFRP5 Level

During the study, untreated rats continued to gain weight, whereas rats that received the adenine diet consumed less food and lost body weight (Table 2). Animals fed adenine had dry hair and decreased activity. At the end of week 8, 1 rat had died in each adenine-fed group, except for the normal diet group.

As shown in Table 2, serum BUN and Cr concentrations at week 8 were increased significantly ($p < 0.05$), complicated by hypocalcaemia and hyperphosphataemia ($p < 0.05$) in adenine-fed rats compared with control rats. Additionally, an obvious decrease of SFRP5 was observed in adenine-fed rats (10.92 ± 3.56 vs. 21.33 ± 3.33 ng/mL, $p < 0.01$).

Serum and Local Levels of SFRP5 in Rats by Different Treatments

Because SFRP5 blocks VSMC calcification induced by high phosphate in vitro [12], we tested its therapeutic efficacy to mitigate VC in vivo. To investigate the relationship between SFRP5 and VC in adenine-fed CKD rats, we used an adenovirus to increase expression of SFRP5 as described in the Materials and Methods section. Adenovirus-mediated overexpression of SFRP5 increased the serum concentration of SFRP5 in CKD rats (18.22 ± 4.46 ng/mL), but there was no significant difference in the Ad-GFP group (10.85 ± 1.70 ng/mL) compared with the VC model group (10.92 ± 3.56 ng/mL) (Fig. 1a). The mRNA content of SFRP5 in white adipose tissue was higher than that in aortic tissue (4.51 ± 0.19 vs. 0.73 ± 0.01 , $p < 0.01$). With CKD aggravation, mRNA expression of SFRP5 was decreased. Ad-SFRP5 recovered the SFRP5 mRNA expression, especially in adipose tissue (4.13 ± 0.63) (Fig. 1b). The expression of SFRP5 was localized in both endothelial and smooth muscle cells of vascular. The level in CKD rat aortas was lower than that in untreated rats, while Ad-SFRP5 could recover the SFRP5 expression as well (Fig. 1c). These results suggest that adenovirus-mediated overexpression of SFRP5 improves its level in both aortas and circulation.

Fig. 1. The expression of SFRP5 in adenine-induced CKD rats and the change after adenovirus overexpression of SFRP5. **a** The concentration of SFRP5 in serum of rats tested on days 0, 28, and 56. With the prolongation of CKD, the concentration of SFRP5 decreased gradually, which was significantly lower in the CKD-VC group compared with the control group. The serum level of SFRP5 could be reversed by adenovirus overexpression of SFRP5 (Ad-SFRP5) in CKD rats, while there was no increase in the empty adenovirus (Ad-GFP) group. **b** The levels of SFRP5 mRNA in adipose tissue and aortic tissue on day 28. The SFRP5 mRNA level of white adipose tissue was higher than that of aortic tissue. With the

aggravation of CKD, the expression of SFRP5 mRNA decreased. Ad-SFRP5 could recover the SFRP5 mRNA especially in adipose tissue. **c** SFRP5 expression in rat aortas. Immunohistochemical staining ($\times 400$) of SFRP5 in CKD rat aortas decreased compared to the control group. But, Ad-SFRP5 reversed the expression. Data are expressed as mean $\pm$ SEM from 9 samples per group. **a** $^{\#}p < 0.05$ compared with the control group, $^{*}p < 0.05$ compared with the Ad-GFP group. **b** $^{*}p < 0.05$ compared with the individual controls. SFRP5, secreted frizzled-related protein 5; CKD, chronic kidney disease; VC, vascular calcification.

Changes of VC in Rats by Different Treatments and the Protecting Effect of SFRP5

Figure 2 shows semiquantitative analysis of the aorta stained by the von Kossa method, which revealed the prevalence and degree of VC. In rats fed with adenine at week 8, VC was seen in the media layer of the aorta, whereas the calcification lesion was significantly attenuated after treatment with Ad-SFRP5. Staining was negative in untreated rats (Fig. 2a). To observe the degree of

VC, the CS was calculated in accordance with the percentage of the calcified area in the vascular ring area (Fig. 2b; Table 3). The results also revealed that CS had declined in the Ad-SFRP5-treated group (4.26 ± 1.07 vs. 7.88 ± 0.74 , $p < 0.05$) (Fig. 2c). Consistent with the von Kossa staining results, the aortic calcium content in Ad-SFRP5-treated rats at week 8 was lower than that in model and Ad-GFP-treated rats (4.01 ± 0.41 vs. 7.90 ± 0.31 and 7.21 ± 0.43 , $p < 0.05$) (Fig. 2d).

Fig. 2. SFRP5 attenuates aortic calcification in adenine-fed rats at week 8. **a** Photomicrographs (×200) of the aorta stained by von Kossa staining in each group. Calcification is located in the media of the aorta. Representative micrographs showing that SFRP5 ameliorated VC in CKD. **b** Representative photomicrographs of the abdominal aorta stained using the von Kossa method and semiquantification of the degree of VC. No calcification: calcified area is absent; mild calcification: calcified area covers 1–50% of the

aortic ring; and severe calcification: calcified area covers 51–100% of the aortic ring (original magnification, ×40). **c** The semiquantitative prevalence and degree of VC showed by calcification score. **d** The effect of SFRP5 on quantification of Ca content in the aorta. Bars are the mean ± SEM of 9 samples per group. * $p < 0.05$ compared with the individual controls. SFRP5, secreted frizzled-related protein 5; CKD, chronic kidney disease; VC, vascular calcification.

Changes of Expression of Osteogenic Markers in the Aorta of Rats and the Inhibiting Effect of SFRP5

Figure 3 shows representative images of immunohistochemical staining and Western blot analysis of osteogenic markers Runx2 and BMP-2 in the aorta at week 8. In the group with intense calcification (model group), substantial expression of Runx2 (Fig. 3a) and BMP-2 (Fig. 3b) was observed in the aortic media. As expected, SFRP5 attenuated the induction of their expression (BMP-2, 1.81 ± 0.19 vs. 2.96 ± 0.25 , $p < 0.05$, Fig. 3b).

Inhibition of the Wnt/ β -Catenin Pathway by SFRP5 against VC

Our previous studies have shown that the protective effect of SFRP5 against calcification induced by high phosphate is mediated through inhibition of the canonical Wnt/ β -catenin signalling pathway. In vivo experiments showed that SFRP5 suppressed β -catenin upregulation in calcification of the aorta in CKD rats (1.78 ± 0.33 vs. 3.19 ± 0.31 , $p < 0.05$, Fig. 4a). Therefore, we determined whether SFRP5 also influenced downstream target genes of the Wnt/ β -catenin signalling pathway in vivo. As

Fig. 3. SFRP5 inhibits the expression of osteogenic markers Runx2 and BMP-2 in the aorta of adenine-fed rats at week 8. **a** Representative photomicrographs ($\times 400$) of immunohistochemistry for Runx2. **b** Western blotting demonstrated that SFRP5 reversed the increased expression of BMP-2. Bars are the mean $\pm$ SEM of 9 samples per group. $*p < 0.05$ compared with the individual controls. SFRP5, secreted frizzled-related protein 5.

expected, quantitative RT-PCR revealed that increases in c-Myc and cyclin D1 expression in adenine-fed rats were reversed by SFRP5 (c-Myc, 1.08 ± 0.45 vs. 1.98 ± 0.23 , $p < 0.05$, and cyclin D1, 1.32 ± 0.50 vs. 2.01 ± 0.24 , $p < 0.05$, Fig. 4b).

Discussion

In this in vivo study, we established a rat model of VC in CKD induced by a diet with 0.75% adenine and 1.8% phosphorus, which is a common model of chronic renal failure. Previous studies have shown that serum BUN and Cr begin to increase after 2 weeks of adenine administration, and renal failure at 4 weeks cannot be reversed [15]. The animal model of chronic renal failure can be complicated with CKD mineral and bone disorder, that is, low calcium, high phosphorus, and high PTH. The middle layer of the aorta and coronary artery can be calcified at various degrees. Therefore, this model is widely used to study VC of CKD [16]. In this study, compared with the normal diet, serum BUN and Cr in the adenine diet group were increased significantly, and there was low calcium, high phosphorus, and aortic calcification. In the group

Table 3. Quantitative score of aortic calcification

Calcification area/aortic ring area, S %	Calcification score
$0 < S \leq 10$	1
$10 < S \leq 20$	2
$20 < S \leq 30$	3
$30 < S \leq 40$	4
$40 < S \leq 50$	5
$50 < S \leq 60$	6
$60 < S \leq 70$	7
$70 < S \leq 80$	8
$80 < S \leq 90$	9
$90 < S \leq 100$	10

complicated with VC, the aortic histomorphology and calcium content showed that calcification was significant and that the osteogenic trans-differentiation index was high in the VC model group.

Many recent studies have shown that Wnt signalling plays a major role in the progression of cardiovascular disease (CVD) [17], and SFRP5 acts primarily by inhibiting the Wnt signalling pathway, which suppresses the development of diseases [18]. Numerous studies have sup-

Fig. 4. SFRP5 inhibited the activated Wnt/β-catenin pathway in CKD-associated VC. **a** Western blotting revealed that the expression of β-catenin significantly increased in the CKD with VC group compared with the control group, which could be inhibited by SFRP5 overexpression. **b** The mRNA levels of β-catenin nucle-

ar-transcriptional target genes c-Myc and cyclin D1 were consistent with the change of β-catenin. Bars are the mean ± SEM of 9 samples per group. **p* < 0.05 compared with the individual controls. SFRP5, secreted frizzled-related protein 5; CKD, chronic kidney disease; VC, vascular calcification.

ported the protective effect of SFRP5 in the pathophysiology of CVD including VC [7–9]. VC is often detected in patients with CKD, which is highly correlated with its cardiovascular morbidity and mortality. Moreover, the serum concentration of SFRP5 is significantly lower in patients on haemodialysis with VC than in those without VC [13]. Our previous in vitro study showed that SFRP5 interferes with the canonical Wnt pathway and mitigates high phosphate-induced VC [12]. These findings suggest a suppressive effect of SFRP5 on CKD-VC. The effect of SFRP5 in vivo is relatively unexplored, but several recent studies have suggested that this may be an interesting area to investigate [11, 13]. Therefore, we investigated the role of SFRP5 through a series of in vivo experiments with a model of VC in adenine-induced CKD rats. The results showed that SFRP5 prevented CKD-VC and osteogenic differentiation, which was mediated by inhibition of the Wnt/β-catenin signalling pathway.

In the rat model of CKD-VC, we found that the serum concentration of SFRP5 was decreased significantly. Because Ouchi et al. [6] found that SFRP5 is highly expressed in adipose tissue, SFRP5 is considered to be secreted mainly from adipocytes. We measured the expression of SFRP5 in both white adipose and aortic tissues.

The mRNA content of SFRP5 in adipose tissue was higher than that in aortic tissue, which is consistent with previous studies [6, 19]. Initially, SFRP5 was believed to be a secreted protein with autocrine or paracrine functions. Thus, we hypothesized that SFRP5 secreted primarily from adipocytes is released into systemic circulation. Serum SFRP5 levels are inversely correlated with inflammatory cytokine levels [20, 21], whereas decreased local production of SFRP5 in the CKD environment may also contribute to reduction of the serum SFRP5 level. Although the effect of SFRP5 on VC is relatively unexplored, our study suggests that SFRP5 expression changes within calcified vascular tissues, so that not only changes in serum levels of SFRP5, but also paracrine effects in or around vascular tissues are affected.

To investigate whether the decrease of SFRP5 was related to the occurrence of CKD-related VC, we used an adenovirus to overexpress SFRP5 and increase the level of SFRP5 in serum as well as adipose and aortic tissues. The results demonstrated that SFRP5 prevented CKD-VC. Von Kossa staining and calcium content analysis showed an increase in aortic calcification in CKD rats compared with controls. Moreover, SFRP5 treatment attenuated VC, and we confirmed the specificity of this ef-

fect compared with the empty adenovirus (Ad-GFP), which suggested that SFRP5 suppresses CKD-VC by inhibiting calcium deposition. VC is a multifaceted active, cell-mediated process including transformation of VSMCs to osteoblast-like cells. BMP-2, a member of the transforming growth factor- β superfamily, is expressed and secreted from various cell types, including VSMCs, endothelial cells, and osteoblasts, which promotes calcification of adjacent cells in a paracrine manner [22]. BMP-2 induces VC and accelerates phosphate uptake [23], and its downstream effects are exerted by upregulating Runx2. Runx2 is a transcription factor that controls the expression of calcification and other osteoblastic markers. In our model, calcification was associated with upregulation of osteogenic factors as reported previously [24]. We found that CKD increased the expression of these molecules in VC and that this effect was specifically reduced by SFRP5 treatment, which indicated that the attenuation of VC by SFRP5 occurs through inhibition of the osteogenic programme.

Next, we explored the related signalling pathways. The canonical Wnt/ β -catenin pathway, a critical mediator of osteogenic differentiation, is activated during the development of CKD-VC in vivo and in vitro [25, 26]. In the present study, we examined whether the Wnt/ β -catenin pathway was regulated by SFRP5 in vivo. Previously, we found activation of β -catenin in the CKD-VC model. Furthermore, activation of β -catenin-dependent signalling was demonstrated by enhanced expression of its transcriptional target genes *c-Myc* and *cyclin D1*. Conversely, the preventive effect of SFRP5 was associated with inhibition of β -catenin signalling. Thus, all the aforementioned changes elicited in VC were counteracted by co-treatment with SFRP5. Although we did not explore the influence of the non-canonical pathway on inhibition of VC in CKD by SFRP5, it is noteworthy that the specificity of the inhibiting effect of SFRP5 on the Wnt/ β -catenin pathway was demonstrated using Ad-GFP versus Ad-SFRP5. Therefore, on the basis of our results, it can be concluded that upregulation of the canonical Wnt/ β -catenin pathway is involved in CKD-VC, whereas downregulation of this pathway is related to attenuation of calcification elicited by SFRP5. This is coincident with inhibition of Wnt/ β -catenin signalling observed with the prevention of calcium deposition by potentially anti-calcifying molecules such as ginsenoside Rb1 in a similar animal model [26].

The inhibiting effects of SFRP5 are mostly involved in non-canonical Wnt signalling by antagonizing Wnt5a in

metabolic syndrome-related diseases [27, 28]. The results of this study highlight a novel protective effect of SFRP5 against VC of CKD through inhibition of the canonical Wnt/ β -catenin pathway. The cellular and signalling actions of SFRP5 appear to depend on the type of tissue as well as its inflammatory and metabolic states [29]. A previous study has suggested the Wnt β -catenin pathway promotes retinal vascularization and is accompanied by enhancement of β -catenin expression in the nucleus. A target gene of Wnt encodes angiogenic factor VEGF, which further validates the role of this pathway in the vascular system. Another study has reported that SFRP5 may inhibit VSMC proliferation, migration, and inflammation by suppressing Wnt/ β -catenin [11]. SFRP5 also confers protection against oxidative stress-induced apoptosis through inhibition of β -catenin activation in human aortic endothelial cells [30].

Both plasma SFRP5 levels and SFRP5 expression in rat aortas increase with age via negative feedback and might play a protective role in vascular ageing. However, another recent study showed reduction of SFRP5 protein in the aorta of an animal model of adenine-induced CKD with VC, which was associated with activation of the non-canonical Wnt pathway [13]. Additionally, SFRP5 has an inhibitory effect on VSMC trans-differentiation into osteoblast-like cells, which is also involved in regulation of the Wnt3a-mediated non-canonical signalling pathway through inhibition of Rho/ROCK/JNK signalling. However, they also found β -catenin expression was also increased in CKD rats with VC. Unfortunately, this study did not further examine the effect of SFRP5 treatment on VC and the Wnt signalling pathway in animal models. This discrepancy may be partly due to the different methods to establish the model used in the 2 studies, among which high phosphorus is an important factor to induce VC in CKD. Additionally, the complex crosstalk between Wnt/ β -catenin and non-canonical signalling pathways should be a focus. Therefore, further studies are required to better understand the crosstalk between these complicated signalling pathways in cells. Thus, in subsequent research, we will aim to determine how SFRP5 influences non-canonical Wnt signalling and conduct a more detailed analysis of the pathway components.

Conclusion

In summary, our results have shown that SFRP5 attenuates VC in an animal model of adenine-induced CKD and that this effect correlates with suppressed ex-

pression of calcification and osteoblast markers (BMP-2 and Runx2) through inhibition of the Wnt/ β -catenin signalling pathway. The present study may facilitate elucidation of the role of SFRP5 in the pathophysiology of VC in CKD, which might be a therapeutic target for prevention or treatment of CKD-VC.

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Statement of Ethics

The experimental protocol was established according to the ethical guidelines of experimental animals and was approved by the Animal Ethics Committee of Beijing Friendship Hospital (No. YYYYDWSY191025).

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Dai Deng, Zongli Diao, and Wenhui Liu designed the experiment. Dr. Deng carried out and was a major contributor in manuscript writing. Xue Han performed the histological examination of the vascular. All authors read and approved the final manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its supplementary material files. Further enquiries can be directed to the corresponding author.

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