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Reticulocalbin 2 enhances osteogenic differentiation of human vascular smooth muscle cells in diabetic conditions

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ABSTRACT

Aim: Diabetes accelerates pro-atherogenic and pro-osteogenic phenotypes of vascular smooth muscle cells (VSMCs), an important process for vascular calcification. Reticulocalbin 2 (RCN2) is a candidate gene for atherosclerosis and involved in vascular remodeling in hypertension. However, the role of RCN2 in VSMCs calcification under diabetic conditions is unclear.

Materials and methods: Expression of RCN2 and Runt-related transcription factor 2 (Runx2) in femoropopliteal arterial plaques was compared between type 2 diabetes mellitus (DM) and non-DM patients using immunohistochemical staining (IHCS). Human aortic VSMCs (HAVSMCs) were analyzed under RCN2 gene knockdown and overexpression conditions. Alizarin red staining and intracellular calcium deposition quantification were used to observe calcification induced *in vitro* under normal glucose or high glucose combined with β-glycerol phosphoric acid conditions. The cells were investigated for gene modulation of osteogenic differentiation markers using Western blotting.

Key findings: The expression of RCN2 and Runx2 in femoropopliteal artery plaques was significantly higher in DM than in non-DM patients. In addition, a significant positive correlation was observed between RCN2 and Runx2 levels. RCN2 was highly expressed when HAVSMCs were treated with high glucose and the expression levels correlated with the calcification characteristics. RCN2 upregulated osteogenic transformation markers Runx2 and Osterix in HAVSMCs and downregulated contractile phenotype markers α-SMA and SM22α.

Significance: The results from this study indicate RCN2 is a major factor in mediating the calcification process of HAVSMCs in diabetic conditions. Thus, RCN2 may serve as a future therapeutic target for vascular calcification in diabetes.

1. Background

Calcification aggravates vascular stenosis, which leads to ischemia and infarction of the corresponding organs, such as cerebral infarction, myocardial infarction, and peripheral arterial diseases [1–3]. Arterial calcification was previously considered a physiological phenomenon, however, increased research gradually showed that calcification is an active and regulated process affected by various physiological/pathological factors [4]. Diabetes mellitus (DM) is a main factor promoting vascular calcification, however, the specific mechanism remains unclear [2,5,6].

Vascular smooth muscle cells (VSMCs) contribute to the development of atherosclerotic lesions through increased migration, proliferation, secretion of matrix components, osteogenic differentiation and the associated calcification [7]. During this process, the differentiated VSMCs undergo de-differentiation and subsequently osteogenic transition that results in vascular calcification [8]. Studies of animal models have greatly contributed to the identification of genes and pathways involved in numerous complex human diseases, including atherosclerosis and vascular calcification [9–11]. Reticulocalbin 2 (RCN2, also named ERC-55), is a candidate gene for atherosclerosis [9]. Notably, RCN2 was shown involved in the regulation of cytokine expression in an atherosclerotic mouse model [9]. In another study, RCN2 was shown to regulate blood pressure and contribute to hypertension by affecting the endothelial NO synthases [12]. RCN2 is a 55-kDa Ca^{2+}-binding protein that may regulate calcium metabolism. Calcium and phosphorus metabolism disorders are important causes of vascular calcification [13]. To date, the expression level and potential function of RCN2 in vascular calcification remain unclear.

In this study, whether RCN2 influences vascular calcification enhanced by high glucose and contributes to calcification of human aortic VSMCs (HAVSMCs) by affecting the process of osteogenic

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differentiation was investigated.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Ethics Committee of Shengjing Hospital of China Medical University (Approval number: 2017PS115K). Written informed consent was obtained from the patients participating in the study.

2.2. Immunohistochemical staining (IHCS)

Femoropopliteal artery plaque specimens were obtained from 4 peripheral arterial disease (PAD) patients with type 2 DM and 4 PAD patients without DM using directional atherectomy (TurboHawk Plaque Rotation System). The number of lesion passes was left to the discretion of the interventionist. The material obtained was dissected into 5 mm segments and pools of fragments were formalin-fixed and paraffin-embedded. Paraffin blocks were serially sectioned at 6 μm thickness for immunohistochemical staining (IHCS). Tissue sections were deparaffinized in xylene and incubated with primary antibody overnight at 4 °C. The primary antibodies used against RCN2 and Runt-related transcription factor 2 (Runx2) included rabbit polyclonal antibodies (Orb373714, 1:100; Biorbyt, UK and ab23981, 1:200; Abcam, UK, respectively). Then, samples were incubated with HRP-conjugated goat-α-rabbit secondary antibody (31460, 1:250; Thermo Scientific, USA). Immunoreactivity was visualized with the Diaminobenzidine Substrate Kit (Sigma). Using a low power microscope, the same 3 random fields initially selected were examined, and the percentage of RCN2- and Runx2-positive regions in each section was analyzed using ImageJ V1.8.0 software (National Institutes of Health, Bethesda, MD, USA).

2.3. Cell culture and calcification determination

HAVSMCs were purchased from iCell Bioscience Inc. (Shanghai, China). The cells were cultured in DMEM medium (25 mM glucose) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified atmosphere of 5% CO^2 and 95% air. The cells were cultured until 80% confluent, then transferred to either DMEM containing 5 mM glucose (normal glucose group) or DMEM containing 25 mM glucose (high glucose group). To determine the effects of high glucose on the intracellular calcium content, calcification-inducing medium containing 10 mmol/L β-glycerol phosphoric acid was added to the medium in each group. Media were replaced with fresh agents every 2–3 days. After 14 days of incubation, the experiment was terminated and the cells analyzed. The calcification status was detected by staining with Alizarin Red according to the protocol supplied with the Alizarin Red S staining kit (Solarbio, China) [14]. Intracellular calcium quantification was determined using a calcium assay kit (Nanjing Jiancheng Bioengineering Institute, China) as previously described [15]. The calcium content of the cells was normalized to the cellular protein of the culture and expressed as millimolar per milligram (mM/mg) of protein.

2.4. Silencing or forced expression of target genes

For the generation of shRNA expression vectors, a specific sequence targeting the human RCN2 mRNA sequence (5′-CAGATGTATGATCGT GTGA-3′) was cloned into the pLV-U6-EGFP lentiviral RNAi vector (Wanlei Bio, Shenyang, China). For overexpression, the coding sequence was amplified from cDNA derived from HAVSMCs using primers (RCN2-sense: 5′-CAAGCTAGCATGCGGCTGGGCCCGAGGAC-3′; RCN2-antisense: 5′-CGCACCGGTTTAAAGCTCATCATGATAG-3′) and cloned into the pLV-CMV-EGFP-Pure lentiviral vector (Wanlei Bio, Shenyang, China). Four different types of packaged lentivirus were used to infect HAVSMCs according to the manufacturer's protocols and included RCN2 (RCN2 overexpression), empty vector (EV, RCN2 control), shRCN2 (RCN2 silencing), and shRNA (shRCN2 control).

2.5. Reverse transcription polymerase chain reaction (RT-PCR)

Briefly, total RNA was extracted from HAVSMCs using TRIpure (BioTeke, China) and purity, quality, and concentration assessed based on optical density (OD) at 260/280 nm (Nanodrop2000, Thermo, USA). The cDNA was used for reverse transcription polymerase chain reaction (RT-PCR) analysis to evaluate the relative expression levels of RCN2. The real-time PCR (qPCR) was cycled on a PCR machine (Exicycler™ 96, BIONEER, Korea). In this experiment, 4 parallel experiments were performed for each gene in each sample. The primer sequences were as follows: 5′-CCACGAAGAGCAGCAA-3′ and 5′-ATCCCAAGTCACAGTA TCA-3′ for RCN2. For β-actin, the sequences were as follows: 5'-GGCA CCCAGCACAATGAA-3′ and 5′-TAGAAGCATTTGCGGTGG-3′. RCN2 expression was normalized to β-Actin and the relative expression was calculated using the comparative CT ($2^{-\Delta\Delta CT}$) method.

2.6. Western blot analysis

Proteins from the different conditions were separated on 5–15% SDS-PAGE gels and then transferred to PVDF membranes. After blocking with 1% BSA, the membranes were incubated with the following primary antibodies at 4 °C overnight: anti-RCN2 (Orb373714, 1:1,000; Biorbyt, UK), anti-α-SMA (23081-1-AP, 1:1000; Proteintech, China), anti-SM22α (10493-1-AP, 1:1000; Proteintech), anti-Runx2 (ab23981, 1:1,000; Abcam, UK), anti-Osterix (Bs-1110R, 1:1,000; Bioss, China), and anti-β-actin (60008-1-lg, 1:5,000; Proteintech). After rinsing with TBST, the membranes were incubated with either horseradish peroxidase (HRP)-labeled goat anti-rabbit or rabbit anti-goat IgG at 37 °C for 45 min. The protein bands were visualized using the ECL reagent and imaged (WD-9413B, LIUYI, China). Densitometry was performed using Gel-Pro-Analyzer software (Media Cybernetics, USA) with all samples normalized to β-actin.

2.7. Statistical analysis

The expression of RCN2 and Runx2 in femoropopliteal artery plaques in DM and non-DM patients was compared using independent sample *t*-test. Spearman's correlation coefficient was calculated to evaluate the correlation between RCN2 and Runx2. All *in vitro* experiments were repeated at least 3 times. Results of functional assays are presented as mean ± standard deviation (SD) and data analyzed using one-way ANOVA. Statistical significance was defined as a P-value < 0.05. All analyses were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) and SPSS version 22 (IBM, Armonk, NY, USA).

3. Results

3.1. The expression of RCN2 and Runx2 in femoropopliteal artery plaques was higher in DM than in non-DM patients

IHCS was used to investigate the effects of DM on the expression of RCN2 and Runx2, markers of osteogenic differentiation of VSMCs. The IHCS results are shown in Fig. 1. The DM group had significantly increased RCN2 and Runx2 protein OD values compared with the non-DM group (all $P < 0.05$). These results preliminarily indicated DM promotes the expression of RCN2 and Runx2 in arterial plaques. The correlation between RCN2 and Runx2 was further analyzed and a significant positive correlation was observed between RCN2 and Runx2.

RCN2 is highly expressed when HAVSMCs are subjected to high glucose conditions.

To confirm RCN2 was highly expressed during calcification of the

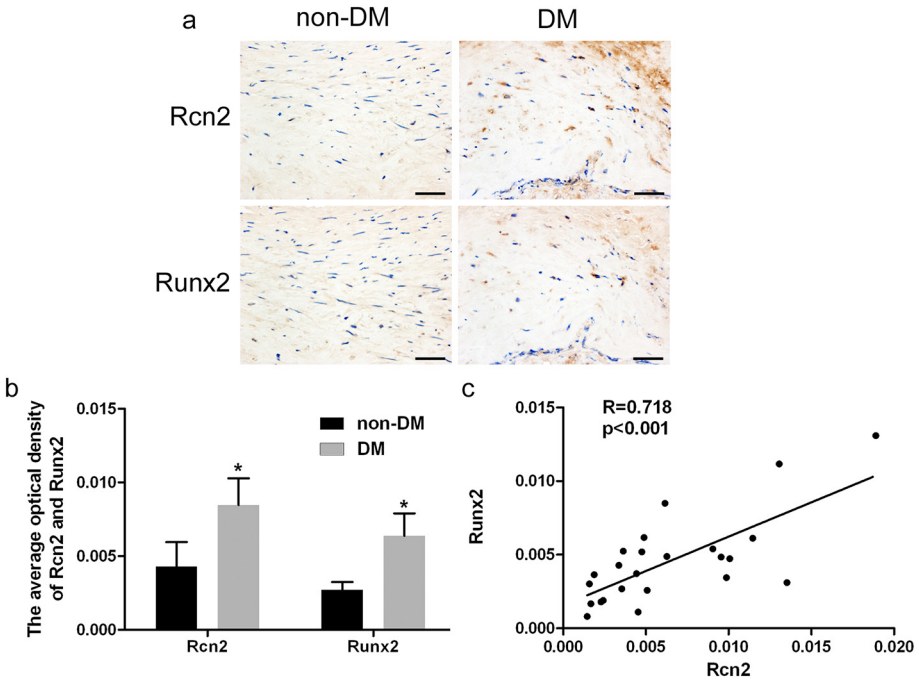

Fig. 1. Increased expression of RCN2 and Runx2 in femoropopliteal artery plaques in DM patients. (a) IHCS results of RCN2 and Runx2 in each group (× 400); (b) Histogram of the RCN2 and Runx2 average OD; (c) RCN2 expression was positively correlated with Runx2. Data were obtained from the results of IHCS analysis of arterial plaques in 4 DM and 4 non-DM patients. Three randomized fields on one section for each patient were analyzed. Scale bar, 50 μm; * $P < 0.05$

OD, optical density; RCN2, Reticulocalbin 2; Runx2, Runt-related transcription factor 2; DM, diabetes mellitus; IHCS, immunohistochemical staining.

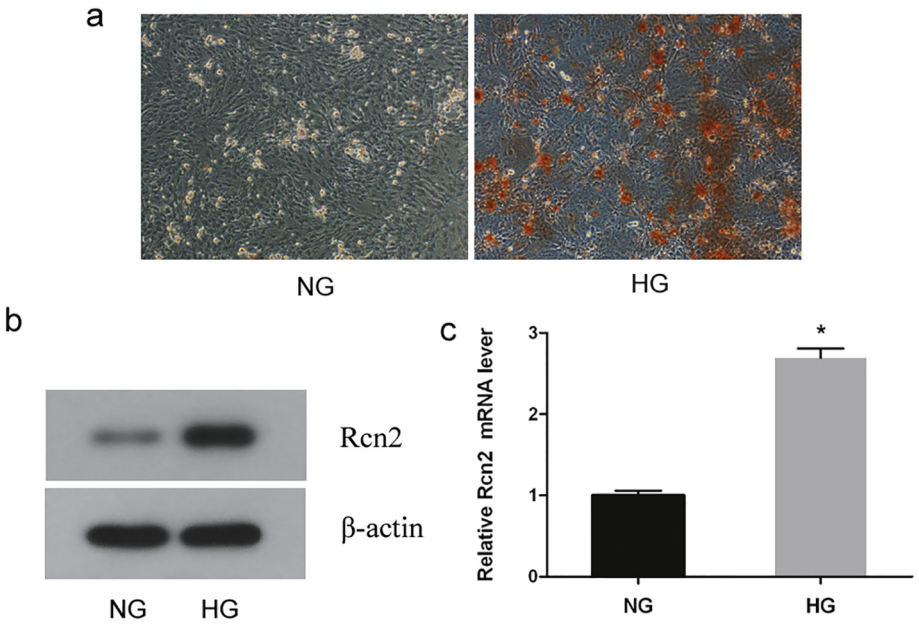

Fig. 2. High glucose enhanced RCN2 expression. (a) HAVSMCs were treated with normal glucose or high glucose concentrations in calcification-inducing media (β-glycerol phosphoric acid, 10 mM) for 14 days, and the calcium deposition was analyzed using Alizarin red staining; (b) Representative Western blotting depicting the effects on RCN2 protein expression; (c) RCN2 mRNA was evaluated using RT-PCR analysis. Values are expressed as mean ± SD, *$P < 0.05$.

RCN2, Reticulocalbin 2; NG, Normal glucose; HG, High glucose; HAVSMCs, human aortic vascular smooth muscle cells; RT-PCR, reverse transcription polymerase chain reaction; SD, standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

VSMC matrix, HAVSMCs were subjected to normal and high glucose media for 14 days. As shown in Fig. 2, HAVSMC calcification induced by high glucose resulted in increased RCN2 mRNA expression. Western blotting for RCN2 confirmed a similar pattern of increased expression in the high glucose group.

3.2. RCN2 is required for in vitro calcification of HAVSMCs

Because RCN2 was highly expressed when HAVSMCs undergo calcification, the functional role of RCN2 in HAVSMCs calcification was further explored. HAVSMC models of RCN2 overexpression or knockdown were established. As shown in Fig. 3, HAVSMCs under RCN2 overexpression and silencing conditions showed significantly increased and decreased mRNA and protein levels, respectively. High glucose promoted intracellular calcium deposition compared with the normal glucose group, which was further enhanced by overexpression of RCN2. Specifically, RCN2 knockdown resulted in significantly decreased cell calcification in HAVSMCs, which was opposite to RCN2 overexpression. These results indicated high RCN2 expression is required for HAVSMC calcification induced by high glucose.

3.3. RCN2 promotes HAVSMC calcification by regulating osteogenic differentiation-related genes

To investigate the correlation between RCN2 and osteogenic differentiation related-genes for promoting calcification capability of HAVSMCs, several osteogenic differentiation factors, such as Runx2 and Osterix, were analyzed in HAVSMCs under RCN2 overexpression and knockdown conditions. In addition, the expression of contractile markers of HAVSMCs, such as SM22α and α-SMA, were simultaneously analyzed. The results indicated osteogenic differentiation markers (Runx2 and Osterix) were upregulated and contractile markers (SM22α

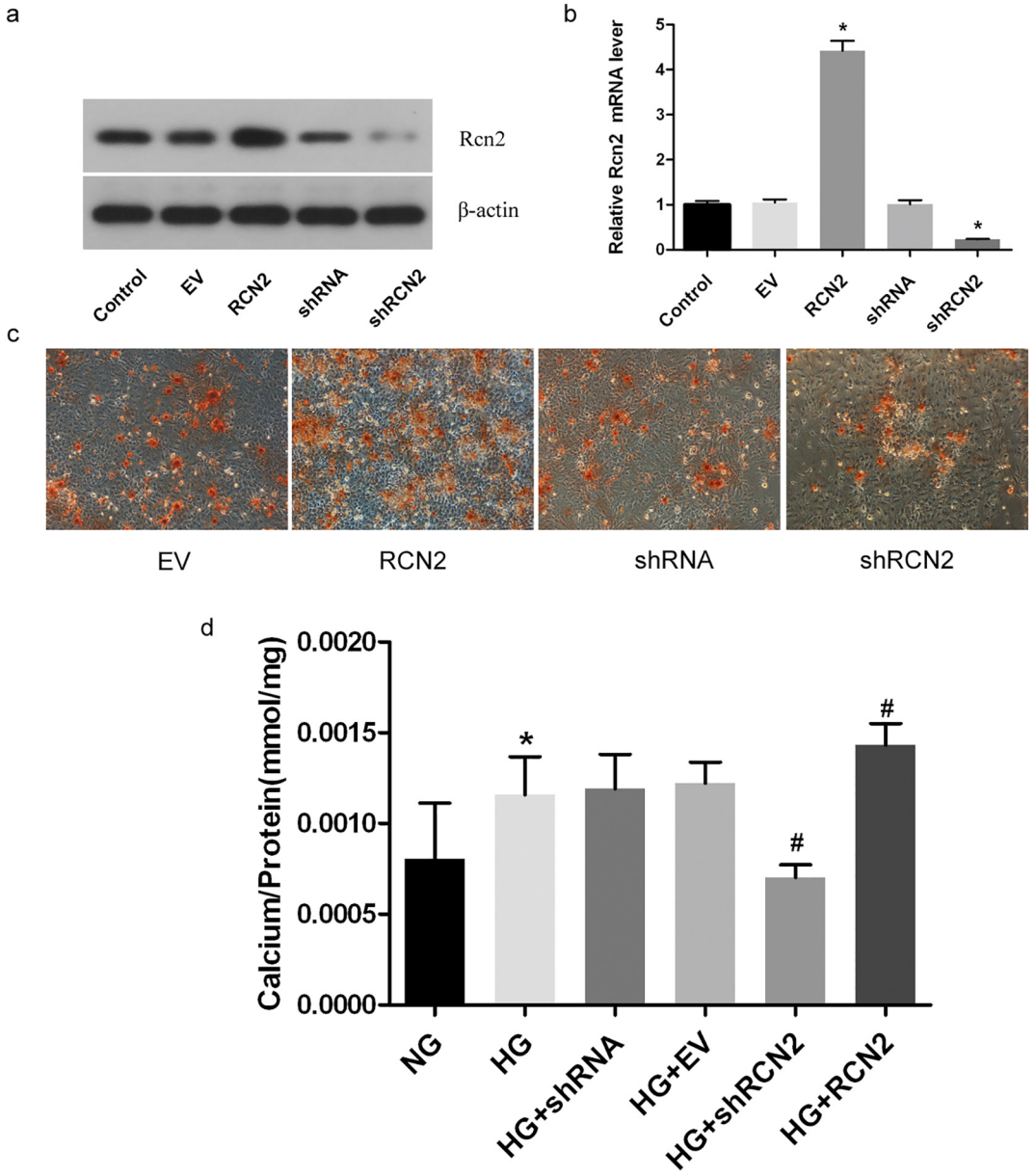

Fig. 3. RCN2 is required for *in vitro* HAVSMC calcification. (a and b) RCN2 overexpression and silencing showed significantly increased and decreased mRNA and protein levels, respectively, in HAVSMCs; (c and d) RCN2 knockdown significantly decreased cell calcification in HVSMCs, opposite to RCN2-overexpression based on Alizarin red staining and intracellular calcium deposition quantification. Data are expressed as mean ± SD. *$P < 0.05$ *vs.*NG; #P < 0.05 *vs.* HG
RCN2, Reticulocalbin 2; NG, Normal glucose; HG, High glucose; HAVSMC, human aortic vascular smooth muscle cell. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

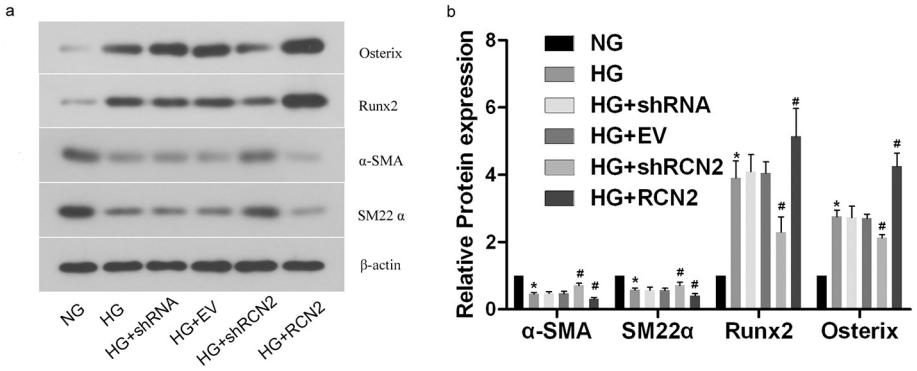

Fig. 4. RCN2 promotes HAVSMC calcification by regulating osteogenic differentiation-related genes. (a) The expression of HAVSMC contractile markers, SM22α and α-SMA, and osteogenic differentiation-related markers, Runx2 and Osterix, in HAVSMCs under RCN2 overexpression and knockdown conditions were analyzed using western blot. (b) The relative protein levels were calculated and β-actin was used as the internal control. Data are expressed as mean ± SD. *$P < 0.05$ *vs.* NG; #$P < 0.05$ *vs.* HG.
HAVSMC, human aortic vascular smooth muscle cell; SD, standard deviation; NG, normal glucose; HG, high glucose; RCN2, Reticulocalbin 2; Runx2, Runt-related transcription factor 2; shRCN2, shRNA expression vector against RCN2; shRNA, shRCN2 control; RCN2, expression vector encoding RCN2; EV, empty vector (RCN2 control).

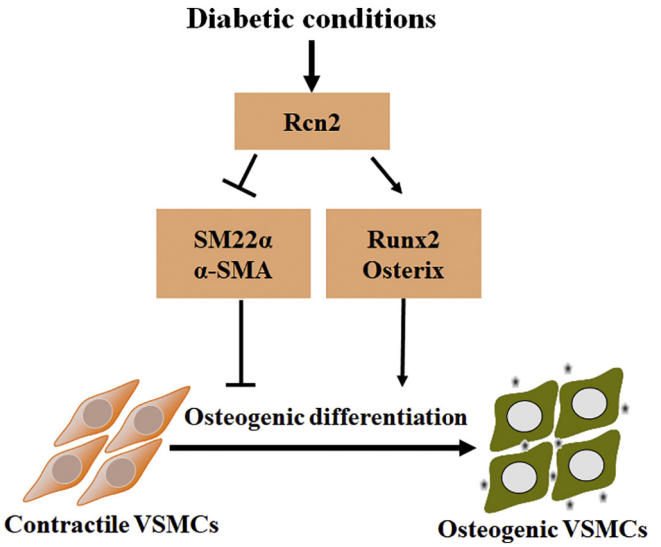

Fig. 5. The schematic representation of osteogenic differentiation markers regulated by RCN2 for HAVSMC calcification in diabetic conditions.
RCN2, Reticulocalbin 2; HAVSMC, human aortic vascular smooth muscle cell.

and α-SMA) were downregulated in HAVSMCs when RCN2 was overexpressed. Similarly, RCN2 knockdown resulted in HAVSMCs with repressed osteogenic differentiation markers and enhanced protein expression levels of contractile markers (Fig. 4).

4. Discussion

Arterial calcification not only aggravates arteriosclerosis occlusion of the lower extremities, but also affects endovascular treatment. Calcification can reduce the technical success rate [16,17], affect the complete expansion of stents or balloons [18], and inhibit drug absorption after drug-eluting balloon expansion [19]. Exploring the mechanisms of different factors affecting calcification will be of great clinical value for intervening or delaying arterial calcification.

With the application of plaque atherectomy, obtaining plaque tissue was possible. In several studies, the protein and gene expression in plaques was analyzed to explore the characteristics of different plaque mechanisms. For example, $P2Y_{12}$ receptor may play a role in coronary plaque destabilization [20]. In another study, gene expression profiles in plaque material from PAD patients showed the existence of a gene signature associated with Notch activation by specific ligands [10]. These results indicate plaque tissue can be used for analysis of differing RCN2 expressions in diabetic and non-diabetic patients.

In the present study, the RCN2 expression in femoropopliteal artery plaques in PAD patients with DM was significantly higher than in non-DM patients. In addition, RCN2 expression significantly correlated with Runx2 expression. The expression level of Runx2 is consistent with the degree of osteogenic differentiation and calcification of VSMCs [4]. Based on the results, RCN2 may play an important regulatory role in the process of hyperglycemia promoting arterial calcification in the lower extremities.

The most important finding from this study was the validation of the role of RCN2 in high glucose-induced osteogenic differentiation and calcification of HAVSMCs *in vitro*. The RCN2 protein level was unregulated in HAVSMCs cultured in high glucose media. Silencing of RCN2 prevented the expression of the osteogenic phenotype in high glucose-stimulated HAVSMCs, and overexpression of RCN2 promoted this process, indicating RCN2 may be a promising therapeutic target for prevention of arterial calcification.

In the process of arterial calcification, the phenotype transition of VSMCs is accompanied by loss of VSMC contractile markers and overexpression of osteogenic profile genes [10,13,21,22]. The results from the present study showed the protein levels of contractile phenotype markers (SM22α and α-SMA) were significantly lower during the process of high glucose-induced calcification, and the osteogenic phenotype markers were significantly higher. In addition, upregulation or downregulation of RCN2 could promote or inhibit the above process. These results indicated RCN2 was responsible for calcification of HAVSMCs by regulating the genes essential for osteogenic differentiation.

In many studies, the regulatory network involved in osteogenic differentiation of VSMCs in diabetic conditions has been explored [21–23]. The role of endoplasmic reticulum stress (ERS) in this process has attracted increasing attention. Death-associated protein kinase 3 deficiency alleviates vascular calcification *via* AMPK-mediated inhibition of ERS [24]. In several studies, activation of transcription factor 4 was shown involved in ERS, contributing to vascular calcification [25,26]. RCN2 is expressed in the endoplasmic reticulum [9] and may be involved in ERS. Further research is necessary to determine whether RCN2 regulates osteogenic differentiation and calcification of VSMCs caused by ERS.

Calcification is a well-recognized complication of atherosclerotic lesions in diabetic patients. The diabetic milieu contributes to the development of calcification in vessels *via* multiple mechanisms. In addition to inducing osteogenic differentiation of VSMCs, calcification also includes hyperglycemia-induced increases in oxidative stress, endothelial dysfunction, renal function-induced alterations in mineral metabolism, and increased inflammatory cytokine production [2]. Increased understanding of diabetic vascular diseases will eventually lead to development of better treatment strategies to delay the progress of calcification and improve clinical outcomes of affected patients.

5. Conclusion

In conclusion, the results from this study revealed the important role of RCN2 in promoting arterial calcification in a high glucose condition. RCN2 enhanced HAVSMC calcification by regulating genes essential for osteogenic differentiation (Fig. 5).

Abbreviations

DM	diabetes mellitus
ERS	endoplasmic reticulum stress
FCS	fetal calf serum
HAVSMCs	human aortic vascular smooth muscle cells
IHCS	immunohistochemical staining
RCN2	Reticulocalbin 2
Runx2	Runt-related transcription factor 2
VSMCs	vascular smooth muscle cells

Authors' contributions

ZC and GY performed the experiments. ZC analyzed and validated the data under supervision of JZ and ZL. HY collected tissue specimens and completed immunohistochemical staining and analysis. JZ contributed to analysis and discussion and wrote the paper. ZC and ZL conceived and designed the study. ZL prepared and submitted the manuscript. All authors read and approved the final manuscript.

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Not applicable.

Declaration of competing interest

The authors declare no competing interests.

Consent for publication

Written informed consent for publication was obtained from all patients participating in the study.

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References

[1] S. Pikija, J. Magdič, T. Hojs-Fabjan, Calcifications of vertebrobasilar arteries on CT: detailed distribution and relation to risk factors in 245 ischemic stroke patients, Biomed. Res. Int. 2013 (2013) 1–7.
[2] K. Yahagi, F.D. Kolodgie, C. Lutter, H. Mori, M.E. Romero, A.V. Finn, R. Virmani, Pathology of human coronary and carotid artery atherosclerosis and vascular calcification in diabetes mellitus, Arterioscler. Thromb. Vasc. Biol. 37 (2) (2017) 191–204.
[3] S.L. Zettervall, A.P. Marshall, P. Fleser, R.J. Guzman, Association of arterial calcification with chronic limb ischemia in patients with peripheral artery disease, J. Vasc. Surg. 67 (2) (2018) 507–513.
[4] A. Shioi, Y. Ikari, Plaque calcification during atherosclerosis progression and regression, J. Atheroscler. Thromb. 25 (4) (2018) 294–303.
[5] A. Mary, A. Hartemann, S. Liabeuf, C.E. Aubert, S. Kemel, J.E. Salem, P. Cluzel, A. Lenglet, Z.A. Massy, J.D. Lalau, et al., Association between metformin use and below-the-knee arterial calcification score in type 2 diabetic patients, Cardiovasc. Diabetol. 16 (1) (2017) 24.
[6] J. Mosch, C.A. Gleissner, S. Body, E. Aikawa, Histopathological assessment of calcification and inflammation of calcific aortic valves from patients with and without diabetes mellitus, Histol. Histopathol. 32 (3) (2017) 293–306.
[7] P. Lacolley, V. Regnault, P. Segers, S. Laurent, Vascular smooth muscle cells and arterial stiffening: relevance in development, aging, and disease, Physiol. Rev. 97 (4) (2017) 1555–1617.
[8] M. Li, P. Wu, J. Shao, Z. Ke, D. Li, J. Wu, Losartan inhibits vascular calcification by suppressing the BMP2 and Runx2 expression in rats in vivo, Cardiovasc. Toxicol. 16 (2) (2016) 172–181.
[9] A. Manichaikul, Q. Wang, Y.L. Shi, Z. Zhang, N. Leitinger, W. Shi, Characterization of Ath29, a major mouse atherosclerosis susceptibility locus, and identification of Rcn2 as a novel regulator of cytokine expression, Am. J. Physiol. Heart Circ. Physiol. 301 (3) (2011) H1056–H1061.
[10] G. Aquila, C. Fortini, A. Pannuti, S. Delbue, M. Pannella, M.B. Morelli, C. Caliceti, F. Castriota, M. de Mattei, A. Ongaro, et al., Distinct gene expression profiles associated with Notch ligands Delta-like 4 and Jagged1 in plaque material from peripheral artery disease patients: a pilot study, J. Transl. Med. 15 (1) (2017).
[11] Z. Chang, C. Huangfu, A.T. Grainger, J. Zhang, Q. Guo, W. Shi, Accelerated atherogenesis in completely ligated common carotid artery of apolipoprotein E-deficient mice, Oncotarget 8 (66) (2017) 110289–110299.
[12] J. Li, S. Cechova, L. Wang, B.E. Isakson, T.H. Le, W. Shi, Loss of reticulocalbin 2 lowers blood pressure and restrains angiotensin II-induced hypertension in vivo, Am J Physiol Renal Physiol 316 (6) (2019) 1141–1150.
[13] W.L. Lau, J.H. Ix, Clinical detection, risk factors, and cardiovascular consequences of medial arterial calcification: a pattern of vascular injury associated with aberrant mineral metabolism, Semin. Nephrol. 33 (2) (2013) 93–105.
[14] A. Ndip, A. Williams, E.B. Jude, F. Serracino-Inglott, S. Richardson, J.V. Smyth, A.J. Boulton, M.Y. Alexander, The RANKL/RANK/OPG signaling pathway mediates medial arterial calcification in diabetic Charcot neuroarthropathy, Diabetes 60 (8) (2011) 2187–2196.
[15] Y. Wang, J. Shan, W. Yang, H. Zheng, S. Xue, High mobility group box 1 (HMGB1) mediates high-glucose-induced calcification in vascular smooth muscle cells of saphenous veins, Inflammation 36 (6) (2013) 1592–1604.
[16] N.K. Itoga, T. Kim, A.M. Sailer, D. Fleischmann, M.W. Mell, Lower extremity computed tomography angiography can help predict technical success of endovascular revascularization in the superficial femoral and popliteal artery, J. Vasc. Surg. 66 (3) (2017) 835–843 (e831).
[17] Z. Chang, J. Zheng, Z. Liu, Subintimal angioplasty for lower limb arterial chronic total occlusions, Cochrane Database Syst. Rev. 11 (2016) CD009418.
[18] H.P. He, J.C. Weng, Y. Zhao, S.H. Cai, X.L. Zhang, H.H. Yin, Impact of plaque calcification and stent oversizing on clinical outcomes of atherosclerotic femoropopliteal arterial occlusive disease following stent angioplasty, Eur. J. Vasc. Endovasc. Surg. 58 (2) (2019) 215–222.
[19] A.R. Tzafriri, F. Garcia-Polite, B. Zani, J. Stanley, B. Muraj, J. Knutson, R. Kohler, P. Markham, A. Nikanorov, E.R. Edelman, Calcified plaque modification alters local drug delivery in the treatment of peripheral atherosclerosis, J. Control. Release 264 (2017) 203–210.
[20] C.W. Lee, I. Hwang, C.S. Park, H. Lee, D.W. Park, S.J. Kang, S.W. Lee, Y.H. Kim, S.W. Park, S.J. Park, Comparison of differential expression of P2Y(1)(2) receptor in culprit coronary plaques in patients with acute myocardial infarction versus stable angina pectoris, Am. J. Cardiol. 108 (6) (2011) 799–803.
[21] M.A. Reddy, S. Das, C. Zhuo, W. Jin, M. Wang, L. Lanting, R. Natarajan, Regulation of vascular smooth muscle cell dysfunction under diabetic conditions by miR-504, Arterioscler. Thromb. Vasc. Biol. 36 (5) (2016) 864–873.
[22] F. Bartoli-Leonard, F.L. Wilkinson, A. Schiro, F.S. Inglott, M.Y. Alexander, R. Weston, Suppression of SIRT1 in diabetic conditions induces osteogenic differentiation of human vascular smooth muscle cells via RUNX2 signalling, Sci. Rep. 9 (1) (2019) 878.
[23] L. Bessueille, M. Fakhry, E. Hamade, B. Badran, D. Magne, Glucose stimulates chondrocyte differentiation of vascular smooth muscle cells and calcification: a possible role for IL-1beta, FEBS Lett. 589 (19 Pt B) (2015) 2797–2804.
[24] C. Gilliland, J. Shah, J.G. Martin, M.J. Miller Jr., Acute limb ischemia, Tech. Vasc. Interv. Radiol. 20 (4) (2017) 274–280.
[25] X.H. Duan, J.R. Chang, J. Zhang, B.H. Zhang, Y.L. Li, X. Teng, Y. Zhu, J. Du, C.S. Tang, Y.F. Qi, Activating transcription factor 4 is involved in endoplasmic reticulum stress-mediated apoptosis contributing to vascular calcification, Apoptosis 18 (9) (2013) 1132–1144.
[26] M. Furmanik, C.M. Shanahan, ER stress regulates alkaline phosphatase gene expression in vascular smooth muscle cells via an ATF4-dependent mechanism, BMC Res Notes 11 (1) (2018) 483.