Protective effects of irisin as a myokine on vascular calcification in chronic kidney disease model

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I'd like to dedicate this paper to all of you with all of my heart.
Thanks everyone.

Seung Jun Kim

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Protective effects of irisin as a myokine on vascular calcification in chronic kidney disease model

Background: Vascular calcification is the prevalent complication and an independent predictor of cardiovascular mortality in chronic kidney disease (CKD) patients. The pathogenesis of vascular calcification in CKD is a complex process involving active cell-mediated mechanisms, particularly the transformation of vascular smooth muscle cells (VSMCs) into osteoblast-like cells. The renin-angiotensin system (RAS) plays a significant role in the pathogenesis of osteogenic transdifferentiation and vascular calcification in CKD, with angiotensin II implicated in its development and progression. Meanwhile, CKD often leads to progressive sarcopenia due to declining renal function, which may further increase cardiovascular risk in this population. The prevention and treatment of muscle wasting in CKD patients should be considered as an important target. Irisin is an exercise-induced myokine derived from its precursor protein, fibronectin type III domain-containing protein 5 (FNDC5). Studies have shown an inverse relationship between serum irisin levels and cardiovascular mortality, suggesting a potential protective role for irisin in vascular health. Irisin may play a critical role in preserving the functional integrity of the vascular endothelium and preventing vascular calcification. Therefore, the present study is aimed to elucidate the potential therapeutic benefits of irisin in mitigating vascular calcification in CKD.

Methods: In vitro study, VSMCs were subjected to treatment with or without angiotensin II. The expression levels of osteogenic genes and proteins, including Runx2, BMP2, and OPN, were assessed using real-time PCR and Western blot analysis. Calcium deposition was evaluated using alizarin red staining. VSMCs were cultured with or without angiotensin II, recombinant irisin, and siFndc5 to investigate the effects of irisin on osteogenic transdifferentiation and calcium deposition. CKD mice model was induced by 0.2% adenine diet for up to 6 weeks. Aortic sections were stained with von Kossa to assess calcium deposition. The expression levels of osteogenic genes and proteins

were assessed in control and CKD mice. For ex vivo experiments, aortas of CKD mice were harvested and divided into control group (n=4) and calcification induction group (n=16). The aortas of calcification induction group were cultured in calcification induction media for 14 days with or without angiotensin II and recombinant irisin to identify the protective effects of irisin to vascular calcification. Calcium deposition and indicators of osteogenic transdifferentiation were analyzed in aortas of mice.

Result: In VSMCs, exposure to angiotensin II led to increased expression of osteogenic genes and proteins, such as Runx2, BMP2, and OPN, as well as enhanced calcium deposition. These changes were significantly mitigated by treatment with recombinant irisin. Additionally, knocking down FNDC5 as a precursor of irisin exacerbated osteogenic transdifferentiation and calcium deposition in VSMCs. In vivo, CKD mice exhibited significantly higher levels of calcium deposition in their aortas compared to control mice. The increased expression of osteogenic markers and the decreased expression of FNDC5 were observed in the aortas of CKD mice. Ex vivo, aortic calcium contents were elevated in calcification induction media with angiotensin II and were alleviated by the presence of recombinant irisin. The exposure to calcification media containing angiotensin II led to an elevation in the levels of osteogenesis-related genes and proteins, however, treatment with recombinant irisin significantly improved these indicators.

Conclusion: Present study demonstrated that irisin treatment alleviated calcification and osteogenic transdifferentiation in VSMCs in vitro and ex vivo experiments with separated aortas from CKD mice. These data suggest that irisin could serve as a potential therapeutic strategy for vascular calcification in CKD.

Key words : irisin, vascular calcification, chronic kidney disease

I. INTRODUCTION

Cardiovascular disease remains the main cause of morbidity and mortality in patients with chronic kidney disease (CKD).[1,2] Vascular calcification is the prevalent complication and an independent predictor of cardiovascular mortality in CKD patients.[3,4] Pathogenesis of vascular calcification in CKD is an active cell-mediated process, including transformation of vascular smooth muscle cells (VSMCs) to osteoblast-like cells, increased uptake of calcium and phosphate by the VSMCs, apoptotic cell death, release of VSMC-derived vesicles, and deficiency of calcification inhibitors.[5,6] Upregulation of osteogenic transcription factors, including cbfa1 (core-binding factor 1α)/runt-related transcription factor 2 (Runx2), MSX-2 and bone morphogenetic protein 2 (BMP-2) contributes to the differentiation of VSMCs into an osteoblastic phenotype. Moreover, another mechanism contributing to vascular mineralization is loss of calcification inhibitors, such as fetuin-A, matrix Gla protein, pyrophosphate and osteopontin (OPN).[7] Vascular calcification in CKD is associated not only with traditional cardiovascular risk factors like advanced age, hypertension, diabetes, and dyslipidemia but also with nontraditional risk factors including activation of the renin-angiotensin system (RAS), imbalanced calcium/phosphorus metabolism, uremic toxins, and oxidative stress. In particular, the RAS plays a significant role in the pathogenesis of vascular calcification in CKD, with angiotensin II implicated in its development and progression.[8,9] While some studies have demonstrated the contribution of angiotensin II to vascular calcification, others have failed to find reductions in vascular calcification with RAS inhibitors.[10-12] This inconsistency emphasizes the complexity of the mechanisms involved in vascular calcification and the need for further research to elucidate the exact contribution of the RAS. Furthermore, other effective therapeutic approaches beyond the RAS to attenuate the progression of vascular calcification in CKD remain insufficient. There is a need for further research to better understand the underlying mechanisms and to develop novel therapeutic strategies to target vascular calcification and reduce cardiovascular risk in CKD patients.

The progressive loss of muscle mass, known as sarcopenia, is a common complication of CKD attributed to declining renal function.[13] Sarcopenia not only affects physical function but also contributes to increased cardiovascular risk in CKD patients.[14] As such, preventing and treating muscle wasting in CKD is essential for improving patient outcomes. Exercise is a potential

intervention for managing sarcopenia in CKD patients.[15] However, the evidence regarding the benefits of exercise in CKD, particularly its effects on cardiovascular outcomes and vascular calcification, has been inconsistent in previous studies.[16-19] Some studies have shown that exercise, such as wheel running, can improve physical function and cardiovascular outcomes in CKD rodent models.[20,21] On the other hand, other studies have not consistently demonstrated beneficial cardiovascular effects of exercise in similar CKD rodent models.[22] Therefore, further research is needed to elucidate the potential benefits of exercise for vascular calcification in CKD. Ultimately, a better understanding of the role of exercise in managing sarcopenia and mitigating cardiovascular risk in CKD patients can inform the development of effective therapeutic strategies to improve patient outcomes.

The discovery of irisin, an exercise-induced myokine, has garnered significant interest due to its potential role in cardiovascular disease. Irisin is cleaved from its precursor protein, fibronectin type III domain-containing protein 5 (FNDC5), and secreted into circulation in response to exercise.[23] Several studies have reported an inverse relationship between serum irisin levels and cardiovascular mortality, suggesting a protective effect of irisin on cardiovascular disease.[4,24-26] Some previous researches have also demonstrated various cardio-protective effects of irisin, including its ability to ameliorate angiotensin II-induced cardiomyocyte apoptosis, atrial fibrillation, and cardiac fibrosis.[27-29] However, the therapeutic effect of irisin specifically on angiotensin II-induced vascular calcification has been largely unknown.

Therefore, the present study aims to investigate whether irisin can attenuate angiotensin II-related vascular calcification and osteogenic transdifferentiation in a CKD animal model and VSMCs. This research could provide valuable insights into the potential use of irisin as a novel therapeutic approach for managing vascular calcification in patients with CKD.

II. MATERIALS AND METHODS

2.1. Clinical data analysis

The Cardiovascular and Metabolic Diseases Etiology Research Center (CMERC) was designed for community-dwelling individuals without a history of myocardial infarction, heart failure, or stroke. The study's rationale, design, methods, and protocols have been previously outlined in detail. Initially, a subset of 630 participants, randomly selected for irisin measurement using baseline serum sample, underwent screening. After excluding individuals with missing coronary artery calcification score (CCS) data (n=90), with eGFR >60 mL/min/1.73 m2 (n=88), and with end stage renal disease (n=177), the final analysis was conducted on 275 individuals. The coronary calcium scores were categorized as follows: 0-1, 1-100, 100-400, >400. Baseline characteristics were compared according to the CCS category using trend analyses. Cuzick's test was employed for continuous variables with skewed deviation, while the Cochran-Armitage test was utilized for categorical variables. A scatter plot was depicted for the natural logarithm values of CCS and irisin. Linear regression analysis was performed to assess the statistical significance.

2.2. Cell culture

Human aortic smooth muscle cells (HASMCs) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). HASMCs were cultured in vascular smooth muscle cell medium (ATCC) supplemented with 5% fetal bovine serum (FBS), 9.5 ng/ml rh FGF-basic, 5 μg/ml rh Insulin, 5 ng/ml rh EGF, 10 mM L-glutamine and 50 μg/ml ascorbic acid. The culture conditions were maintained at 37℃ in a humidified atmosphere containing 5% CO_2. HASMCs used in these experiments were between the 5th and 7th passage.

2.3. Calcification induction and calcium quantification

The cells were seeded in 24-well plates and cultured until the cells were grown to 80-90 % confluence. For induction of calcification of VSMCs, β-glycerophosphate (β-GP; Sigma-Aldrich, St. Louis, MO, USA) and $CaCl_2$ (Tech&Innovation; Gangwon-do, Republic of Korea) was added to vascular smooth muscle cell basal medium (15% FBS, 100 U/ml penicillin, and 100 μg/ml

streptomycin) to final concentrations of 4 mM and 2.5 mM. Medium was replaced at 2 day-intervals for up to 14 days with or without 500 nM angiotensin II (Ang II) (Tocris, Bristol, UK). To investigate the effects of irisin, 10 nM recombinant Irisin (Novoprotein, China) and 10 nM siFNDC5 (Invitrogen, Carlsbad, CA, USA) were treated in VSMCs. siFNDC5 was transfected with Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol.

The treated VSMCs were washed twice with cold PBS and decalcified by treating them with 0.6 N HCl for 24 hours at room temperature to quantify calcium deposition. The calcium content in the supernatant was measured using the QuantiChromeTM Calcium Assay Kit (BioAssay Systems, Hayward, CA, USA). The calcium levels were determined against the concentration of remaining cellular protein.

2.4. Induction of CKD mice

Male C57BL/6 mice (Jackson Laboratory, BarHarbor, ME, USA) weighing 20~25g were used. The animals were maintained in a temperature-controlled room (22℃) in a 12 hours light/dark cycle. One week after arrival, animals were divided into control group (n = 6) and CKD group (n = 6). To induce CKD model, mice were fed with 0.2% adenine diet for up to 6 weeks. After 6 weeks, the mice were euthanized to collect aorta and serum samples.

All animal experiments were conducted following approval from the Institutional Animal Care and Use Ethics Committee of Yonsei University (approval reference number: 2020-0075). The procedures adhered to the guidelines outlined in the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication no. 85-23, revised 1985).

2.5. Ex vivo calcification assays in mouse aorta

For ex vivo experiments, mice were deeply anesthetized by intraperitoneal injection with Zoletil (30 mg/kg) and Rompun (10 mg/kg). They were perfused with ice-cold PBS into the heart to flush out the remaining blood. Entire aortas were carefully harvested from mice. Using a dissecting microscope, the aortas were cleaned. The separated aortas were then plated in cell culture plates (6 well) and cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The aortas of CKD mice were divided into control group (n=4) and calcification induction group (n=16). The aortas of calcification induction group were cultured in calcification induction media (DMEM with 15% FBS, 4 mM β-GP, 2.5 mM CaCl2, 100 U/ml penicillin, and 100

μg/ml streptomycin) for up to 14 days with or without 500 nM angiotensin II and 10 nM recombinant irisin to identify the protective effects of irisin to vascular calcification. The culture medium were changed every 2 days. At the end of the experimental period, the aortas were washed twice with cold PBS. The aortic rings were then decalcified by incubating them with 0.6 N HCl for 24 hours at 37℃. The calcium contents were measured using the QuantiChromeTM Calcium Assay Kit (BioAssay Systems). After decalcification, aortic rings were dried for 24 hours at 55℃ and then weighted. The amounts of calcium extracted from aortic rings were normalized for aortic rings weight (μg/mg).

2.6. Alizarin red staining and calcium quantification

Calcium deposition was visualized using alizarin red staining in VSMCs. After the induction of calcification, the cells were washed twice with cold PBS and fixed with ice-cold 70% ethanol for 30 minutes. The fixed cells were rinsed 3 times with cold PBS and stained with 2% alizarin red solution (pH 4.1-4.3; Sigma-Aldrich) for 10 minutes. After staining, the cells were rinsed with distilled water to remove excess dye. The mineralized nodules were observed as red deposits. For quantification, the alizarin red stain was eluted from the cells using 10% cetylpyridinium chloride monohydrate (Thermo Fisher Scientific, Waltham, MA, USA) for 20 minutes. For quantitative analysis of alizarin red staining, the absorbance was measured at 595 nm.

2.7. Von-kossa staining

Calcium deposits in aortas of mice were visualized by von Kossa staining. For tissue staining, aortic arch sections were fixed in 10 % formalin and embedded in paraffin. The tissue sections were cut into 4 μm-thick slices and dewaxed. To visualize calcium deposition, sections of the aortic arches were stained with 5% silver nitrate ($AgNO_3$; Sigma-Aldrich) under ultraviolet light for 2 hours. After staining, unreacted silver was removed by treating the sections with 5% sodium thiosulfate ($Na_2S_2O_3$; Sigma-Aldrich) for 5 minutes.

2.8. Total RNA extraction

Total RNA was extracted from whole kidney. A piece of the whole kidney was snap-frozen in liquid nitrogen and pulverized with a mortar and pestle while keeping it frozen. The tissues was suspended in 100 μl of RNA STAT-60 reagent (Tel-Test, Friendswood, TX, USA), which was lysed

by freezing and thawing three times. Another 700 μl of RNA STAT-60 reagent was added. The mixture was vortexed and then stored at room temperature for 5 minutes. 160 μl of chloroform was added and the mixture was shaken vigorously for 30 seconds. After 3 minutes, it was centrifuged at 12,000 g for 15 minutes at 4°C. The upper aqueous phase, which contains the extracted RNA, was transferred to a new tube. RNA was precipitated from the aqueous phase by adding 400 μl of isopropanol. The mixture was centrifuged at 12,000 g for 30 minutes at 4°C to pellet the RNA. The RNA precipitate was washed with 70% ice-cold ethanol and dried using a Speed Vac. The dried RNA was dissolved in DEPC-treated distilled water. RNA yield and quality were assessed by measuring absorbance at 260 and 280 nm using a spectrophotometer.

2.9. Reverse transcription

First strand cDNA was made by using a Takara cDNA synthesis kit (Takara Bio Inc., Otsu, Shiga, Japan). Two micrograms of total RNA extracted from tissues or cultured cells were reverse transcribed using 10 μM random hexanucleotide primer, 1 mM dNTP, 8 mM MgCl2, 30 mM KCl, 50 mM Tris·HCl, pH 8.5, 0.2 mM dithiothreithol, 25 U RNAse inhibitor, and 40 U PrimeScript reverse transcriptase. The mixture was incubated at 30°C for 10 minutes and at 42°C for 1 hour. The enzyme was inactivated by heating the mixture at 99°C for 5 minutes.

2.10. Real-time PCR

The primer sequences used in this study were described in Table 2. cDNAs from 25 ng RNA of tissues or cultured cells per reaction tube were used for amplification. Using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), quantitative PCR was performed with a total volume of 20 μl in each well, which contained 10 μl of SYBR Green PCR Master Mix (Applied Biosystems), 5 μl of cDNA, and 5 pM sense and antisense primers. Primer concentrations were determined through preliminary experiments that analyzed the optimal concentrations of each primer. The PCR conditions are as follows: 35 cycles of denaturation at 94.5°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 1 minute. Initial heating at 95°C for 9 minutes and final extension at 72°C for 7 minutes was performed for all PCR reactions. Each sample was run in triplicate in separate tubes and a control without cDNA was also run in parallel with each assay. After real-time PCR, the temperature was increased from 60 to 95°C at a rate of 2°C/min to construct a melting curve. The cDNA content of each specimen was

determined using a comparative CT method with 2−ΔΔCT. The results were given as relative expression normalized to the expression of 18s rRNA and expressed in arbitrary units.

Table 1. Sequences of oligonucleotide primers used for qPCR.

Gene	**Sequence (5' → 3')**	
18S (human)	Forward	ACC GCG GTT CTA TTT TGT T
	Reverse	CGG TCC AAG AAT TTC ACC TC
Fndc5 (human)	Forward	TCGTGGTCCTGTTCATGTG
	Reverse	TCTGGTGTGCTGGTTTCTG
Runx2 (human)	Forward	AGG CGC ATT TCA GGT GCT TC
	Reverse	TAG TGC ATT CGT GGG TTG GAG
Bmp2 (human)	Forward	AGA ATG CAA GCA GGT GGG AA
	Reverse	CCA CTT CCA CCA CGA ATC CA
OPN (human)	Forward	GCA GCT TTA CAA CAA ATA CCC AG
	Reverse	TTA CTT GGA AGG GTC TGT GGG
18S (mouse)	Forward	CGC TTC CTT ACC TGG TTG AT
	Reverse	GGC CGT GCG TAC TTA GAC AT
Fndc5 (mouse)	Forward	AAGGAGATGGGGAGGAACCAG
	Reverse	TGGCGGCAGAAGAGAGCTATAA
Runx2 (mouse)	Forward	CACCGACAGTCCCAACTTCC
	Reverse	CTCTCCGAGGGCTACAACCT
Bmp2 (mouse)	Forward	GAAGAAGCCGTGGAGGAACT
	Reverse	TGAGAAACTCGTCACTGGGG
OPN (mouse)	Forward	CTCCATCGTCATCATCATCG
	Reverse	TGCACCCAGATCCTATAGCC

2.11. Western blot analysis

Pieces of tissues and harvested cultured cells were lysed in SDS sample buffer containing 2% SDS, 10 mM Tris·HCl, pH 6.8, 10% (vol/vol) glycerol. The lysates were treated with Laemmli sample buffer and heated at 100°C for 5 minutes. The denatured proteins were separated by electrophoresis in a 12% acrylamide denaturing SDS-polyacrylamide gel. Proteins were then transferred from gel to a Hybond-ECL membrane using a Hoeffer semidry blotting apparatus (Hoeffer Instruments, San Francisco, CA). The membrane was incubated in blocking buffer A (1× TBS, 0.1% Tween 20, and 5% nonfat milk) for 1 hour at room temperature, followed by an overnight incubation at 4°C in a 1:1,000 dilution of polyclonal antibodies to Fndc5, Runx2, BMP2, OPN (Abcam, Cambridge, MA, USA) and β-actin (Sigma-Aldrich, St. Louis, MO, USA). The membrane was washed once for 15 minutes and twice for 5 minutes in 1× TBS with 0.1% Tween 20. The membrane was incubated in buffer A containing a 1:2,000 dilution of horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody or anti-rabbit IgG antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The membrane was washed again and developed using a chemiluminescent agent (ECL; Amersham Life Science, Inc., Arlington Heights, IL, USA). The densities of protein bands were measured using Image J software (National Institutes of Health, Bethesda, MA, USA; online at http://rsbweb.nih.gov/ij) was used. The optical densities of the bands from treated samples were compared to those from control samples to determine relative changes in protein expression.

2.12. Statistical analysis

All values were expressed as the mean ± standard error of the mean (SEM). The statistical package SPSS for Windows Ver. 20.0 (SPSS, Inc., Chicago, IL, USA) was utilized for data analysis. The Kruskal-Wallis non-parametric test for multiple comparisons was used to assess whether there were significant differences among the groups. Significant differences detected by the Kruskal-Wallis test were further confirmed using the Mann-Whitney U-test. P-values less than 0.05 were considered statistically significant.

III. RESULTS

3.1. Serum irisin levels had a negative correlation with coronary artery calcification

Before experimental study, clinical data from CMERC (Cardiovascular and Metabolic Disease Etiology Research Center) study at Yonsei University Health System were analyzed to investigate the relationship between serum irisin levels and coronary artery calcification. In 275 patients with CKD, serum irisin levels had a negative correlation with coronary artery calcification scores, highlighting a potential role for irisin in preventing vascular calcification in CKD patients (Fig. 1 and Table 1).

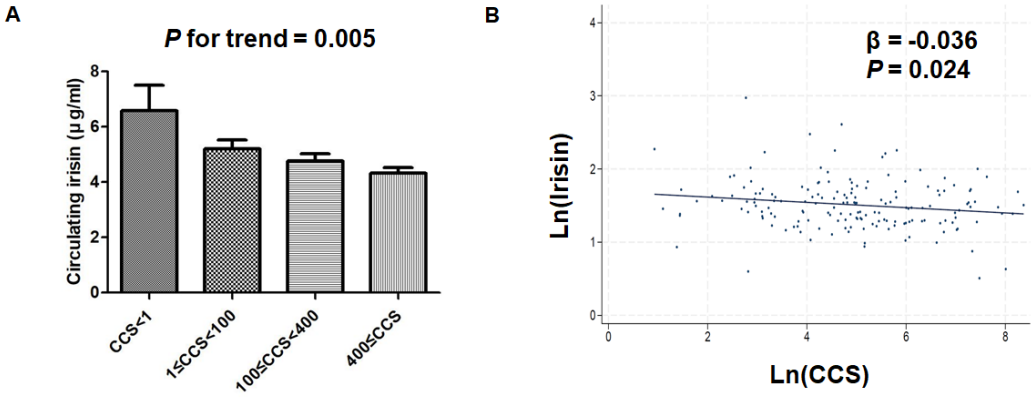

Figure 1. Serum irisin levels and coronary artery calcification scores (CCS) in CKD patients. (A) Serum irisin levels in CKD patients were decreased according to CCS (*P* for trend=0.005). (B) There was a negative correlation between serum irisin levels and coronary artery calcification (*P*=0.024).

Table 2. Clinical and biochemical characteristics according to coronary artery calcification scores

	Total (N=275)	CCS <1 (N=107)	1≤CCS<100 (N=67)	100≤CCS<400 (N=56)	400≤CCS (N=45)	*P* for trend
Demographics						
Age	61.1±11.5	55.4±12.3	62.4±10.0	66.1±9.0	66.3±7.8	<0.001
Sex, male	170 (61.8%)	58 (54.2%)	42 (62.7%)	34 (60.7%)	36 (80.0%)	0.007
BMI	25.3±4.0	24.8±4.0	25.9±4.1	25.2±3.4	25.8±4.1	0.21
SBP	130.2±17.5	124.9±14.7	132.3±15.0	136.0±19.8	132.7±20.7	0.002
DBP	75.8±10.4	77.3±10.4	76.4±10.6	74.7±9.2	72.7±11.1	0.03
Comorbidities						
Hypertension	253 (92.0%)	102 (95.3%)	60 (89.6%)	50 (89.3%)	41 (91.1%)	0.24
Diabetes	123 (44.7%)	27 (25.2%)	34 (50.7%)	32 (57.1%)	30 (66.7%)	<0.001
Dyslipidemia	140 (50.9%)	54 (50.5%)	28 (41.8%)	30 (53.6%)	28 (62.2%)	0.19
CAD	13 (4.7%)	1 (0.9%)	3 (4.5%)	5 (8.9%)	4 (8.9%)	0.009
CVA	11 (4.0%)	2 (1.9%)	4 (6.0%)	4 (7.1%)	1 (2.2%)	0.49
IHD	17 (6.2%)	4 (3.7%)	3 (4.5%)	5 (8.9%)	5 (11.1%)	0.05
Laboratory data						
Hemoglobin	12.6±2.0	12.9±2.0	12.8±1.9	12.3±2.1	12.2±2.1	0.02
Calcium	9.1±0.5	9.1±0.4	9.2±0.5	9.0±0.4	9.0±0.5	0.049
Phosphorus	3.6±0.5	3.6±0.5	3.7±0.5	3.7±0.6	3.6±0.5	0.81
Glucose	111.9±34.9	105.3±26.9	116.0±37.2	114.2±26.2	118.4±52.1	0.007
BUN	30.4±10.9	29.3±10.7	31.0±11.2	30.5±12.0	32.2±9.2	0.07
Creatinine	1.92±0.72	1.85±0.72	1.96±0.74	1.89±0.68	2.03±0.72	0.12
eGFR, mean	37.3±13.5	38.8±13.8	36.6±14.5	36.9±13.5	35.0±11.4	0.13
CRP	3.7±11.5	4.6±13.4	4.1±14.4	1.7±2.2	3.2±8.6	0.4
CCS	261.9±598.5	0.0	38.2±27.6	198.9±83.0	1296.0±937.5	<0.001
Irisin	5.5±6.2	6.6±9.6	5.2±2.5	4.8±1.9	4.3±1.3	0.005

CCS, coronary artery calcium score; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; CAD, coronary artery disease; CVA, cerebral vascular accident; IHD, ischemic heart disease; BUN, blood urea nitrogen; eGFR, estimated glomerular filtration rate; CRP, C-reactive protein.

Data are presented as n (%) or mean ± SD.

3.2. Angiotensin II induced VSMCs calcification and osteogenic transdifferentiation.

To investigate the relationship with vascular calcification and angiotensin II, VSMCs were treated either with or without angiotensin II. The results indicated that the administration of angiotensin II led to significant increases in the expression of osteogenesis-related genes as observed through real-time PCR analysis. Additionally, protein expressions associated with osteogenesis, such as Runx2, BMP2, and OPN, were also upregulated, as demonstrated by Western blot analysis. Furthermore, it was found that angiotensin II treatment resulted in a decrease in the expression level of FNDC5, which is the precursor of irisin (Fig. 2). This result suggests a potential mechanism through which angiotensin II may influence vascular calcification by modulating irisin. Moreover, calcium deposition was found to be more pronounced in VSMCs exposed to angiotensin II compared to control cells, as indicated by alizarin red staining (Fig. 3).

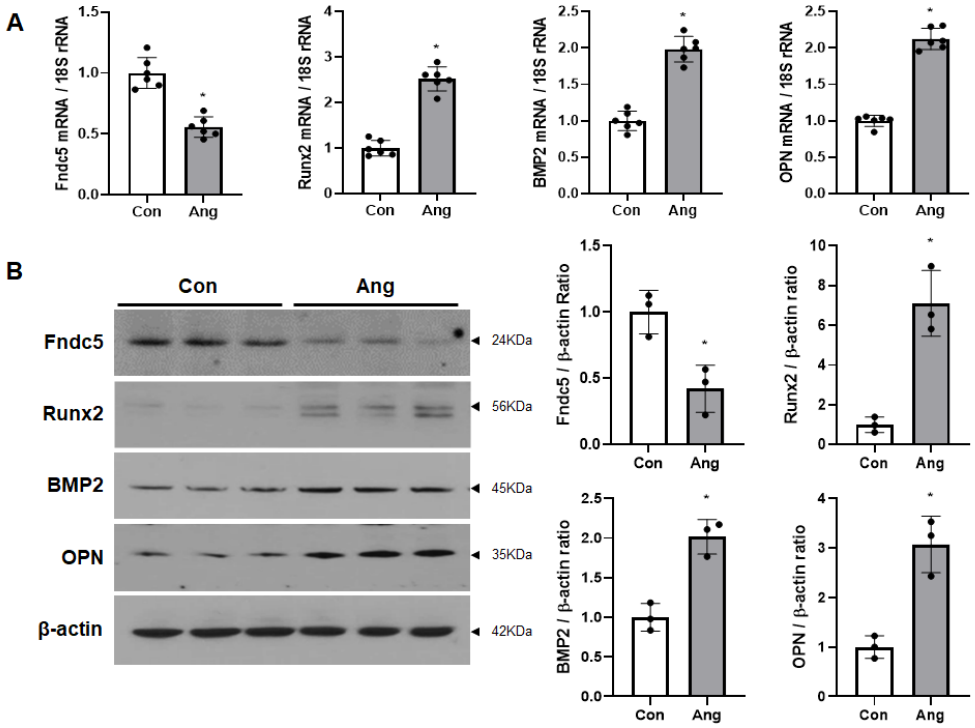

Figure 2. Representative images of real-time PCR and western blot of Fndc5, Runx2, BMP2, and OPN in control (Con) and angiotensin II-induced calcification group (Ang) in VMSCs. (A) The administration of angiotensin II led to significant increases in the expression of osteogenesis-related genes and decrease in the expression of FNDC5 as observed through real-time PCR analysis. (B) Protein expressions associated with osteogenesis were upregulated, as demonstrated by Western blot analysis. *; $P < 0.05$ vs. Con group. N=6 in each group

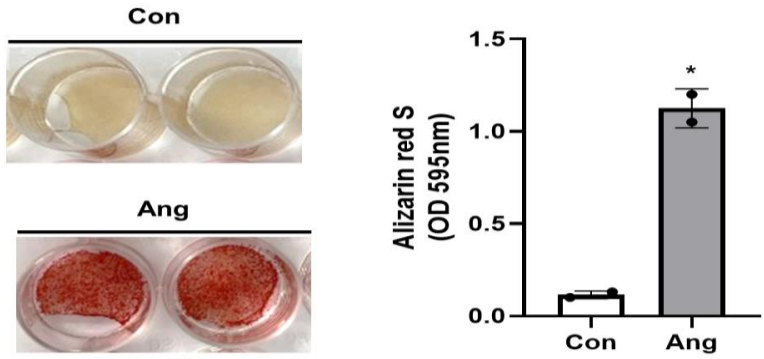

Figure 3. Representative images of Alizarin red staining in control (Con) and angiotensin II-induced calcification group (Ang). Calcium deposition was found to be more pronounced in VSMCs exposed to angiotensin II compared to control cells. Data are shown as mean ± SEM. N=3. *; $P < 0.05$ vs. Con group.

3.3. Irisin alleviated VSMCs calcification and osteogenic transdifferentiation.

I investigated the protective effect of irisin against VSMCs calcification and osteogenic transdifferentiation. The results revealed that the increased calcium deposition and expressions of osteogenesis-related genes and proteins were induced by angiotensin II, and that these changes were significantly mitigated by treatment with recombinant irisin (Fig. 4 and 5). This suggests that irisin has a counteractive effect against the osteogenic transdifferentiation promoted by angiotensin II in VSMCs.

Moreover, I found that knocking down FNDC5 as a precursor of irisin using siFndc5 exacerbated angiotensin II-induced osteogenic transdifferentiation and calcium deposition in VSMCs (Fig. 6 and 7). This further supports the notion that irisin plays a crucial role in preventing VSMCs from undergoing osteogenic differentiation and accumulating calcium deposits, which are key events in vascular calcification.

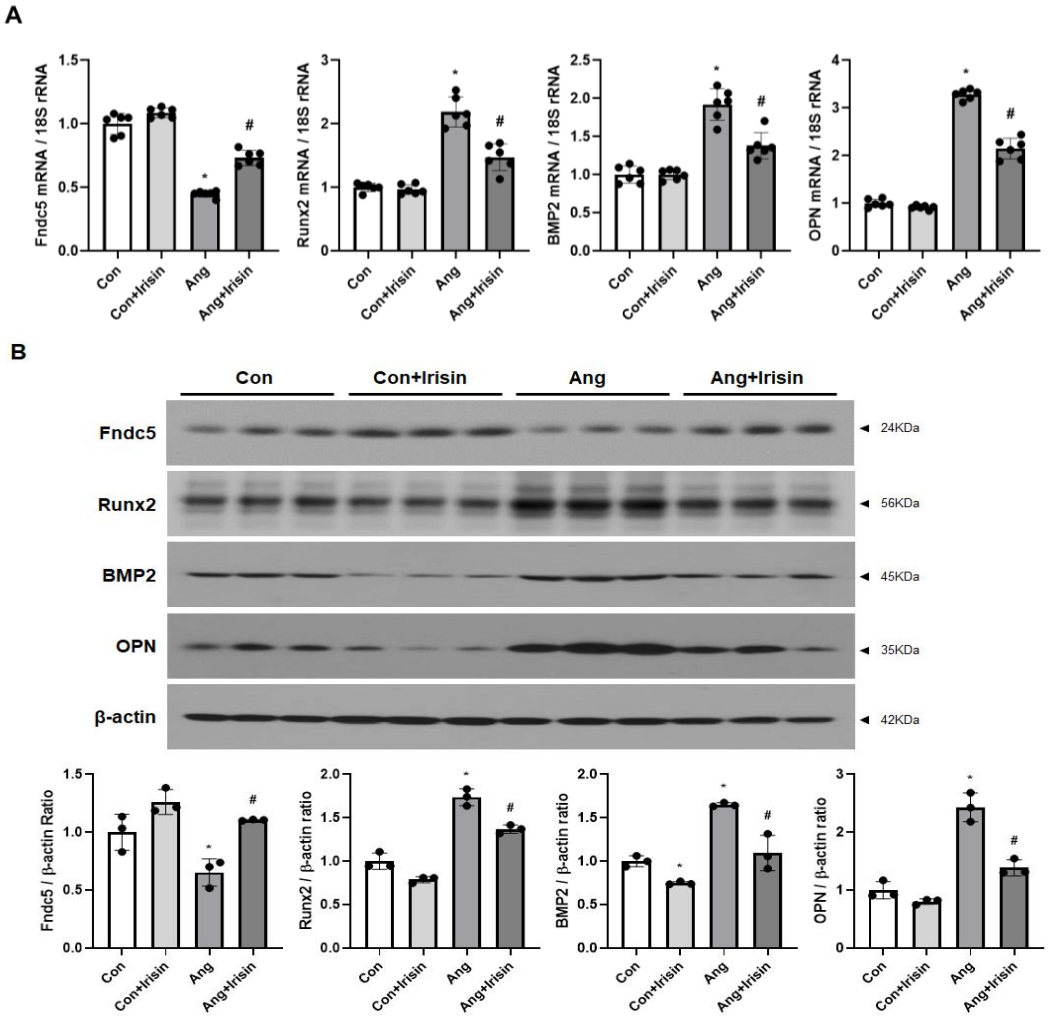

Figure 4. Representative images of real-time PCR and western blot of Fndc5, Runx2, BMP2, and OPN in control (Con), Con + recombinant irisin (Irisin), angiotensin II-induced calcification (Ang), and Ang + Irisin groups. The increased expressions of osteogenesis-related (A) genes and (B) proteins were induced by angiotensin II, and these changes were significantly mitigated by treatment with recombinant irisin. The decrease expressions of FNDC5 were induced by angiotensin II, and these changes were increased by treatment with recombinant irisin. Data are shown as mean ± SEM. N=6. *; $P < 0.05$ vs. Con group, #; $P < 0.05$ vs. Ang group.

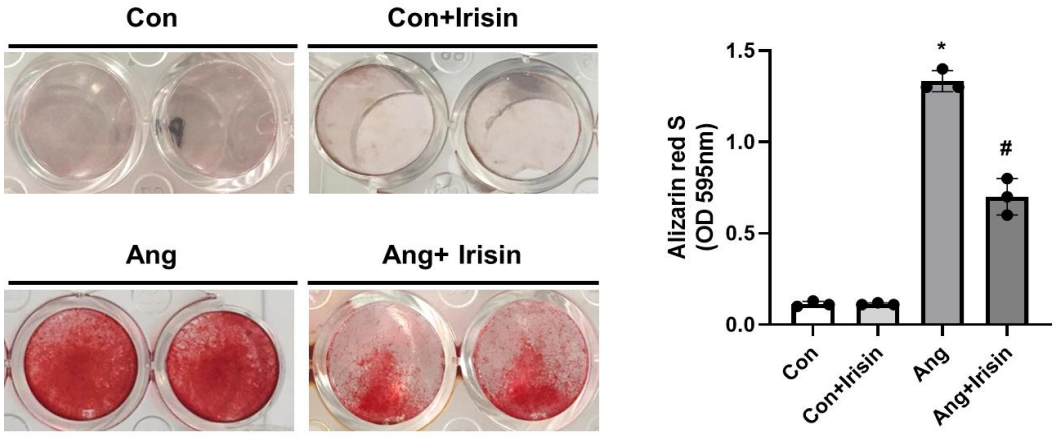

Figure 5. Representative images of Alizarin red staining in control (Con), Con + recombinant irisin (Irisin), angiotensin II-induced calcification (Ang), and Ang + Irisin groups. Calcium deposition was found to be more pronounced in VSMCs exposed to angiotensin II compared to control cells, and these changes were significantly mitigated by treatment with recombinant irisin. Data are shown as mean ± SEM. N=3. *; $P < 0.05$ vs. Con group, #; $P < 0.05$ vs. Ang group.

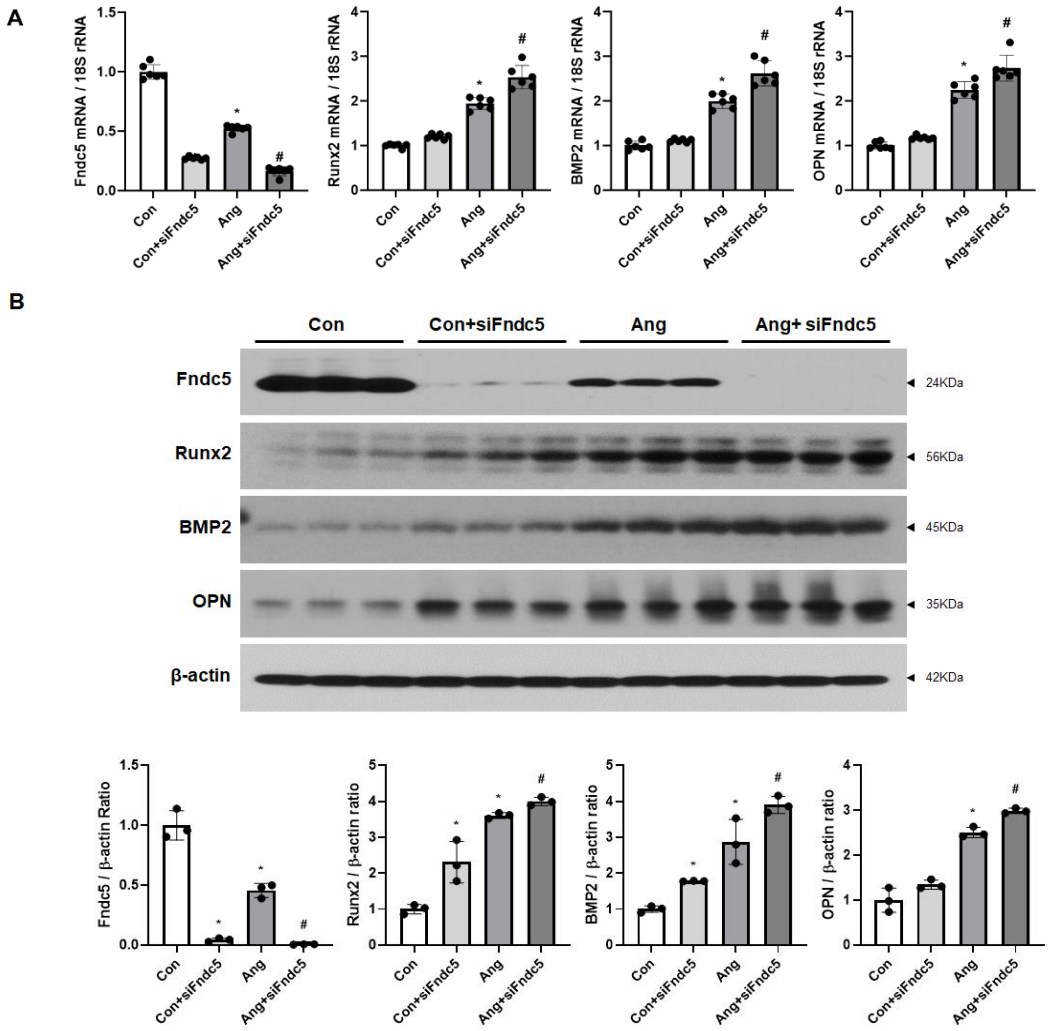

Figure 6. Representative images of real-time PCR and western blot of Fndc5, Runx2, BMP2, and OPN in control (Con), Con + siFndc5, angiotensin II-induced calcification (Ang), and Ang + siFndc5 groups. The increased expressions of osteogenesis-related (A) genes and (B) proteins were induced by angiotensin II, and these changes were significantly exacerbated by treatment with siFndc5. The decrease expressions of FNDC5 were induced by angiotensin II, and these changes were more decreased by treatment with siFndc5. Data are shown as mean ± SEM. N=6. *; $P < 0.05$ vs. Con group, #; $P < 0.05$ vs. Ang group.

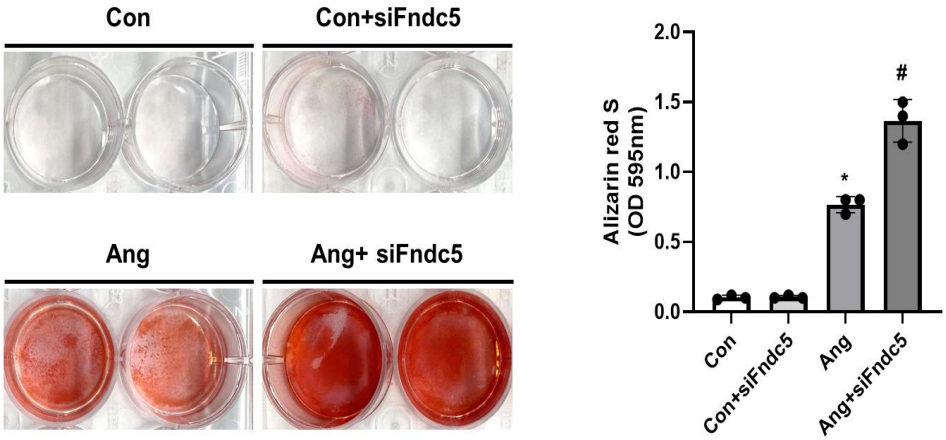

Figure 7. Representative images of Alizarin red staining in control (Con), Con + siFndc5, angiotensin II-induced calcification (Ang), and Ang + siFndc5 groups. Calcium deposition was found to be more pronounced in VSMCs exposed to angiotensin II compared to control cells, and these changes were significantly aggravated by treatment with siFndc5. Data are shown as mean ± SEM. N=3. *; $P < 0.05$ vs. Con group, #; $P < 0.05$ vs. Ang group.

3.4. Vascular calcification and osteogenic transdifferentiation were pronounced in CKD mice.

Kidney injury was induced in animals by administering a 0.2% adenine diet over a period of 6 weeks. Aortic sections were stained with von Kossa to assess calcium deposition in control and CKD mice. CKD mice exhibited significantly higher levels of calcium deposition in their aortas compared to control mice (Fig. 8A).

I investigated the expression of genes and proteins associated with osteogenesis in the aortas of CKD mice. Using real-time PCR and western blot analysis, increased expression of osteogenic markers including Runx2, BMP2, and OPN was observed in CKD mice. These findings suggest that CKD may promote osteogenic transdifferentiation in the vasculature, contributing to vascular calcification. Additionally, the study found that the expression of FNDC5 was decreased in CKD mice (Fig. 8B and 8C). This observation suggests that FNDC5 may play a role in the pathogenesis of vascular calcification in CKD. Overall, the findings of the study indicated that vascular calcification and osteogenic transdifferentiation were more pronounced in CKD.

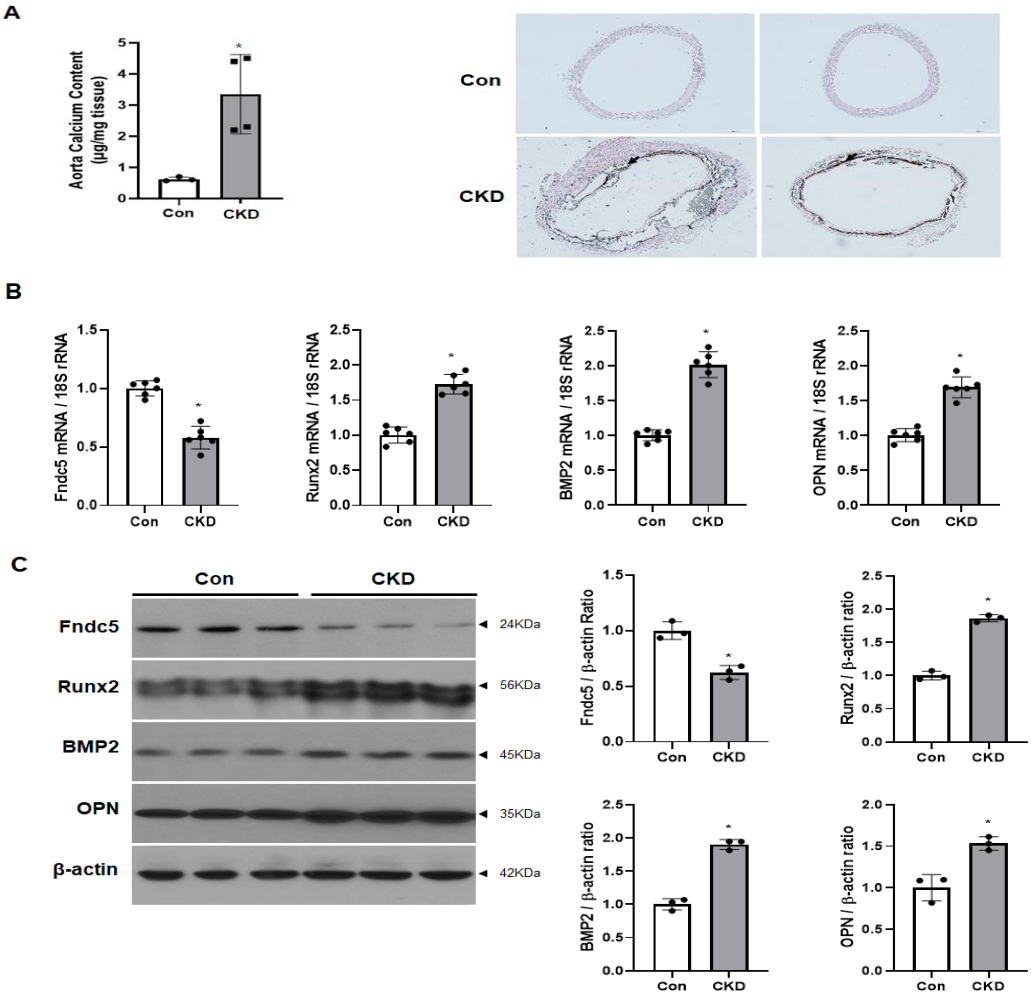

Figure 8. Representative images of real-time PCR and western blot of Fndc5, Runx2, BMP2, and OPN, and von Kossa staining in control (Con) and chronic kidney disease (CKD) mice. (A) CKD mice displayed heightened levels of calcium deposition in their aortas. (B) The expressions of osteogenesis-related genes were increased and the expression of FNDC5 was decreased in CKD mice, as observed through real-time PCR analysis. (C) Protein expressions associated with osteogenesis were upregulated in CKD mice, as demonstrated by western blot analysis. Data are shown as mean ± SEM. N=6. *; $P < 0.05$ vs. Con group

3.5. Irisin attenuated aortic calcification ex vivo.

For ex vivo experiments, aortas of CKD mice were harvested and divided into control and calcification induction group. The aortas of calcification induction group were cultured in calcification induction media for 14 days with or without angiotensin II and recombinant irisin to identify the protective effects of irisin to vascular calcification.

The results indicated that aortic calcium contents were elevated in calcification induction media. Moreover, the addition of angiotensin II further increased the levels of calcium deposition in the aortas, suggesting a synergistic effect between calcification induction media and angiotensin II in promoting vascular calcification. In addition, the increased calcium contents observed in calcification media supplemented with angiotensin II was alleviated by the presence of recombinant irisin (Fig. 9). This suggests a protective role for irisin against angiotensin II-induced vascular calcification, potentially through mechanisms that counteract the effects of angiotensin II on calcium deposition in the aortas.

I further analyzed indicators of osteogenic transdifferentiation in the aortas of mice, particularly focusing on the effects of calcification media containing angiotensin II and the potential protective effects of recombinant irisin treatment. The results showed that exposure to calcification media containing angiotensin II led to an elevation in the levels of osteogenesis-related genes and proteins, including Runx2, BMP2, and OPN, in the aortas. Furthermore, the results showed improvements in osteogenic transdifferentiation in the aortas with recombinant irisin treatment. The decreased expression of FNDC5 observed in the presence of calcification media with angiotensin II was elevated when treated with recombinant irisin (Fig. 10). Overall, these results suggested that irisin treatment might have therapeutic potential in reducing vascular calcification by mitigating osteogenic transdifferentiation in CKD mice.

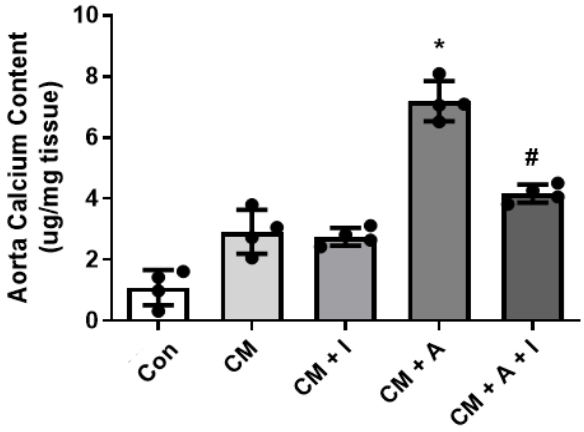

Figure 9. A representative image of calcium contents in control (Con), calcification media (CM), calcification media + recombinant irisin (CM + I), calcification media + angiotensin II (CM + A), and calcification media + angiotensin II + recombinant irisin (CM + A + I). The increased calcium contents observed in calcification media supplemented with angiotensin II was alleviated by the presence of recombinant irisin. Data are shown as mean ± SEM. N=4. *; $P < 0.05$ vs. Con group, #; $P < 0.05$ vs. CM + A group.

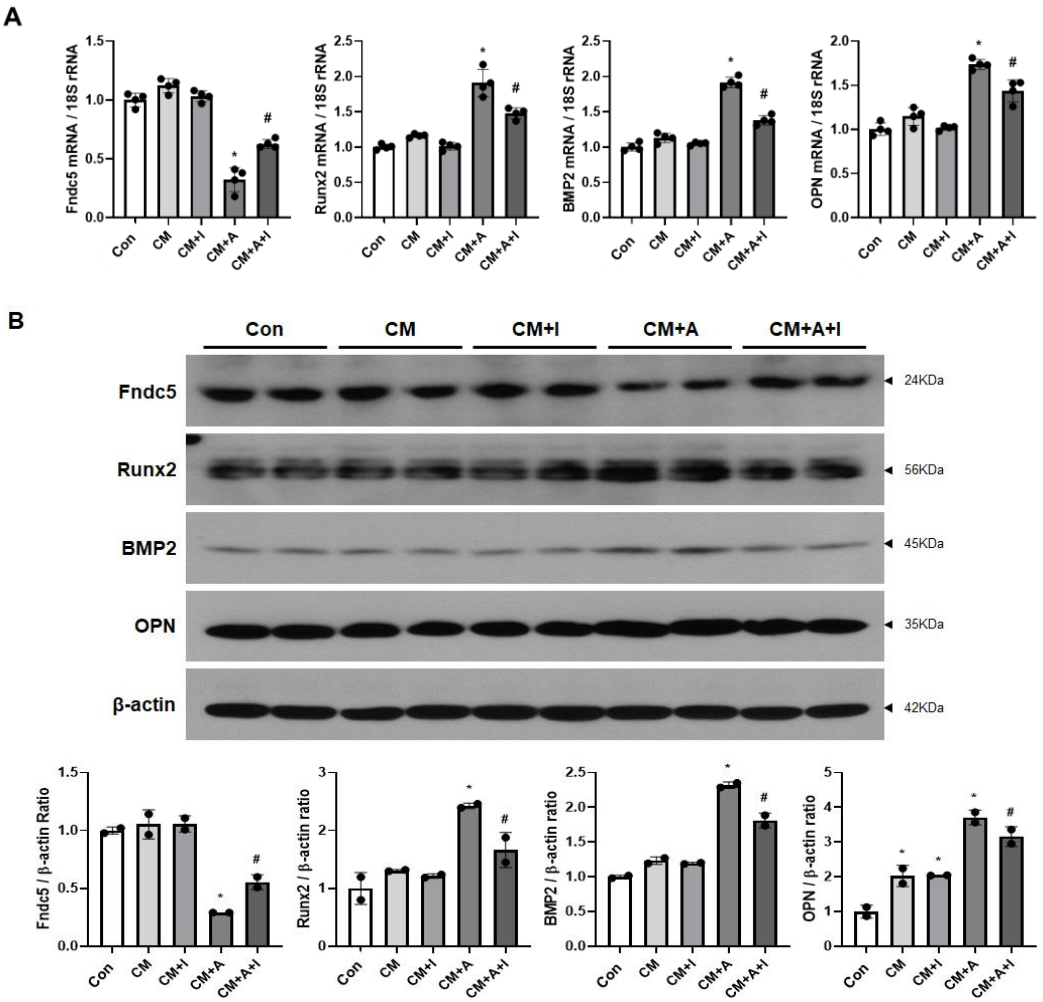

Figure 10. Representative images of real-time PCR and western blot of FNDC5, Runx2, BMP2, and OPN in control (Con), calcification media (CM), calcification media + recombinant irisin (CM + I), calcification media + angiotensin II (CM + A), and calcification media + angiotensin II + recombinant irisin (CM + A + I). (A) The administration of angiotensin II led to significant increases in the expression of osteogenesis-related genes and decrease in the expression of FNDC5 as observed through real-time PCR analysis. (B) Protein expressions associated with osteogenesis were upregulated, as demonstrated by western blot analysis. These changes were improved with recombinant irisin treatment. Data are shown as mean ± SEM. N=4 *; $P < 0.05$ vs. Con group, #; $P < 0.05$ vs. CM + A group.

IV. DISCUSSION

The results of present study showed that angiotensin II induced osteogenic transdifferentiation and the deposition of cellular calcium in VSMCs. Moreover, this study demonstrated that treatment with recombinant irisin significantly alleviated these changes induced by angiotensin II, while knocking down irisin using siFndc5 worsened the angiotensin II-induced calcium deposition in VSMCs. CKD mice by administering a 0.2% adenine diet exhibited significantly higher levels of calcium deposition in their aortas compared to control mice. The increased expression of osteogenic markers including Runx2, BMP2, and OPN and the decreased expression of FNDC5 were observed in CKD mice. In the ex vivo study, aortic calcium contents were elevated in calcification induction media with angiotensin II. The increased calcium contents observed in calcification media supplemented with angiotensin II was alleviated by the presence of recombinant irisin. The exposure to calcification media containing angiotensin II led to an elevation in the levels of osteogenesis-related genes and proteins. However, treatment with recombinant irisin improved these indicators, suggesting that irisin could serve as a potential therapeutic strategy for vascular calcification in CKD.

Cardiovascular disease stands as the primary cause of mortality among patients with CKD, with vascular calcification playing a significant role in the heightened cardiovascular mortality observed in this population.[1-3] Vascular calcification in CKD is a multifaceted and dynamic process, involving various mechanisms that stimulate VSMCs to differentiate into an osteoblastic phenotype.[7] The crucial mechanism driving this process is the upregulation of transcription factors such as Runx2, MSX2, and BMP2, leading to the deposition of calcium in the vascular walls. Additionally, the loss of calcification inhibitors further exacerbates vascular mineralization in CKD. Proteins such as fetuin-A, matrix Gla protein, pyrophosphate, and OPN normally act to inhibit the formation and growth of calcium deposits in the vasculature. Furthermore, the activity of these inhibitors may be compromised in CKD, allowing for vascular calcification to occur.[9] However, OPN, despite being an important negative regulator of calcification, is paradoxically highly expressed in calcified plaques. This phenomenon may represent a compensatory mechanism attempting to counterbalance vascular calcification.[30] In present study, the increased expressions of osteogenic transdifferentiation-related genes and proteins including Runx2, BMP2, and OPN were observed in the angiotensin II-induced calcification models. Overall, vascular calcification in CKD is a complex process involving dysregulation of various molecular pathways and imbalance between calcification

promoters and inhibitors. Understanding these mechanisms is important for developing effective therapeutic strategies aimed at mitigating vascular calcification and reducing cardiovascular mortality in CKD patients.

The role of the RAS in vascular calcification has been the subject of considerable research interest. Angiotensin II, a key component of the RAS, has been implicated in the upregulation of osteogenic proteins and signaling pathways, thereby promoting vascular calcification.[8] For instance, angiotensin receptor blockade has been shown to inhibit arterial calcification by disrupting osteogenesis in atherogenic rabbit models.[10] Additionally, stimulation of the angiotensin II type 2 receptor (AT2) has been implicated in inhibiting phosphate-induced vascular calcification in AT2-overexpressing mice.[12] However, conflicting results have also emerged from other studies. In 5/6 nephrectomy rat models, treatment with enalapril, an angiotensin-converting enzyme (ACE) inhibitor, did not have beneficial effect on vascular calcification.[11] This suggests that the therapeutic effects of blocking the RAS may not always be consistent across different experimental settings. In the present study, it was demonstrated that angiotensin II-induced calcification in VSMCs led to a significant increase in the expression of osteogenic genes and proteins, including Runx2, BMP2, and OPN. These findings further emphasized the involvement of the RAS in promoting vascular calcification. However, the effectiveness of RAS blockade as a therapeutic strategy for vascular calcification will be investigated in further research.

While sarcopenia tends to worsen with reduced renal function and is associated with increased cardiovascular risk, the efficacy of exercise as a preventive or therapeutic measure in CKD patients remains uncertain due to inconsistent findings across studies. Some preclinical studies have shown promising results regarding the benefits of exercise in CKD models. For instance, in a spontaneous progressive cystic kidney disease model, wheel running improved muscle strength and mitigated left ventricular hypertrophy and aortic calcification.[21] However, other animal studies have suggested that exercise may not effectively ameliorate certain cardiovascular parameters, such as increased systolic pressure induced by renal failure.[22] Clinical studies investigating the effects of exercise in CKD patients have yielded mixed results. While some observational studies, like the Comprehensive Dialysis Study, have found strong associations between low physical activity levels and increased mortality among dialysis patients, others comparing the effects of exercise training interventions have not consistently demonstrated improvements in physical function or vascular parameters.[17-19] Inconsistency in these studies highlights the need for further research to better understand the

therapeutic effects of exercise in CKD patients.

Irisin, as an exercise-induced peptide hormone, may play a role in mitigating cardiovascular risk, including vascular calcification.[23] Higher serum irisin levels were associated with less burden of coronary atherosclerosis in prospective, population-based study of Japanese men.[25] In addition, an observation study in patient with stage 3-5 CKD showed that serum irisin in CKD patients was significantly decreased than in healthy subjects, and that cardiovascular events had been more developed in patients with lower level of irisin.[31] This relationship might be considered as an indirect assessment for the effect of skeletal muscle activity on the cardiovascular risk. In the present study, while the direct effect of exercise on vascular calcification in CKD mice was not confirmed, recombinant irisin was found to alleviate angiotensin II-associated VSMCs calcification and aortic calcification in CKD mice. Because irisin was regarded as a marker of indirect assessment for skeletal muscle activity, it could be suggested that exercise might have a positive effect on vascular calcification in CKD. Further research is needed to clarify the effect of exercise in CKD-associated vascular calcification.

Irisin has been reported to have various cardio-protective effects in angiotensin II-related cardiovascular diseases. It was reported that irisin attenuated angiotensin II-induced cardiac fibrosis via Nrf2 mediated inhibition of TGFβ1-Smad3 signaling.[29] Other study showed that angiotensin II-induced atrial fibrillation was ameliorated by irisin treatment via same signaling pathway.[28] In another experimental study, overexpression of irisin reduced cardiomyocyte apoptosis and alleviated myocardial hypertrophy.[27] However, there was no study which established the beneficial effect of irisin on angiotension II-associated vascular calcification. In this study, recombinant irisin was found to alleviate angiotensin II-associated VSMCs calcification and aortic calcification in CKD mice. Furthermore, future researches are needed to elucidate the relevant signaling pathways of irisin in preventing or mitigating angiotensin II-associated vascular calcification.

The present study has several limitations. Firstly, while the study demonstrated that irisin treatment alleviated calcification and osteogenic transdifferentiation in VSMCs in vitro and ex vivo experiments with separated aortas from CKD mice, it did not directly demonstrate the improvement of vascular calcification by irisin treatment in vivo. Further studies should aim to validate these findings in animal models of CKD by assessing vascular calcification with irisin treatment. Secondly, the study did not elucidate the specific signaling pathways through which irisin exerts its inhibitory effects on vascular calcification. Further investigations are needed to determine the molecular

mechanisms underlying the protective effects of irisin treatment on vascular calcification, including the potential involvement of angiotensin II-associated signaling pathways such as MAP kinases, tyrosine kinases, tyrosine phosphatases, and Rho/Rho kinase. Thirdly, the study did not directly assess the effects of exercise on vascular calcification in CKD animal models. While irisin was used as a marker of indirect assessment for skeletal muscle activity, future studies should investigate the impact of exercise interventions, such as voluntary wheel running, on both irisin levels and vascular calcification in CKD animal models. This will help to establish the potential therapeutic benefits of exercise in mitigating vascular calcification in CKD. Addressing these limitations through further research will provide valuable insights into the therapeutic potential of irisin and exercise in preventing or treating vascular calcification in CKD, ultimately contributing to the development of novel therapeutic strategies for this clinically important condition.

V. CONCLUSION

To clarify the potential role of irisin in mitigating vascular calcification in CKD, I conducted experiments both in vitro using VSMCs and ex vivo using isolated aortas from CKD mice. I assessed the effects of irisin treatment on calcification and osteogenic transdifferentiation.

1. Angiotensin II-induced calcification in VSMCs led to a significant increase in the expression of osteogenic genes and proteins, including Runx2, BMP2, and OPN. Calcium deposition was found to be more pronounced in VSMCs exposed to angiotensin II compared to control cells, as indicated by alizarin red staining.
2. The increased expressions of osteogenesis-related genes and proteins induced by angiotensin II were significantly mitigated by treatment with recombinant irisin. Knocking down FNDC5 as a precursor of irisin using siFndc5 exacerbated osteogenic transdifferentiation and calcium deposition in VSMCs.
3. Kidney injury was induced in animals by administering a 0.2% adenine diet over a period of 6 weeks. Compared to the control group, CKD mice displayed heightened levels of calcium deposition in their aortas, as indicated by von Kossa staining. The increased expression of osteogenic markers including Runx2, BMP2, and OPN and the decreased expression of FNDC5 were observed in CKD mice.
4. In the ex vivo study, aortas from mice with CKD were harvested and cultured. Aortic calcium contents were elevated in calcification induction media with angiotensin II. The increased calcium contents observed in calcification media supplemented with angiotensin II was alleviated by the presence of recombinant irisin.
5. The exposure to calcification media containing angiotensin II led to an elevation in the levels of osteogenesis-related genes and proteins such as Runx2, BMP2, and OPN. However, treatment with recombinant irisin improved these indicators.

In conclusion, I demonstrated that irisin treatment alleviated calcification and osteogenic transdifferentiation in VSMCs in vitro and ex vivo experiments with separated aortas from CKD mice. These data suggest that irisin could serve as a potential therapeutic strategy for vascular calcification in CKD. Further research is warranted to elucidate the underlying mechanisms of irisin in alleviating vascular calcification.

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Abstract in Korean

만성 신질환 모델에서 마이오 카인의 일종인 Irisin의 혈관 석회화 억제 효과

배경: 혈관의 석회화는 만성 신질환 환자들에게 있어서 흔한 합병증이고 심혈관계 사망률을 예측하는 주요한 인자로 알려져 있다. 만성 신질환에서 혈관의 석회화는 능동적인 세포를 매개로 하는 과정을 거쳐서 발생하는데 특히 혈관 평활근 세포(vascular smooth muscle cells)가 조골세포로 분화하는 과정을 포함한다. 이러한 조골세포로의 분화 (osteogenic transdifferentiation)에 있어서 angiotensin II 가 중요한 역할을 하는 것으로 확인되었다. 한편, 신기능 악화에 따라서 근감소증을 동반하는 경우가 많은데, 근감소증은 심혈관계 사망률의 증가와 관련이 있고 이에 대한 치료가 만성 신질환 환자에 있어서 중요한 목표로 여겨진다. 운동에 의해서 분비되는 마이오 카인의 일종인 Irisin 은 전구체인 fibronectin type III domain-containing protein 5 (FNDC5)으로부터 분리되어서 체내로 분비되는데 이전 연구를 통해서 Irisin 의 혈중 농도가 심혈관계 사망률과 음의 상관 관계가 있으며 심혈관 건강에 있어서 중요한 역할을 하는 것을 확인할 수 있었다. 따라서 이번 연구에서는 만성 신질환 모델에서 Irisin 이 혈관 석회화에 대해서 억제 효과가 있는지 확인함으로써 향후 만성 신질환 환자의 혈관 석회화에 대한 치료 후보물질로서의 가능성을 확인하고자 한다.

방법: 생체 외 실험으로는 혈관 평활근 세포를 대조군과 angiotensin II 투여군으로 나눠서 조골세포 분화 신호전달체계의 표지자인 Runx2, BMP2, OPN 의 발현 정도를 real-time PCR 과 western blot analysis 로 확인하였고 칼슘 침착은 alizarin red 염색으로 관찰하였다. 혈관 평활근 세포에 recombinant irisin 와 siFndc5를 추가로 투여함으로써 칼슘 침착과 조골세포 분화에 대해서 Irisin 이 억제 효과를 갖고 있는지 확인하였다. 동물 실험을 위해서 마우스에게 0.2% 아데닌 사료를 6주간 투여하여 만성 신질환 모델을 유발하였다. 대조군과 만성 신질환군의 대동맥내 칼슘 침착을 확인하기 위해서 von Kossa 염색을 실시하였고 두 군간 조골분화 정도를 real-time PCR 과 western blot analysis 로 비교하였다. 만성 신질환 마우스의 대동맥을 분리하여서 배양하는 ex vivo 실험을 실시하였는데 대조군과 석회화 유도군으로 나누고, 석회와 유도군은 recombinant irisin 과 angiotensin II 투여 여부에 따라서 4개의 세부군으로 나눠서 칼슘 침착과 조골세포 분화에 대해서 비교 분석하였다.

결과: 혈관 평활근 세포를 이용한 실험에서 대조군과 비교했을 때 angiotensin II 투여군에서 조골세포 분화 신호전달체계의 표지자인 Runx2, BMP2, OPN 의 발현과 칼슘 침착이 증가함을 확인할 수 있었다. 이러한 변화는 recombinant irisin 투여에

의해서 완화되었고 siFndc5 투여에 의해서 악화됨을 확인할 수 있었다. 동물 실험을 실시하여 대조군에 비해서 만성 신질환 마우스에서 혈관 석회화와 조골세포 분화 신호전달체계의 표지자들의 발현이 증가하여 있음을 확인하였다. 만성 신질환 마우스에서 분리한 대동맥을 이용한 ex vivo 실험에서 angiotensin II 투여시 칼슘 침착 및 조골분화 관련 인자들의 발현이 증가함을 확인할 수 있었고 recombinant irisin 투여에 의해서 그 변화가 완화됨을 확인하였다.

결론: 이상의 결과를 종합하여 볼 때, 만성 신질환 모델에서 Irisin 치료가 혈관 석회화를 완화시킬 수 있음을 확인 할 수 있었다. Irisin 이 만성 신질환 환자들의 혈관 석회화를 억제시킬 수 있는 치료 후보 물질로 여겨지며 향후 추가 연구를 통하여 관련 기전을 규명할 수 있을 것으로 생각된다.

핵심되는 말 : Irisin, 혈관 석회화, 만성 신질환