

RESEARCH

Open Access



Exosomes from hyperglycemia-stimulated vascular endothelial cells contain versican that regulate calcification/senescence in vascular smooth muscle cells

Shuang Li[†], Jun-Kun Zhan[†], Yan-Jiao Wang, Xiao Lin, Jia-Yu Zhong, Yi Wang, Pan Tan, Jie-Yu He, Xing-Jun Cui, Yi-Yin Chen, Wu Huang and You-Shuo Liu^{*†} 

Abstract

Background: To determine whether and how exosomes from human umbilical vein endothelial cells (HUVEC-Exos) regulates vascular smooth muscle cells (VSMCs) calcification/senescence in high glucose condition.

Methods: HUVEC-Exos were isolated from normal glucose (NG) and high glucose (HG) stimulated HUVECs (NG/HG-HUVEC-Exos) by super speed centrifugation. HUVEC-Exos were identified by transmission electron microscopy and Western blot of CD63. Protein profile in HUVEC-Exos was examined to screen the candidate molecules that mediate HUVEC-Exos function. VSMCs were incubated with HUVEC-Exos. A series of functional assays in vitro were performed to assess the effects of HUVEC-Exos on the calcification/senescence of VSMCs. The role of the candidate protein in HUVEC-Exos-induced VSMCs dysfunction was assessed.

Results: Exosomes isolated from HG-HUVEC-Exos induced calcification/senescence in VSMCs as assessed by Alizarin Red Staining, senescence-associated β -galactosidase (SA- β -gal) staining, and the expression of ALP and p21. HG-HUVEC-Exos significantly increased LDH activity, as well as the product of lipid peroxidation (MDA content), and decreased oxidative stress marker activity, as compared with NG-HUVEC-Exos. Moreover, mechanism studies showed that mitochondrial membrane potential and the expression levels of mitochondrial function related protein HADHA and Cox-4 were significantly decreased in HG-HUVEC-Exos compared to controls. Proteomic analysis showed that HG-HUVEC-Exos consisted of higher level of versican (VCAN), as compared with NG-HUVEC-Exos. Observation under laser confocal microscopy revealed that most green fluorescence of VCAN could overlap with the red fluorescence came from mitochondria, indicating VCAN is mainly localized to the mitochondria of VSMCs. Knockdown of VCAN with siRNA in HUVECs, inhibited HG-HUVEC-Exos-induced mitochondrial dysfunction and calcification/senescence of VSMCs.

Conclusions: Our data indicate an intracellular role for VCAN in VSMCs. VCAN participates in hyperglycemia-induced calcification/senescence via modulation of mitochondrial function in VSMCs.

Keywords: Vascular smooth muscle cells, Calcification, Aging, Exosomes, VCAN, Mitochondria

*Correspondence: liuyoushuo@csu.edu.cn

[†]Shuang Li and Jun-Kun Zhan contributed equally to this work
Department of Geriatrics, Institute of Aging and Geriatrics, The Second Xiangya Hospital, Central South University, 139 Renmin Road, Changsha 410011, Hunan, People's Republic of China



Background

In 2013, it was estimated that 382 million people had diabetes worldwide, and by 2035, this was predicted to rise to 592 million [1]. Diabetes and associated complications give rise to a tremendous burden on the healthcare and present major challenges to patients and national economies. Morbidity and mortality of diabetic patients are substantially aggravated by vascular complications including coronary artery, cerebrovascular, and peripheral artery disease. Vascular calcification/aging could influence the threshold, process, severity and prognosis of diabetic vascular complications [2, 3]. A major determinant of vascular aging is vascular calcification, characterized by vascular smooth muscle cells (VSMCs) calcification (Monckeberg's calcification). Transdifferentiation of VSMCs into osteoblasts is considered to be the most critical pathophysiological of VSMCs calcification [4–7]. There is accumulating evidence suggesting that VSMCs calcification/senescence have central roles in the development and progression of diabetes-related cardiovascular disorders [4, 8].

The vascular response to hyperglycemia is a multifactorial process involving endothelial cells (ECs) and VSMCs, although the mechanism by which the information in circulating blood are transferred from ECs to VSMCs is yet to be understood. Signaling between ECs and VSMCs is crucial for the pathogenesis of diabetic vascular calcification/aging. However, how does circulating high glucose affect the calcification/senescence of VSMCs that are not directly contact with the blood? Exosomes, small vesicles with a diameter of 40–100 nm released from various cell types, have gained much attention for their role in intercellular communication [9–11]. Exosomes can transfer active proteins, lipids, small molecules and RNAs from their cell of origin to the target cell [9]. ECs have been demonstrated to secrete exosomes [12, 13], and the transfer of signaling molecules by exosomes may thus provide a way for communicating between ECs and VSMCs. Similarly, prior study has demonstrated that exosomes from senescent ECs promotes VSMCs calcification [14].

Oxidative stress plays a pivotal role in the pathogenesis of diabetic vascular disease. Mitochondria are important cellular organelles, as well as major sites of reactive oxygen species (ROS) generation. Hyperglycemia-induced mitochondrial dysfunction promotes ROS accumulation that, in turn, causes cellular damage [15, 16]. Several studies have linked mitochondrial dysfunction to VSMCs calcification/senescence [17, 18]. Cellular senescence shows a series of changes in morphology and physiology, including a flat and enlarged morphology, cell replication stop, changes in aging-related proteins expression, such as p53, p21 and p16 [19, 20]. VSMCs senescence in vitro enhances calcification and osteogenic markers including

alkaline phosphatase (ALP), collagen 1, and Runt-related transcription factor (Runx2) expression [6, 7, 10].

Therefore, we proposed that exosomes from human umbilical vein endothelial cells (HUVEC-Exos) release their contents into VSMCs, modulating VSMCs calcification/senescence through regulating mitochondrial function. The aims of the present study were to determine: (1) whether HUVEC-Exos regulate VSMCs calcification/senescence, (2) which contents of such HUVEC-Exos might be involved and the associated mechanism of mitochondrial function.

Methods

Cell culture

The HUVECs and VSMCs were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The HUVECs were maintained in F12 K (Hyclone, Logan, Utah, USA) with 10% fetal bovine serum (Sigma, St. Louis, MA, USA) and 1% penicillin and streptomycin. The VSMCs were maintained in F12 (Hyclone, Logan, Utah, USA) with 10% fetal bovine serum and 1% penicillin and streptomycin. These cells were maintained under standard cell culture conditions of 37 °C, 5% CO₂ and 95% humidity.

Isolation of exosomes from HUVECs

HUVECs were seeded in 55 cm² culture flasks. After incubation in exosomes-free medium with normal glucose (5 mmol/l, NG) or high glucose (30 mmol/l, HG) conditions for 48 h, the cell culture supernatant were collected. The supernatant containing exosomes were centrifuged at 2000g for 20 min, 10,000g for 30 min and 14,000g for 20 min. Then, exosomes were isolated with Exosome Isolation Kit (SBI, Palo Alto, CA, USA) according to the manufacturer's protocol. All centrifugations were done at 4 °C. Exosomes were stored at –80 °C or used for the downstream experiments.

Identification of exosomes

The size and morphology feature of exosomes were examined by transmission electron microscopy as described previously in detail [21]. Briefly, exosomes suspension was mixed with an equal volume of 4% paraformaldehyde and deposited on Formvar-carbon-coated EM grids. Images were acquired with a transmission electron microscope (Hitachi, Tokyo, Japan). Exosomal surface marker protein CD63 was identified by Western blot.

Calcification assays

VSMCs calcification was assessed by Alizarin Red Staining. Following co-culture with HUVEC-Exos for 10 days, VSMCs were washed twice with PBS and fixed with 4%

paraformaldehyde. The cells were exposed to 0.2% Alizarin red (pH 8.3, Solarbio, Beijing, China). Subsequent to washing with PBS, cells were visualized by phase microscopy using an inverted microscope (Olympus Corporation, Tokyo, Japan). The ALP protein expression level was detected using Western blot.

Senescence analysis

After incubation with HUVEC-Exos for 48 h, VSMCs were fixed in 2% formaldehyde and 0.2% glutaraldehyde for 10 min at room temperature and then washed with PBS. VSMCs senescence was determined with senescence-associated β -galactosidase (SA- β -gal) Staining Kit (Solarbio, Beijing, China) according to the manufacturer's protocol. The p21 protein expression level was assayed by Western blot.

Mitochondrial membrane potential assay

The mitochondrial membrane potential was assessed by flow cytometry detection of JC-1 fluorescence (Sigma, St. Louis, MO, USA). After culturing with HUVEC-Exos for 48 h, VSMCs (5×10^5) were harvested by centrifugation (5 min at 500g) and then resuspended in 200 μ l of RPMI medium without fetal bovine serum. According to the manufacturer's protocols, cells were left for 20 min at 37 °C in a humidified atmosphere. After this incubation, JC-1 was added to a final concentration of 2.5 μ mol/l, and cells were shaken in the dark at 37 °C for 15 min. Afterwards, cells were counted in a BD FACS Calibur (BD Bioscience, San Jose, CA, USA).

SOD, LDH and MDA determination

As indicators of cellular damage, malondialdehyde (MDA) content and lactate dehydrogenase (LDH) activity were determined using commercial kits (Beyotime, Beijing, China) according to the manufacturer's instructions. The activity of oxidative stress indicator (superoxide dismutase, SOD) was detected using commercial kit (Beyotime, Beijing, China) according to the manufacturer's instructions.

Exosomes labeling and uptake

Exosomes were labeled with the red fluorescent dye CellTracker DiD (AAT Bioquest, Sunnyvale, CA, USA) as described in previous study [22]. Exosomes labeling with CellTracker DiD were performed following the manufacturer's procedures. Exosomes from 1.5×10^8 HUVECs were resuspended in 200 μ l PBS with 12 μ l/ml diluted CellTracker DiD. After 20 min of incubation at room temperature, VSMCs were incubated with the CellTracker DiD-labeled HUVEC-Exos at 37 °C for 2 h. VSMCs were then washed with PBS and fixed with 4% paraformaldehyde for 20 min. After washing with PBS,

nuclei were stained with DAPI (Invitrogen, Carlsbad, CA, USA). The signals were analyzed with a fluorescence microscope.

Proteomic analysis of HUVEC-Exos

The HUVEC-Exos samples were processed for iTRAQ-based quantitative proteomic analysis by Jingjie PTM BioLab (Hangzhou, China). We compared the proteomic content of HUVEC-Exos in HG with NG conditions, using high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS). Gene Ontology (GO) analysis was performed to classify all identified proteins into three categories (cell component, molecular function and biological process) using the UniprotKB database (<http://www.uniprot.org/>), InterProScan (<http://www.ebi.ac.uk/interpro/>) and GO annotation (<http://geneontology.org/>). Differentially expressed proteins were identified with a cutoff of absolute fold change ≥ 1.3 . For each category, a two-tailed Fisher's exact test was employed to test the enrichment of the differentially expressed protein against all identified proteins. The GO with a corrected p value < 0.05 was considered significant.

Immunofluorescent staining

VSMCs and HG-HUVEC-Exos were co-cultured in glass coverslips that had been placed in 6-well culture dishes for 48 h, and then were washed with PBS prior to fixation with 4% formaldehyde. After fixation, cells were washed with PBS and then blocked for 20 min with goat serum. Mitochondria were labeled with fluorescent mitochondrial indicator (ATT, Sunnyvale, CA, USA) according to the manufacturer's protocol. The cells were washed with PBS and incubated overnight at 4 °C with the VCAN antibody (R&D, Minneapolis, Minnesota, USA) at a concentration of 1:200. Cells were then washed with PBS and incubated with Alexa Fluor goat anti-rabbit IgG at a concentration of 1:100 for 40 min at 37 °C. After that, cell nuclei were stained with DAPI. Images were captured using a Leica TSC-SP5 laser confocal scanning microscope.

Western blot analysis

Cells were lysed in a buffer containing 50 mmol/l TrisHCl, 150 mmol/l NaCl, 10 mmol/l EDTA, 1% Triton X-100, 0.2% NaN_3 , 10 μ g/ml Aprotini and protease inhibitors. The lysates were centrifuged at 10,000g for 5 min and supernatants were collected. Protein concentrations were determined, and equal amounts of protein were submitted to SDS-PAGE and transferred onto 0.2 μ m PVDF membranes (Millipore, Temecula, CA, USA). After transfer to PVDF membranes, the membranes were incubated with antibodies that recognize proteins, such as p21 (Cat. No. 60214-1-Ig, 1:1000 dilution),

hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase, alpha subunit (HADHA) (Cat. No. 60250-1-Ig, 1:500) (Proteintech, Rosemont, IL, USA); CD63 (Cat. No. ab59479, 1:500 dilution), ALP (Cat. No. ab67228, 1:500 dilution), VCAN (Cat. No. ab19345, 1:500 dilution), cytochrome oxidase-4 (Cox-4) (Cat. No. ab110261, 1:1000 dilution) and GAPDH (Cat. No. ab125247, 1:4000 dilution) (Abcam, Cambridge, MA, USA) at 4 °C overnight. The membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Cat. No. A32727, Thermo Fisher, Waltham, MA, USA) for 1 h at room temperature. The reaction was visualized with chemiluminescence.

Knockdown of VCAN with siRNA

The specific small interfering RNA (siRNA) and negative control siRNA were synthesized and purchased from Gene Pharmagps (Shanghai, China). The knockdown of the versican (VCAN) gene was performed using siRNA with the following target sequences: VCAN sense, 5'-GAGGCUGGAACUGUUAUUUATT-3'; VCAN antisense, 5'-UAAUAACAGUCCAGCCUCTT-3'; negative control sense, 5'-UUCUCCGAACGUGUCACGUTT-3'; negative control antisense, 5'-ACGUGACACGUUCGGAGAATT-3'. The HUVECs were seeded in six-well plates at 70% confluence. Either VCAN siRNA or negative control siRNA (2 µmol/l) was added to the cells, which underwent transfection using Lipofectamine 3000 Kit (Thermo Fisher, Waltham, MA, USA) according to the manufacturer's protocol. Following transfection for 6 h, the cells were exposed to HG condition.

Real-time PCR analysis

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The reverse transcription reaction was performed using 1 µg of RNA and a RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Burlington, Ontario, CA), according to the manufacturer's protocol. For real-time PCR amplification, the cDNA were amplified using SYBR GreenPCR Master Mix (ABI, New York, NY, USA) and 0.4 µmol/l of each primer pair. Amplification was undertaken using an ABI 7900 real-time PCR system (ABI, New York, NY, USA). Quantitation of the data was performed via the $2^{-\Delta\Delta CT}$ method, using GAPDH gene expression as an endogenous reference. The primer sequences used for the real-time PCR analysis were as follows: VCAN forward, 5'-GTAACCCATGCGCTACATAAAGT-3'; VCAN reverse, 5'-GGCAAAGTAGGCATCGTTGAAA-3'; GAPDH forward, 5'-GGAGCGAGATCCCTCCAAAT-3'; GAPDH reverse, 5'-GGCTGTTGTCATACTTCTCATGG-3'.

Statistical analysis

Results were presented as mean \pm SEM, and analysis was performed with Statistical Product and Service Solutions (version 13.0). Differences between groups were evaluated by one-way analysis of variance (ANOVA), followed by the Bonferroni post hoc test to assess the significance of differences between two groups. The data were based on three independent experiments. A level of $p < 0.05$ was considered significant.

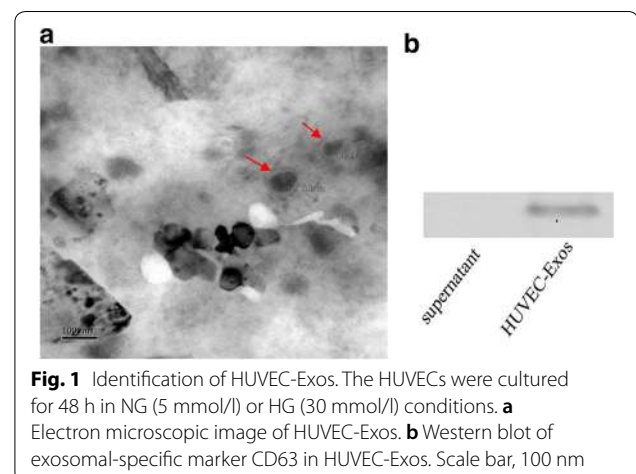
Results

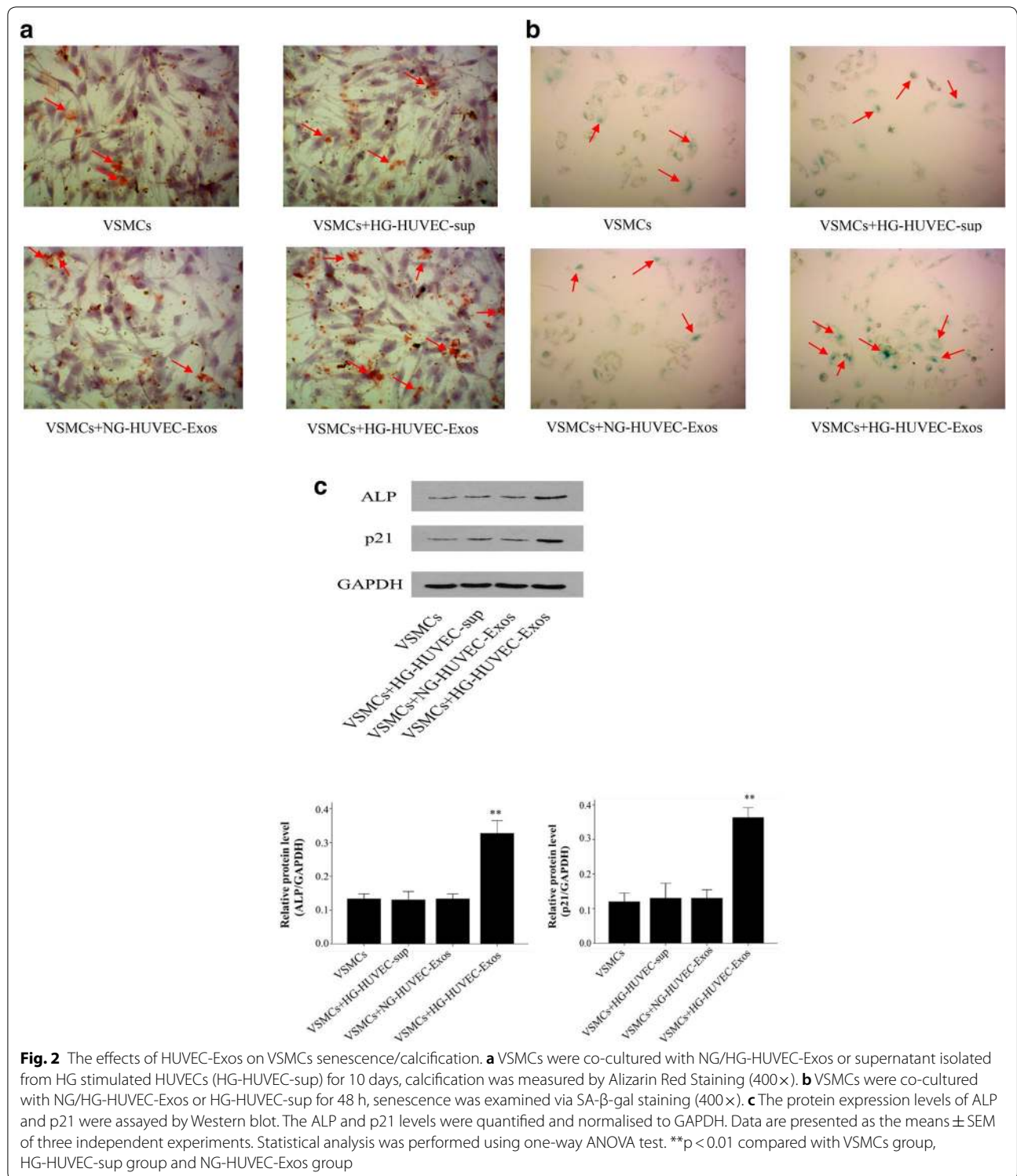
Exosomes extraction and identification

We hypothesized that exosomes were involved in the communication of HUVECs and VSMCs. To test this hypothesis, we first isolated exosomes from the supernatants of NG (5 mmol/l) and HG (30 mmol/l) induced HUVECs. Analysis of transmission electron microscopy showed that the diameter of the HUVEC-Exos were 74.6 ± 8.3 nm (mean \pm SEM) (Fig. 1a). Western blot also confirmed the expression of exosomal-specific marker CD63 on the exosome surface (Fig. 1b).

HG-HUVEC-Exos induce VSMCs calcification/senescence

To determine the ability of HUVEC-Exos in regulating calcification/senescence in hyperglycaemic condition, VSMCs were cultured in the presence of HUVEC-Exos. We found that compared with NG-HUVEC-Exos, HG-HUVEC-Exos induced calcification/senescence of VSMCs as determined by Alizarin Red Staining and SA- β -gal staining, respectively (Fig. 2a, b). These findings were further supported by increased protein levels of ALP and p21 ($p < 0.01$, Fig. 2c), again suggesting that HG-HUVEC-Exos could promote VSMCs calcification/senescence.





HG-HUVEC-Exos induce mitochondrial dysfunction in VSMCs

Oxidative stress is associated with the pathogenesis of vascular calcification/senescence. We next examined

the levels of oxidative stress and cellular damage indicators (LDH, MDA and SOD). As shown in Fig. 3, VSMCs incubated with HG-HUVEC-Exos was associated with a significant up-regulation of LDH activity (*p* < 0.01)

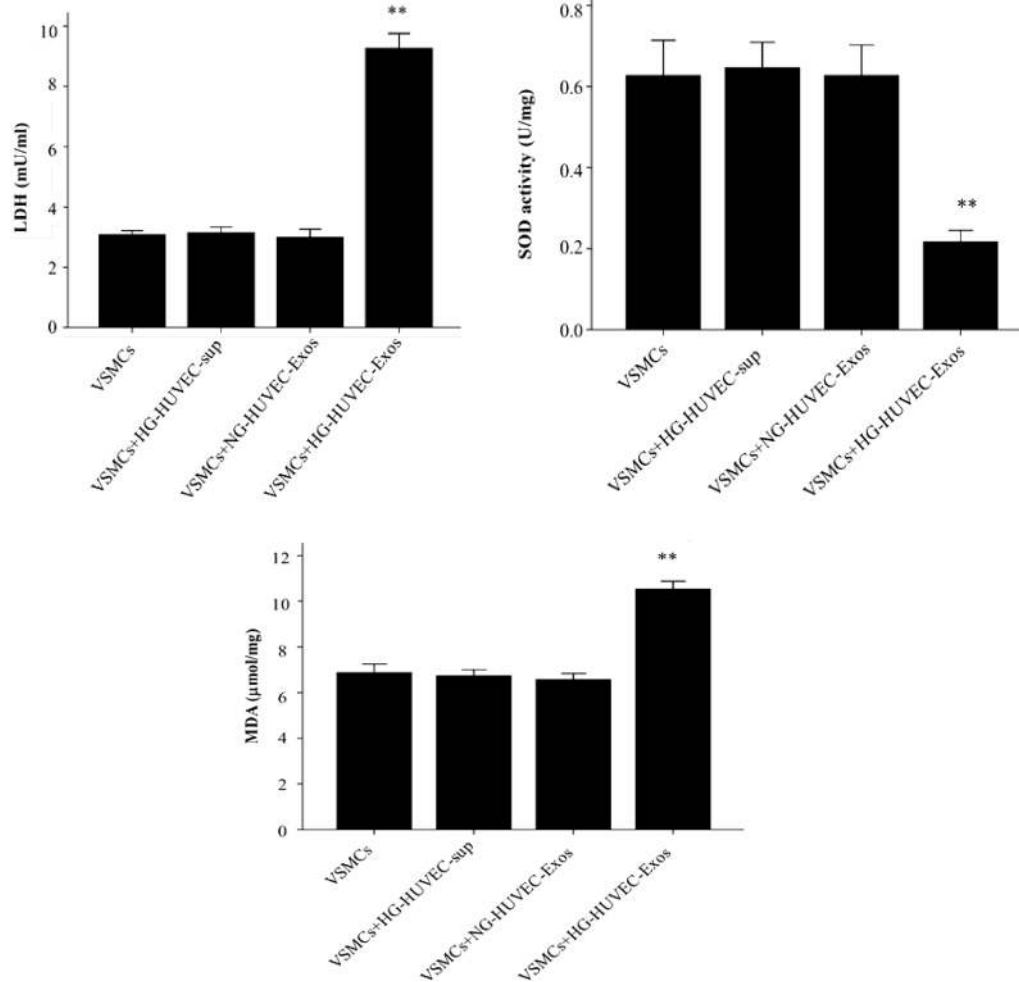


Fig. 3 The effects of HUVEC-Exos on MDA content, LDH and SOD activity. VSMCs were co-cultured with HUVEC-Exos or HG-HUVEC-sup for 48 h. MDA content, LDH and SOD activity were determined by using commercial kits. Data are presented as the means \pm SEM of three independent experiments. Statistical analysis was performed by using one-way ANOVA test. ** $p < 0.01$ compared with VSMCs group, HG-HUVEC-sup group and NG-HUVEC-Exos group

and MDA content ($p < 0.01$) and down-regulation of SOD activity ($p < 0.01$) when compared with NG-HUVEC-Exos group. Mitochondria are a major source of ROS. To determine whether mitochondrial dysfunction mediates HG-HUVEC-Exos induced cellular oxidative stress, we examined mitochondrial membrane potential and mitochondrial function related protein HADHA and Cox-4 expression levels. Compared to NG-HUVEC-Exos group, HG-HUVEC-Exos significantly decreased the mitochondrial membrane potential ($p < 0.01$, Fig. 4a) and protein levels of HADHA and Cox-4 ($p < 0.01$, Fig. 4b).

Capture of HUVEC-Exos by VSMCs

HUVEC-Exos were labeled with CellTracker DiD (red) and cultured for 8 h with VSMCs to determine whether they regulate calcification/senescence by adherence to cell surface and/or by being endocytosed by VSMCs. Confocal microscopy showed the majority of the HUVEC-Exos were located inside the cytoplasmic compartment of VSMCs (Fig. 5).

HUVEC-Exos carry proteins

HUVEC-Exos are able to modulate VSMCs calcification/senescence. It was therefore decided to investigate how they may impact on VSMCs function. After successfully isolated exosomes and extracted exosomal proteins from NG and HG culture conditions, we

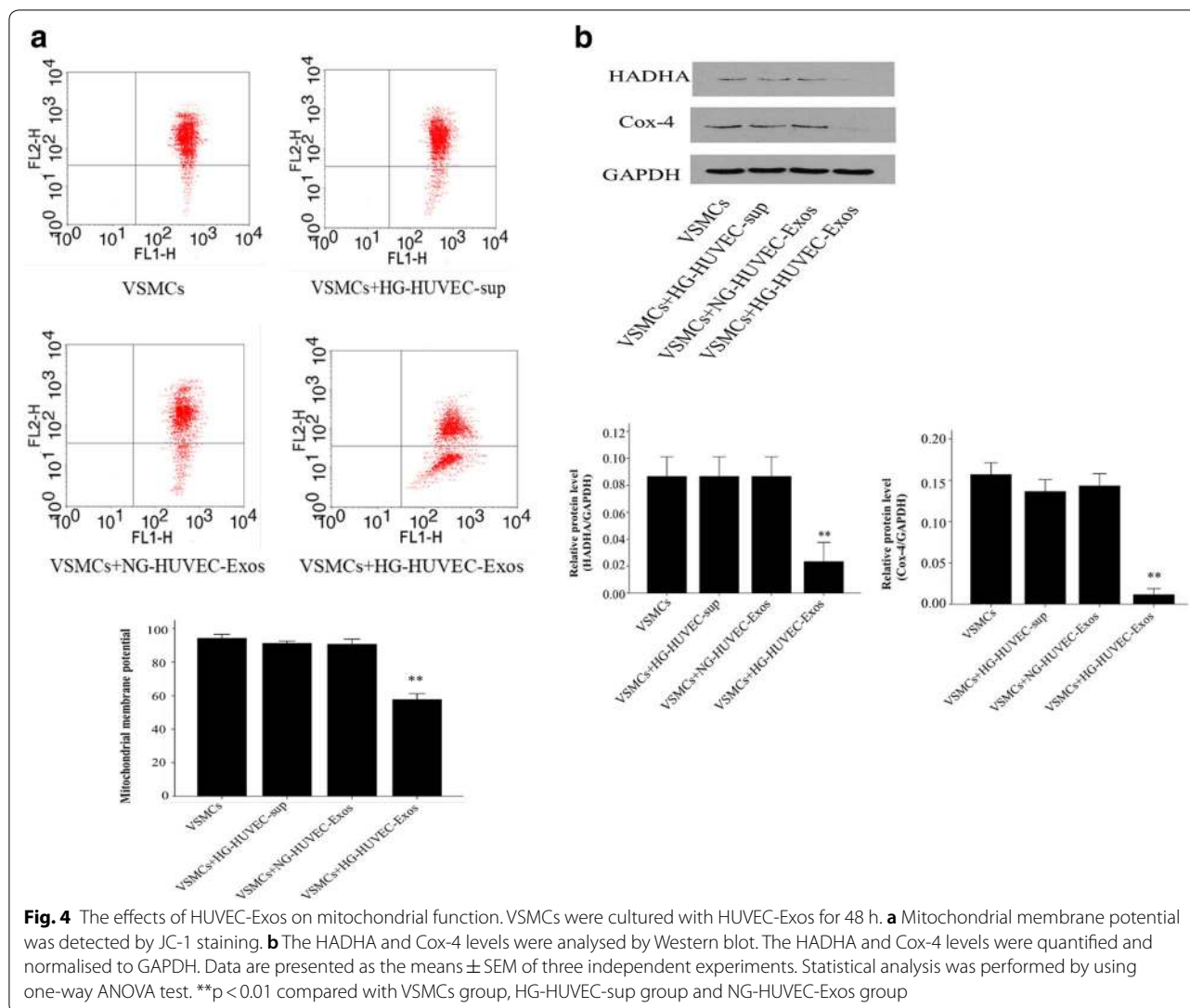


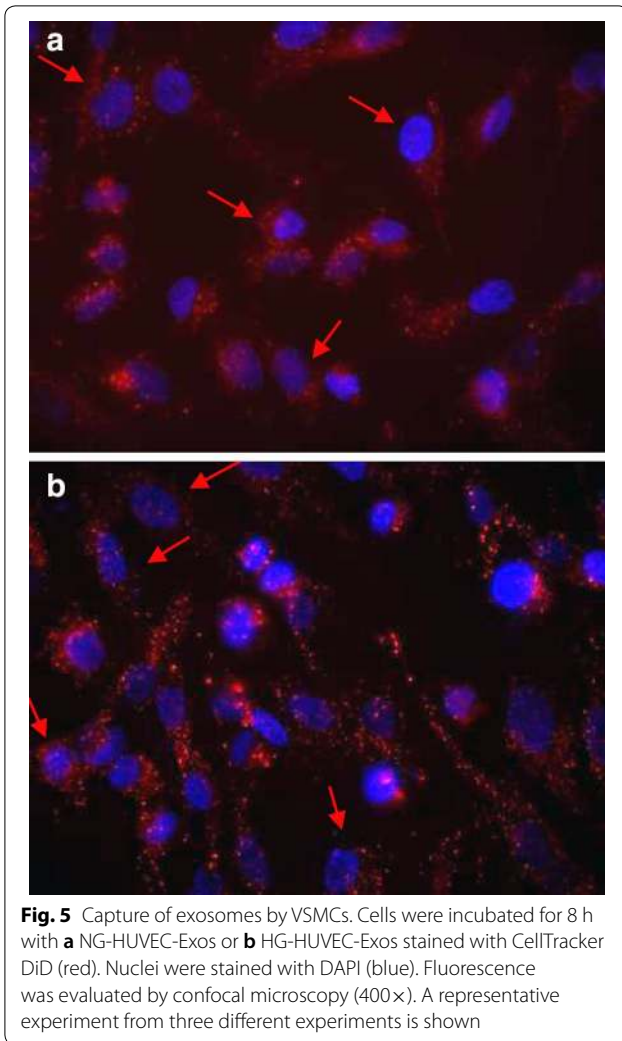
Fig. 4 The effects of HUVEC-Exos on mitochondrial function. VSMCs were cultured with HUVEC-Exos for 48 h. **a** Mitochondrial membrane potential was detected by JC-1 staining. **b** The HADHA and Cox-4 levels were analysed by Western blot. The HADHA and Cox-4 levels were quantified and normalised to GAPDH. Data are presented as the means \pm SEM of three independent experiments. Statistical analysis was performed by using one-way ANOVA test. ** $p < 0.01$ compared with VSMCs group, HG-HUVEC-sup group and NG-HUVEC-Exos group

conducted HPLC–MS/MS analysis and identified a total of 569 distinct proteins. 74 proteins were up-regulated and 105 were down-regulated using the cutoff value of 1.3-fold change. The identified proteins were investigated to assess their subcellular localization and molecular function by UniprotKB. These results helped us to focus on VCAN protein. We found that, unlike NG condition, HG treatment was associated with a significant up-regulation of VCAN (Fig. 6).

VCAN localization and its role in HG-HUVEC-Exos-induced VSMCs calcification/senescence

We have observed that HG-HUVEC-Exos induce mitochondrial dysfunction in VSMCs; thus, we sought to investigate the localization of VCAN in VSMCs. Merged

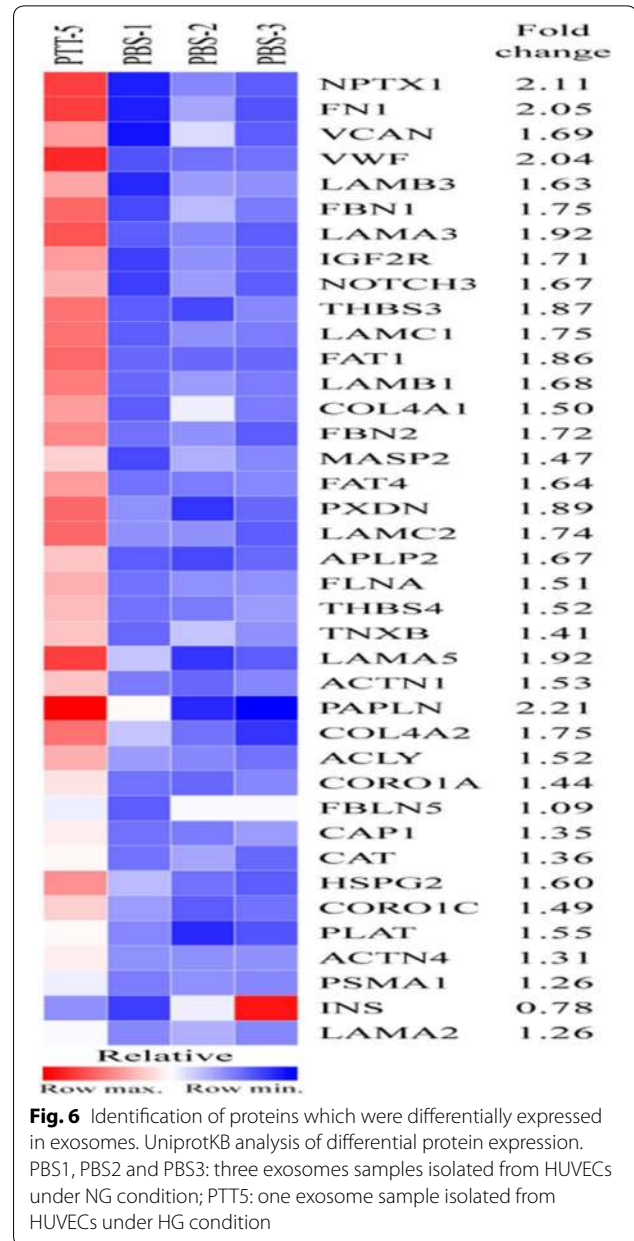
images showed the aggregation of VCAN in the mitochondria (orange), hinting of a mitochondrial localization of VCAN in VSMCs (Fig. 7). To determine the role of VCAN in HG-HUVEC-Exos-induced VSMCs calcification/senescence, VCAN RNA silencing was utilized. As depicted in Fig. 8a and Additional file 1: Figure S1, both VCAN mRNA and protein expression were successfully knocked out by VCAN-siRNA in HUVECs ($p < 0.01$). As expected, HG-HUVEC-Exos with VCAN-depleted were no longer able to either decrease SOD, or increase LDH, MDA levels ($p < 0.01$, Fig. 8b) or induce cellular calcification/senescence in VSMCs (Figs. 9, 10). Knock-down of VCAN upregulated HADHA and Cox-4 protein expression (Fig. 10). Moreover, VCAN knockdown led to upregulation of the mitochondrial membrane



potential (Fig. 11). These results suggest that VCAN might be involved in HG-HUVEC-Exos-induced VSMCs calcification/senescence.

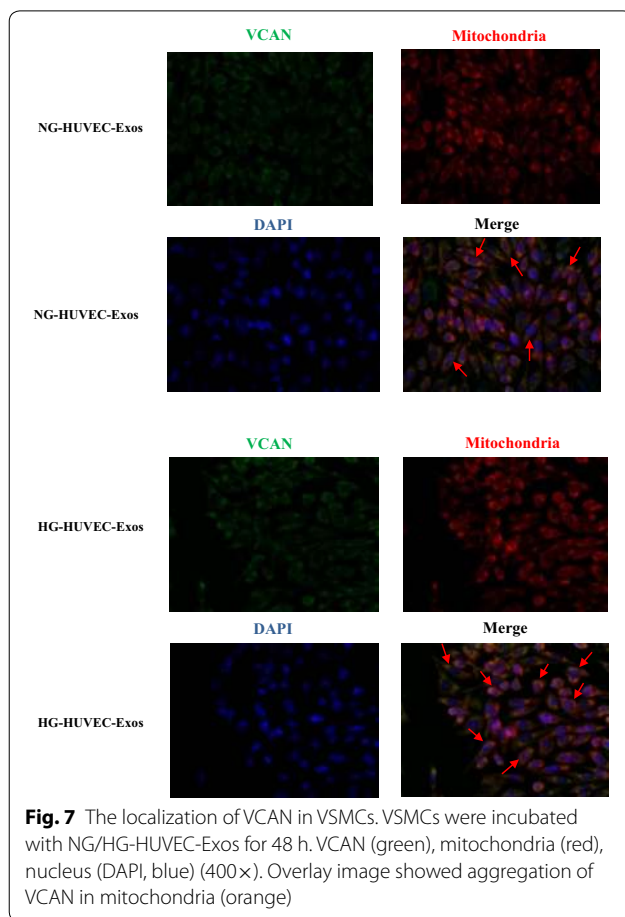
Discussion

Vascular complications account for a substantial proportion of the morbidity and mortality that occurs in patients with diabetes. Vascular calcification/aging are established as severe risk factors for diabetic vascular complications [2, 3]. As a main constituent cell type in tunica-media layer, VSMCs calcification/senescence could be a focal point to explain the pathological progression of diabetic vascular dysfunction. However, how the information in circulating blood is transferred from tunica intima to tunica media? Many studies have found that exosomes can transfer functional molecules to target cells and serve as mediators of cell-to-cell crosstalk under physiological and pathological conditions. Recently, new evidence has revealed that microvesicles produced



by senescent cultured ECs promoted calcification in VSMCs [14]. However, in HG environment, whether exosomes can mediate the interaction between HUVECs and VSMCs and participate in the pathogenesis of diabetic vascular damage remains unclear. Our research reveals that VCAN-rich exosomes were secreted from HG-HUVECs and taken up by recipient VSMCs. Upon uptake by VSMCs, VCAN promoted calcification/senescence of VSMCs by inducing mitochondrial dysfunction.

In diabetic patients, long-term hyperglycaemia can induce vascular medial calcification/aging [23]. Cellular senescence is characterized by changes in cell



morphology and gene expression. During the onset of vascular calcification, VSMCs in the tunica media transdifferentiate into osteoblast-like phenotype [24]. In accordance with previous study [23], our data suggest that HG-HUVEC-Exos induce VSMCs calcification/senescence, as determined by Alizarin Red Staining and SA- β -gal staining. Furthermore, the protein expression of ALP and p21 in VSMCs were also increased in HG-HUVEC-Exos group than that in NG-HUVEC-Exos group.

Excessive ROS is known to mediate VSMCs calcification/senescence [25–27]. Mitochondria are the primary sources of ROS [28]. In fact, it has been proposed that overproduction of ROS that occurs in response to mitochondrial dysfunction in diabetes, is the major mechanism by which hyperglycemia exerts its influence on vascular damage [29–31]. In this regard, we examined mitochondrial function in VSMCs treated with HUVEC-Exos. Our study showed that elevated glucose concentration results in decreased mitochondrial membrane potential in VSMCs. In addition, HG-HUVEC-Exos decreased the expression levels of Cox-4 and HADHA in VSMCs by 15- and 9-fold compared to NG condition,

respectively. Cox is a terminal enzyme of the mitochondrial electron transfer chain. HADHA is the alpha subunit of mitochondrial trifunctional protein. These results above suggest that HG-HUVEC-Exos could induce mitochondrial dysfunction in VSMCs. Our results is consistent with previous studies showing that mitochondrial dysfunction leads to cellular calcification/senescence of VSMCs [25, 32]. MDA, which is produced by lipid peroxidation of polyunsaturated fatty acids, is commonly used for measuring oxidative stress levels [33]. MDA levels increases under oxidative stress [34]. LDH is an intracellular enzyme present in cell cytoplasm, and its leakage from the cells is considered as an important indicator of cellular damage. In this study, treatment with HG-HUVEC-Exos increased the levels of MDA and LDH in VSMCs, as compared with NG-HUVEC-Exos group. The activity of SOD, an important antioxidant enzyme involved in antioxidant defenses, decreased significantly in VSMCs incubating with HG-HUVEC-Exos when compared with NG-HUVEC-Exos. Taken together, these results indicate that mitochondria played a critical role in inducing oxidative stress and subsequent cellular calcification/senescence of VSMCs induced by HG-HUVEC-Exos.

Exosomes are defined as microvesicles of 40–100 nm in diameter. The size distribution (74.6 ± 8.3 nm) and the enrichment of CD63 in isolated vesicles concur with the criteria for defining exosomes [35]. Because the role of exosomes is to transport miRNA, mRNA and protein from secretory cells to recipient cells [36], we conducted HPLC–MS/MS analysis and identified a total of 569 distinct proteins. The proteomic analysis assisted us to focus on VCAN among 179 proteins with altered levels. VCAN is a chondroitin sulphate proteoglycan that is found in the extracellular matrix (ECM) of many tissues in the body. A number of studies have shown that VCAN is associated with cardiovascular diseases including atherosclerosis, postangioplasty restenosis, pulmonary arterial hypertension and others [37–40]. VCAN is known to regulate smooth muscle cell retention of lipoproteins [40], growth and migration [41] and inflammation [42]. VCAN regulate cell adhesion, cell migration, and ECM assembly by direct or indirect interactions with cells and molecules [40]. Because of the overwhelming presence of VCAN in the ECM, there are only a few studies that have reported an intracellular localization of VCAN in different cells [43–45]. In this study, we provided clear evidence of VCAN's presence in the mitochondria of VSMCs. Mitochondria usually act as metabolic headquarters. Molecular fuel, adenosine 5'-triphosphate (ATP) and ROS are produced in the mitochondria. Given the importance of mitochondria on cellular function, it is plausible that VCAN serves a vital role inside VSMCs. Indeed, our

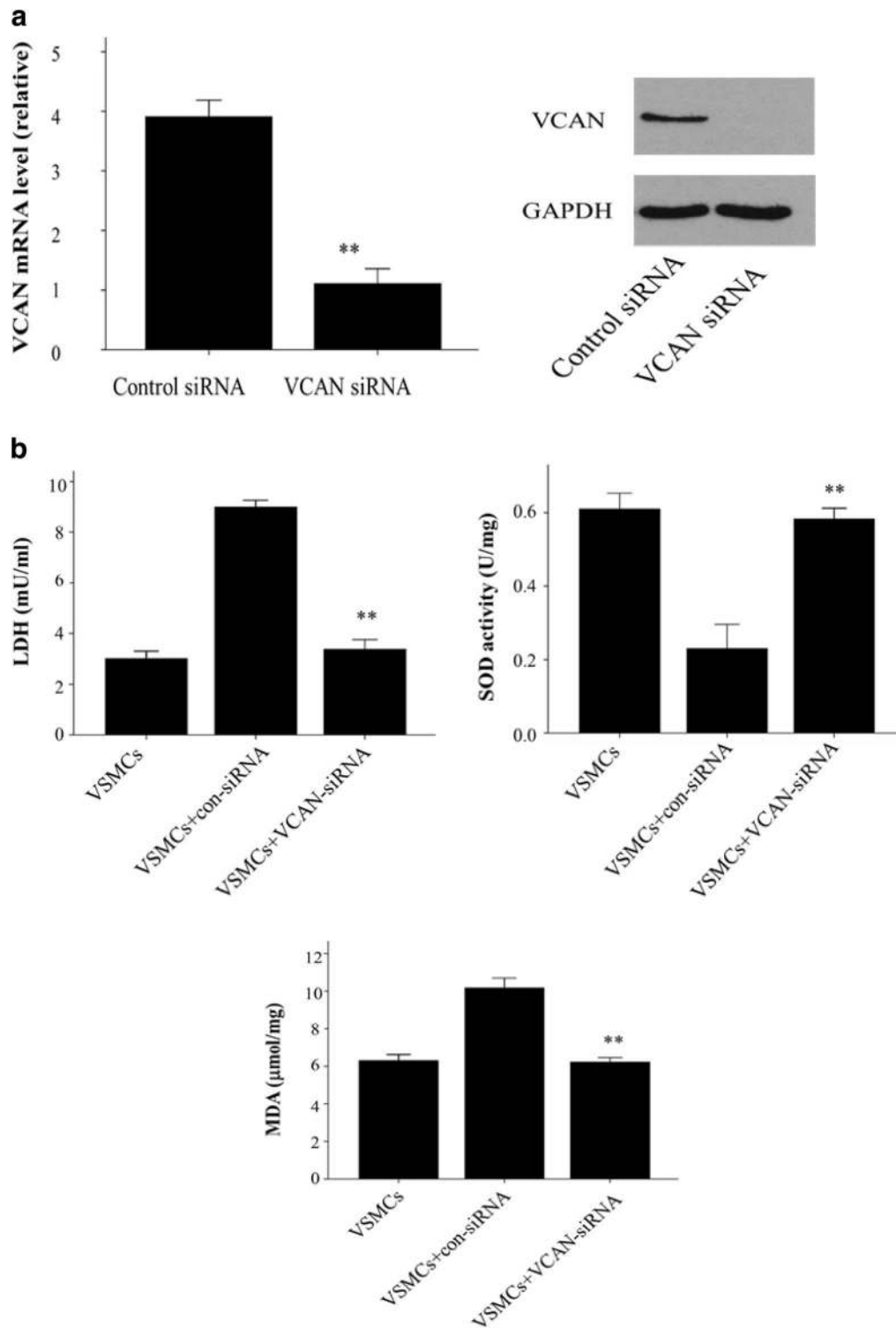


Fig. 8 The role of VCAN in HG-HUVEC-Exos-induced VSMCs senescence/calcification. HUVECs were transfected with negative control siRNA (con-siRNA) or VCAN siRNA (VCAN-siRNA) for 6 h and then exposed to HG condition for 48 h. After that, exosomes were isolated and co-cultured with VSMC for 48 h. **a** VCAN mRNA and protein expression were assessed by qRT-PCR and Western blot. **b** MDA content, LDH and SOD activity were determined by using commercial kits. Data are presented as the means \pm SEM of three independent experiments. Statistical analysis was performed by using one-way ANOVA test. **p < 0.01 compared with con-siRNA group

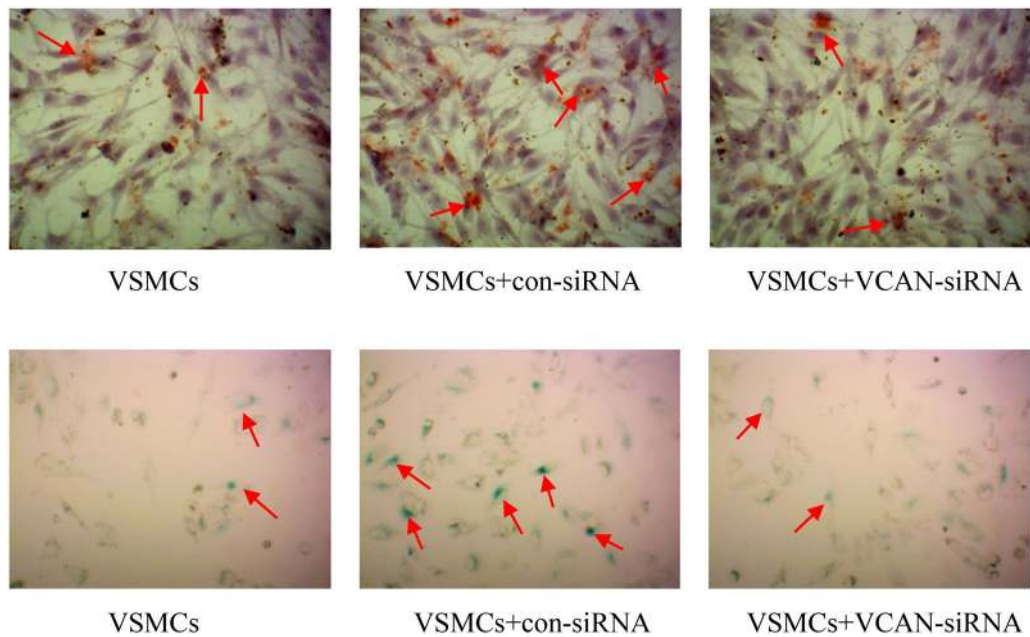


Fig. 9 The role of VCAN in HG-HUVEC-Exos-induced VSMCs senescence/calcification. HUVECs were transfected with negative control siRNA (con-siRNA) or VCAN siRNA (VCAN-siRNA) for 6 h and then exposed to HG condition for 48 h. After that, exosomes were isolated and co-cultured with VSMC for 48 h. VSMCs were co-cultured with HUVEC-Exos for 10 days/48 h, calcification/senescence were measured by Alizarin Red Staining/SA-β-gal staining, respectively (400×)

study suggests a function for VCAN in regulating mitochondrial function of VSMCs. Downregulation of VCAN by siRNA in HUVECs resulted in decreased oxidative stress levels and improved mitochondrial function in VSMCs. Collectively, these findings suggest that VCAN could, at least in part, induce mitochondrial dysfunction in VSMCs. These observations open a new avenue for study of VCAN, implying even more versatile roles for this proteoglycan than previously described.

Given the importance of VCAN to the normal function of ECM, it is difficult to differentiate between intracellular and extracellular functions of this proteoglycan. To this note, one of the limitations of this study is that we cannot rule out the possibility that the changes seen in VSMCs induced by HG-HUVEC-Exos were in fact secondary to altered VCAN in the extracellular environment. In addition, we cannot exclude the possibility that other exosomal proteins with altered levels might play a

minor role in HG-HUVEC-Exos-induced VSMCs dysfunction, which needs future examination.

Despite the limitations, our data suggest a functional role for VCAN inside VSMCs. VCAN carried by HG-HUVEC-Exos promotes VSMCs calcification/senescence, probably by inducing mitochondrial dysfunction. Since VSMCs calcification/senescence could induce vascular dysfunction, blockage of the exosome-mediated transfer of VCAN between these two cells may serve as a potential therapeutic target against diabetic vascular complications. More work will be needed to explore this possibility and to better understand the intracellular roles of VCAN.

Conclusion

In summary, here we report a study revealing that HG-HUVEC-Exos contain functional VCAN that is deliverable to VSMCs to induce calcification/senescence via

(See figure on next page.)

Fig. 10 The role of VCAN in HG-HUVEC-Exos-induced VSMCs senescence/calcification. HUVECs were transfected with negative control siRNA (con-siRNA) or VCAN siRNA (VCAN-siRNA) for 6 h and then exposed to HG condition for 48 h. After that, exosomes were isolated and co-cultured with VSMC for 48 h. The protein expression of ALP, p21, HADHA and Cox-4 were examined by Western blot. The ALP, p21, HADHA and Cox-4 levels were quantified and normalized to GAPDH. Data are presented as the means \pm SEM of three independent experiments. Statistical analysis was performed by using one-way ANOVA test. ** $p < 0.01$ compared with con-siRNA group

modulation of mitochondrial function. A better understanding of the relationship between VCAN and mitochondrial dysfunction will help us to gain insight into the pathogenesis of diabetic vascular damage.

Additional file

Additional file 1: Figure S1. The original film of Fig. 8a. HUVECs were transfected with negative control siRNA (con-siRNA) or VCAN siRNA (VCAN-siRNA) for 6 h. VCAN protein expression was assessed by Western blot. HUVECs (left panel), con-siRNA (middle panel) and VCAN-siRNA (right panel).

Abbreviations

ECs: endothelial cells; VSMCs: vascular smooth muscle cells; ROS: reactive oxygen species; ALP: alkaline phosphatase; Runx2: Runt-related transcription factor; HUVECs: human umbilical vein endothelial cells; HUVEC-Exos: exosomes from human umbilical vein endothelial cells; NG/HG-HUVEC-Exos: exosomes from normal/high glucose stimulated HUVECs; NG: normal glucose; HG: high glucose; (SA- β -gal) staining: senescence-associated β -galactosidase staining; MDA: malondialdehyde; LDH: lactate dehydrogenase; SOD: superoxide dismutase; HPLC-MS/MS: high-performance liquid chromatography tandem mass spectrometry; GO analysis: Gene Ontology analysis; HADHA: hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase, alpha subunit; Cox-4: cytochrome oxidase-4; siRNA: small interfering RNA; VCAN: versican; ANOVA: one-way analysis of variance; ECM: extracellular matrix; HG-HUVEC-sup: supernatant isolated from HG stimulated HUVECs; con-siRNA: negative control siRNA; VCAN-siRNA: VCAN siRNA.

Authors' contributions

Conception and design of the research: SL, JKZ, YSL. Acquisition of data: YJW, XL, JYZ. Analysis and interpretation of the data: YW, PT, JYH. Statistical analysis: XJC, YYC, WH. Drafting of the manuscript: SL, JKZ, YSL. Supervision: YSL. Critical revision of the manuscript: YSL. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data and materials are available upon request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

Funding

This work was supported by the National Natural Science Foundation of China (Grant Nos. 81770833, 81501212) and the Natural Science Foundation of Hunan Province (Grant No. 2018JJ3721).

Publisher's Note

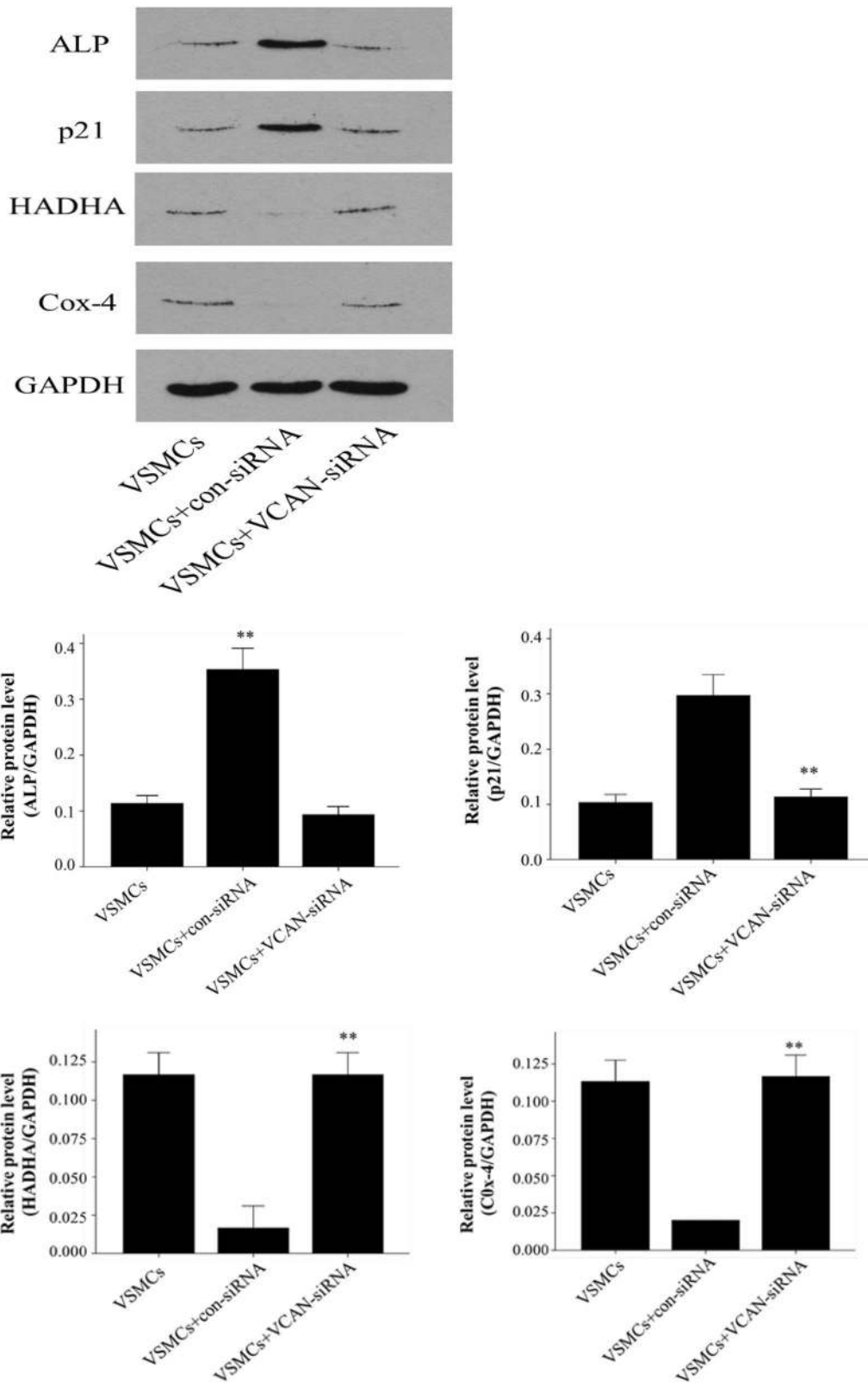
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 14 September 2018 Accepted: 21 December 2018

Published online: 03 January 2019

References

- Nanditha A, Ma RC, Ramachandran A, Snehalatha C, Chan JC, Chia KS, Shaw JE, Zimmet PZ. Diabetes in Asia and the Pacific: implications for the Global Epidemic. *Diabetes Care*. 2016;39(3):472–85.
- Sinha A, Vyavahare NR. High-glucose levels and elastin degradation products accelerate osteogenesis in vascular smooth muscle cells. *Diab Vasc Dis Res*. 2013;10(5):410–9.
- Hayashi T, Kotani H, Yamaguchi T, Taguchi K, Iida M, Ina K, Maeda M, Kuzuya M, Hattori Y, Ignarro LJ. Endothelial cellular senescence is inhibited by liver X receptor activation with an additional mechanism for its atheroprotection in diabetes. *Proc Natl Acad Sci USA*. 2014;111(3):1168–73.
- Zhan JK, Wang YJ, Wang Y, Tang ZY, Tan P, Huang W, Liu YS. The protective effect of GLP-1 analogue in arterial calcification through attenuating osteoblastic differentiation of human VSMCs. *Int J Cardiol*. 2015;189:188–93.
- Zhan JK, Wang YJ, Wang Y, Wang S, Tan P, Huang W, Liu YS. The mammalian target of rapamycin signalling pathway is involved in osteoblastic differentiation of vascular smooth muscle cells. *Can J Cardiol*. 2014;30(5):568–75.
- Zhan JK, Wang YJ, Wang Y, Tang ZY, Tan P, Huang W, Liu YS. Adiponectin attenuates the osteoblastic differentiation of vascular smooth muscle cells through the AMPK/mTOR pathway. *Exp Cell Res*. 2014;323(2):352–8.
- Zhan JK, Tan P, Wang YJ, Wang Y, He JY, Tang ZY, Huang W, Liu YS. Exenatide can inhibit calcification of human VSMCs through the NF- κ B/RANKL signaling pathway. *Cardiovasc Diabetol*. 2014;13:153.
- Zhao L, Li AQ, Zhou TF, Zhang MQ, Qin XM. Exendin-4 alleviates angiotensin II-induced senescence in vascular smooth muscle cells by inhibiting Rac1 activation via a cAMP/PKA-dependent pathway. *Am J Physiol Cell Physiol*. 2014;307(12):C1130–41.
- Yáñez-Mó M, Siljander PR, Andreu Z, et al. Biological properties of extracellular vesicles and their physiological functions. *J Extracell Vesicles*. 2015;4:27066.
- Bobrie A, Théry C. Exosomes and communication between tumours and the immune system: are all exosomes equal? *Biochem Soc Trans*. 2013;41(1):263–7.
- Camussi G, Deregibus MC, Bruno S, Cantaluppi V, Biancone L. Exosomes/microvesicles as a mechanism of cell-to-cell communication. *Kidney Int*. 2010;78(9):838–48.
- Xiao B, Chai Y, Lv S, Ye M, Wu M, Xie L, Fan Y, Zhu X, Gao Z. Endothelial cell-derived exosomes protect SH-SY5Y nerve cells against ischemia/reperfusion injury. *Int J Mol Med*. 2017;40(4):1201–9.
- Lin X, He Y, Hou X, Zhang Z, Wang R, Wu Q. Endothelial cells can regulate smooth muscle cells in contractile phenotype through the miR-206/ARF6&NCX1/exosome axis. *PLoS ONE*. 2016;11(3):e0152959.



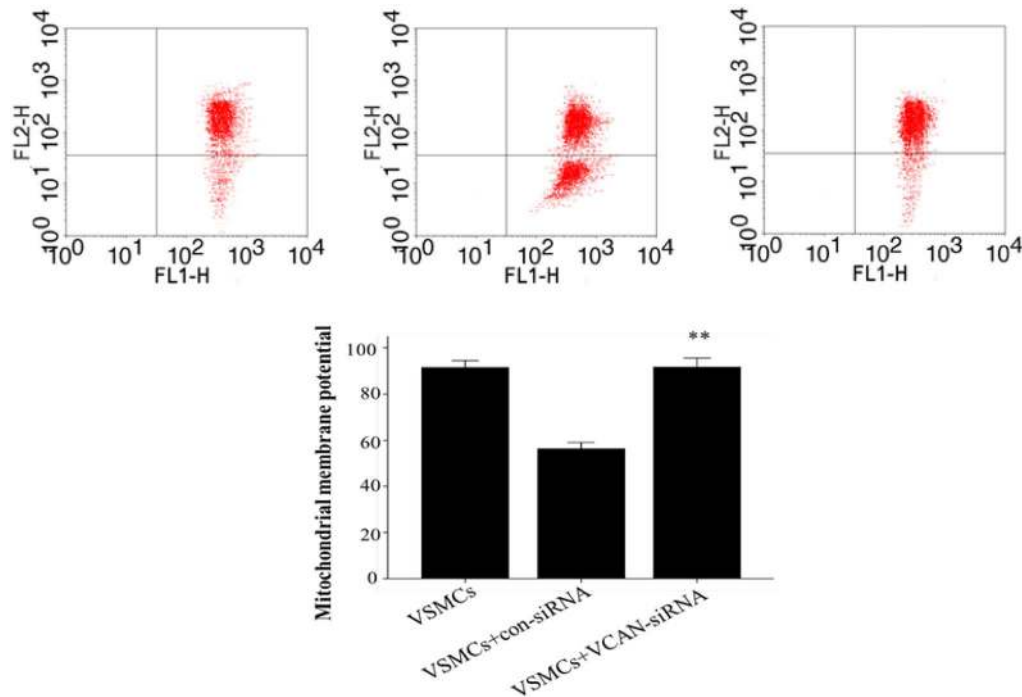


Fig. 11 The role of VCAN in HG-HUVEC-Exos-induced mitochondrial dysfunction. HUVECs were transfected with negative control siRNA (con-siRNA) or VCAN siRNA (VCAN-siRNA) for 6 h and then exposed to HG condition for 48 h. After that, exosomes were isolated and co-cultured with VSMC for 48 h. Mitochondrial membrane potential was detected by JC-1 staining. Data are presented as the means \pm SEM of three independent experiments. Statistical analysis was performed by using one-way ANOVA test. ** $p < 0.01$ compared with con-siRNA group

- Alique M, Ruiz-Torres MP, Bodega G, Noci MV, Troyano N, Bohórquez L, Luna C, Luque R, Carmona A, Carracedo J, Ramírez R. Microvesicles from the plasma of elderly subjects and from senescent endothelial cells promote vascular calcification. *Aging (Albany NY)*. 2017;9(3):778–89.
- Jimenez R, Toral M, Gómez-Guzmán M, Romero M, Sanchez M, Mahmoud AM, Duarte J. The role of Nrf2 signaling in PPAR β / δ -Mediated vascular protection against hyperglycemia-induced oxidative stress. *Oxid Med Cell Longev*. 2018;2018:5852706.
- Fiorentino TV, Prioleta A, Zuo P, Folli F. Hyperglycemia-induced oxidative stress and its role in diabetes mellitus related cardiovascular diseases. *Curr Pharm Des*. 2013;19(32):5695–703.
- Mistry Y, Poolman T, Williams B, Herbert KE. A role for mitochondrial oxidants in stress-induced premature senescence of human vascular smooth muscle cells. *Redox Biol*. 2013;1:411–7.
- Tang Y, Xu Q, Peng H, Liu Z, Yang T, Yu Z, Cheng G, Li X, Zhang G, Shi R. The role of vascular peroxidase 1 in ox-LDL-induced vascular smooth muscle cell calcification. *Atherosclerosis*. 2015;243(2):357–63.
- Unterluggauer H, Hütter E, Voglauer R, Grillari J, Vöth M, Bereiter-Hahn J, Jansen-Dürr P, Jendrach M. Identification of cultivation-independent markers of human endothelial cell senescence in vitro. *BioGerontology*. 2007;8(4):383–97.
- Muñoz-Espín D, Serrano M. Cellular senescence: from physiology to pathology. *Nat Rev Mol Cell Biol*. 2014;15(7):482–96.
- Théry C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol*. <https://doi.org/10.1002/0471143030.cb0322530>
- Tian T, Zhu YL, Zhou YY, Liang GF, Wang YY, Hu FH, Xiao ZD. Exosome uptake through clathrin-mediated endocytosis and macropinocytosis and mediating miR-21 delivery. *J Biol Chem*. 2014;289(32):22258–67.
- Laakso M, Cederberg H. Glucose control in diabetes: which target level to aim for? *J Intern Med*. 2012;272(1):1–12.
- Lin X, Xu F, Cui RR, Xiong D, Zhong JY, Zhu T, Li F, Wu F, Xie XB, Mao MZ, Liao XB, Yuan LQ. Arterial calcification is regulated via an miR-204/DNMT3a regulatory circuit both in vitro and in female mice. *Endocrinology*. 2018;159(8):2905–16.
- Tsai IC, Pan ZC, Cheng HP, Liu CH, Lin BT, Jiang MJ. Reactive oxygen species derived from NADPH oxidase 1 and mitochondria mediate angiotensin II-induced smooth muscle cell senescence. *J Mol Cell Cardiol*. 2016;98:18–27.
- Liu H, Li X, Qin F, Huang K. Selenium suppresses oxidative-stress-enhanced vascular smooth muscle cell calcification by inhibiting the activation of the PI3K/AKT and ERK signaling pathways and endoplasmic reticulum stress. *J Biol Inorg Chem*. 2014;19(3):375–88.
- Tan P, Wang YJ, Li S, Wang Y, He JY, Chen YY, Deng HQ, Huang W, Zhan JK, Liu YS. The PI3K/Akt/mTOR pathway regulates the replicative senescence of human VSMCs. *Mol Cell Biochem*. 2016;422(1–2):1–10.
- Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. *Physiol Rev*. 2014;94(3):909–50.
- Nishikawa T, Araki E. Impact of mitochondrial ROS production in the pathogenesis of diabetes mellitus and its complications. *Antioxid Redox Signal*. 2007;9(3):343–53.
- Joshi MS, Williams D, Horlock D, Samarasinghe T, Andrews KL, Jefferis AM, Berger PJ, Chin-Dusting JP, Kaye DM. Role of mitochondrial dysfunction in hyperglycaemia-induced coronary microvascular dysfunction: protective role of resveratrol. *Diab Vasc Dis Res*. 2015;12(3):208–16.
- Du X, Matsumura T, Edelstein D, Rossetti L, Zsengeller Z, Szabó C, Brownlee M. Inhibition of GAPDH activity by poly(ADP-ribose) polymerase activates three major pathways of hyperglycemic damage in endothelial cells. *J Clin Invest*. 2003;112(7):1049–57.

32. Rogers MA, Maldonado N, Hutcheson JD, Goettsch C, Goto S, Yamada I, Faits T, Sesaki H, Aikawa M, Aikawa E. Dynamin-related protein 1 inhibition attenuates cardiovascular calcification in the presence of oxidative stress. *Circ Res*. 2017;121(3):220–33.
33. Del Rio D, Stewart AJ, Pellegrini N. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutr Metab Cardiovasc Dis*. 2005;15(4):316–28.
34. Dalle-Donne I, Rossi R, Colombo R, Giustarini D, Milzani A. Biomarkers of oxidative damage in human disease. *Clin Chem*. 2006;52(4):601–23.
35. Théry C. Exosomes: secreted vesicles and intercellular communications. *F1000 Biol Rep*. 2011;3:15.
36. Fedele C, Singh A, Zerlanko BJ, Iozzo RV, Languino LR. The alpha_v-beta₆ integrin is transferred intercellularly via exosomes. *J Biol Chem*. 2015;290(8):4545–51.
37. Chang YT, Chan CK, Eriksson I, Johnson PY, Cao X, Westöö C, Norvik C, Andersson-Sjöland A, Westergren-Thorsson G, Johansson S, Hedin U, Kjellén L, Wight TN, Tran-Lundmark K. Versican accumulates in vascular lesions in pulmonary arterial hypertension. *Pulm Circ*. 2016;6(3):347–59.
38. Huang R, Merrilees MJ, Braun K, Beaumont B, Lemire J, Clowes AW, Hinek A, Wight TN. Inhibition of versican synthesis by antisense alters smooth muscle cell phenotype and induces elastic fiber formation in vitro and in neointima after vessel injury. *Circ Res*. 2006;98(3):370–7.
39. Wight TN, Kinsella MG, Evanko SP, Potter-Perigo S, Merrilees MJ. Versican and the regulation of cell phenotype in disease. *Biochim Biophys Acta*. 2014;1840(8):2441–51.
40. Wight TN, Merrilees MJ. Proteoglycans in atherosclerosis and restenosis: key roles for versican. *Circ Res*. 2004;94(9):1158–67.
41. Wang X, Hu G, Zhou J. Repression of versican expression by micro-RNA-143. *J Biol Chem*. 2010;285(30):23241–50.
42. Wight TN, Kang I, Merrilees MJ. Versican and the control of inflammation. *Matrix Biol*. 2014;35:152–61.
43. Carthy JM, Abraham T, Meredith AJ, Boroomand S, McManus BM. Versican localizes to the nucleus in proliferating mesenchymal cells. *Cardiovasc Pathol*. 2015;24(6):368–74.
44. Bhardwaj A, Frankel WL, Pellegata NS, Wen P, Prasad ML. Intracellular versican expression in mesenchymal spindle cell tumors contrasts with extracellular expression in epithelial and other tumors—a tissue microarray-based study. *Appl Immunohistochem Mol Morphol*. 2008;16(3):263–6.
45. Quatresooz P, Paquet P, Hermanns-Lê T, Piérard GE. Immunohistochemical aspects of the fibrogenic pathway in nephrogenic systemic fibrosis. *Appl Immunohistochem Mol Morphol*. 2010;18(5):448–52.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions

