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Exosomal Notch3 from high glucose-stimulated endothelial cells regulates vascular smooth muscle cells calcification/aging

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ABSTRACT

Aims: Vascular calcification/aging can cause different kind of serious diabetic vascular complications. High glucose could induce vascular smooth muscle cells (VSMCs) calcification/aging and then lead to diabetes-related vascular calcification/aging. In this study, we investigated how information in the blood is transmitted to VSMCs and the mechanisms of VSMCs calcification/aging under hyperglycaemic conditions.

Materials and methods: Transmission electron microscopy and molecular size analysis were used to assess the morphology and size of exosomes. Alizarin Red S staining and senescence-associated β galactosidase (SA-β-gal) staining were carried out to detect calcification and senescence in VSMCs, respectively. Proteomics analysis was carried out to detect the different expression of exosomal proteins. Protein levels were measured by western blot analysis.

Key findings: The results show that exosomes isolated from high glucose stimulated human umbilical vein endothelial cell (HG-HUVEC-Exo) exhibited a bilayer structure morphology with a mean diameter of 63.63 ± 2.96 nm. The presence of exosome markers including CD9, CD63 and TSG101 were also detected in HG-HUVEC-Exo. High glucose could induce VSMCs calcification/aging by increasing the expression of osteocalcin (OC) and p21 as well as the formation of mineralised nodules and SA-β-gal positive cells. Fluorescence microscopy verified that the exosomes were taken up by VSMCs and Notch3 protein was enriched in HG-HUVEC-Exo. Most importantly, mTOR signalling was closely related to Notch3 protein and was involved in regulating HG-HUVEC-Exo-induced VSMCs calcification/aging.

Significance: The data demonstrate that Notch3 is required for HG-HUVEC-Exo promoted VSMCs calcification/aging and regulates VSMCs calcification/aging through the mTOR signalling pathway.

1. Introduction

Vascular calcification/aging plays a key role in diabetic vascular-related complications, such as amputation, renal failure and stroke, and contributes to morbidity and mortality [1]. Vascular calcification is mainly characterised as medial aortic calcification (also known as Mönckeberg's calcification), and the transdifferentiation of vascular smooth muscle cells (VSMCs) into osteoblast-like cells is thought to be the most essential pathophysiological factor of vascular calcification [2]. Vascular aging includes physiological aging and premature aging caused by environmental factors, including hyperglycaemia [3]. Studies have found that high glucose levels can cause vascular calcification, subsequent renal failure and other diabetic vascular calcification-related complications [4,5]. However, the mechanisms by which high glucose induces VSMCs aging have not been elucidated.

Endothelial cells (ECs) and VSMCs are the main cells driving pathological changes to the vasculature. ECs, the inner layer of blood vessels, are directly stimulated by hyperglycaemia and other factors, so most studies have focused on studying the effect of hyperglycaemia on ECs [6]. However, the role of high glucose in modulating aging has not been explored fully. Our previous study showed for the first time that high glucose induces VSMCs calcification/senescence, regulated by the lncRNA-ES3/miR-34c-5p/BMF axis [7]. In fact, a large number of studies have found that vascular calcification mainly occurs in VSMCs of the medial arterial membrane in patients with diabetes [8,9]. However, it is not known how information regarding hyperglycaemia in the circulation is transferred to VSMCs and how this induces VSMCs calcification/aging.

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Exosomes of endosomal origin are multivesicular bodies with a diameter of 30–100 nm. They are released into the extracellular matrix through the fusion of intracellular multivesicles with cell membranes. Because of the variety and abundance of specific cargos, including proteins, lipids and nucleic acids, exosomes have emerged as regulators of cell to cell communication during different physiological and pathological processes, including cell proliferation, differentiation, apoptosis and migration [10,11]. For instance, ECs release exosomes carrying miR-214 to stimulate angiogenesis in neighbouring target ECs [11]. Liang et al. demonstrated that exosomes secreted by adipose mesenchymal stem cells can transfer miR-125a to ECs and promote angiogenesis [12]. Our previous study demonstrated that exosomes from hyperglycaemia-stimulated ECs carry versican, which regulated VSMCs calcification/senescence [13]. However, whether other cargos in exosomes play a role in information transmission between ECs and VSMCs under hyperglycaemic conditions is unclear, and the mechanisms involved need to be explored further.

In the present study, to better understand the role of exosomes in mediating intercellular communication between ECs and VSMCs during the process of vascular calcification/aging, we investigated the roles and mechanisms of exosomes derived from high glucose-induced human umbilical vein endothelial cell (HG-HUVEC-Exo) in regulating VSMCs calcification/aging. We observed that HG-HUVEC-Exo induced VSMCs calcification/aging. Further studies showed that Notch3 in HUVEC could be transferred to VSMCs via exosomes and promoted VSMCs calcification/aging through the mTOR signalling pathway.

2. Materials and methods

2.1. Cell culture and transfection

Human VSMCs and HUVEC were purchased from ATCC (ATCC-CRL-1999) and CHI Scientific, Inc. (7–1012), respectively. VSMCs were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM, Hyclone, Logan, USA) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin. HUVEC were cultured in F-12 k medium (Hyclone), supplemented with 10% FBS (Gibco, Invitrogen, New York, USA), 1% penicillin/streptomycin, 0.05 mg/ml endothelial cell growth supplement (ECGS) and 0.1 mg/ml heparin (Sciencell, San Diego, USA). Cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2 and passaged every 3–4 days.

For cell transfection, mTOR small interfering RNA (simTOR) oligos (GenePharma, Shanghai, China) were transfected into VSMCs using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. The cells were cultured in DMEM without FBS for 6 h and then cultured for another 48 h in DMEM supplementated with 10% FBS.

2.2. Extraction of exosomes from HUVEC

HUVEC were treated with 5 mM glucose (pH 7.4) as the normal concentration (NG) or 30 mM (pH 7.4) as the high concentration of glucose (HG). Exosomes were extracted using an ExoQuick-TC Exosome Precipitation Solution kit (System Biosciences, USA). Briefly, the supernatant was centrifuged as follows: 300 *g* for 10 min, 2000 *g* for 30 min and 10,000 *g* for 30 min. An Amicon Ultra-15 Centrifugal Filter Unit (100 kDa; Millipore) was used to concentrate the supernatant. The ultrafiltration liquid and exosome isolation reagent were mixed at a 4:1 ratio and incubated at 4 °C for over 12 h. Finally, the mixture was centrifuged at 1500 *g* for 30 min and the exosome pellets were resuspended in 200 μl of PBS. The protein quantification of exosomes was performed using a BCA kit (Beyotime Biotechnology, Shanghai, China).

2.3. Identification of exosomes

The morphology of exosomes was detected by phosphotungstic acid negative staining as previously described [13]. Briefly, exosomes were fixed with equal volumes of 1% phosphotungstic acid (pH 7.4). After rinsing, a 10 μl of the sample was loaded onto a bronze net with film and left for 10 min at room temperature (RT). Then, 10 μl of the phosphotungstic acid staining solution was added for negative staining and observed under a Hitachi H-7650 transmission electron microscope (Hitachi, Tokyo, Japan). The size distribution of the exosomes was measured by dynamic light scattering using a particle and molecular size analyser (Zetasizer Nano ZS; Malvern Instruments). The expression of the exosomal surface marker proteins CD9 (ab92726, Abcam, USA), CD63 (ab68418, Abcam, USA) and TSG101 (ab125011, Abcam, USA) were analysed by western blot analysis.

2.4. VSMCs uptake and exosome analysis

Exosomes were labelled with a DiD fluorescent probe (AAT-22033, AAT Bioquest. Beijing, China) according to the manufacturer's instructions. Briefly, 5 μl of the DiD fluorescent probe was dissolved in DMSO and incubated with VSMCs at 37 °C for 12 h. After fixing the cells with 4% paraformaldehyde for 30 min at RT and washing the cells three times with PBS, DAPI (Invitrogen, Carlsbad, USA) was added for 5 min. After washing with PBS three times, the staining signals were analysed with a fluorescence microscope (DMI6000B, Leica, Germany).

2.5. Alizarin Red S staining

VSMCs cultured with exosomes derived from normal glucose induced HUVEC (NG-HUVEC-Exo) or HG-HUVEC-Exo for at least 14 days were fixed in 4% paraformaldehyde for 30 min at RT and then stained with 1% (pH 4.2) Alizarin Red S for 1–2 min. Mineralised nodules were assessed and photographed with microscope.

2.6. SA-β-gal staining

SA-β-gal staining was performed using a SA-β-gal staining kit (Beyotime Biotechnology, Shanghai, China) as previously described [14]. Briefly, VSMCs were fixed in β-galactosidase fixation solution for 15 min and washed with PBS three times. The cells were stained in β-galactosidase solution at 37 °C for over 12 h. The number and intensity of SA-β-gal positive cells was determined.

2.7. Proteomic analysis

Exosomes were extracted from NG or HG induced HUVEC and processed for iTRAQ-based quantitative proteomic analysis by Jingjie PTM BioLab (Hangzhou, China). The different proteomic contents of NG-HUVEC-Exo and HG-HUVEC-Exo were assessed using high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS). GO annotation (http://geneontology.org/), InterProScan (http://www.ebi.ac.uk/interpro/) and the UniprotKB database (https://www.uniprot.org/) were used to classify all identified proteins into three categories (molecular function, cell component and biological process). A cutoff of absolute fold change ≥ 1.3 was used for the differentially expressed proteins and a corrected p-value < 0.05 was considered significant.

2.8. Western blot analysis

The expression of proteins was determined by western blot as previously described [15]. Briefly, the concentration of protein was detected using a BCA kit (Beyotime, Shanghai, China). Then, 30 μg of total protein was loaded onto a 10% SDS-PAGE gel and transferred to a PVDF membrane. The membranes were incubated with the primary antibody at 4 °C for over 12 h after blocking with 5% non-fat milk for 1 h. Subsequently, HRP-conjugated goat-anti-rabbit (sc-2004, 1:5000, Santa Cruz) or HRP-conjugated goat-anti-mouse (sc-2005, 1:5000, Santa

Cruz) secondary antibodies were incubated with the membrane at RT for 1 h. The immunoreactive bands were visualised using an ECL Plus western blot detection kit (Amersham Biosciences U.K. Ltd). The relative expression level of target proteins was normalised to the intensity of the β-actin band. Primary antibodies included Notch3 (#2889, 1:1000, CST), Notch3-NIC (55114-1-AP, 1:1000, Proteintech), mTOR (#2972, 1:1000, CST), p-mTOR^{S2448} (ab84400, 1:1000, Abcam), p-mTOR^{T2446} (ab63552, 1:1000, Abcam), p-mTOR^{S2481} (ab137133, 1:1000, Abcam), osteocalcin (OC, ab133612, 1:1000, Abcam), p21(10355-1-AP, 1:1000, Proteintech) and β-actin (ab6276, 1:3000, Abcam).

2.9. Statistical analysis

Data are presented as mean ± standard deviation (SD) and were analysed using Statistical Product and Service Solutions (SPSS, version 17.0). Student's *t*-test was used to analyse the differences between two groups and one-way analysis of variance (ANOVA) and was used for multiple groups; $p < 0.05$ was considered statistically significant. All experiments were repeated at least three times and representative experimental data are shown in the figures.

3. Results

3.1. Identification of exosomes

Exosomes were isolated from the supernatant of HUVEC treated with NG and HG, respectively. Transmission electron microscopy showed that the vesicles had a bilayer structural morphology and the vesicles, detected by molecular size analysis, had a mean diameter of 63.63 ± 2.96 nm (Fig. 1A and B). These features are consistent with previous reports on exosomes [16]. Western blot analysis further showed the presence of exosome markers including CD9, CD63 and TSG101 (Fig. 1C). These three features confirmed that the vesicles were exosomes.

3.2. HG-HUVEC-Exo induce VSMCs calcification/aging

Next, the effects of exosomes on VSMCs calcification/aging were assessed. Firstly, fluorescent microscopy revealed that DiD-labelled HG-HUVEC-Exo could be taken up and incorporated into VSMCs (Fig. 1D). Furthermore, compared with VSMCs treated with NG-HUVEC-Exo, Alizarin Red S staining showed that mineralised nodules were greatly increased in HG-HUVEC-Exo induced VSMCs for 14 days (Fig. 2A). Moreover, SA-β-gal staining showed that the number of SA-β-gal positive cells was also significantly increased (Fig. 2B). The expression of both OC and p21 were greatly increased in VSMCs treated with HG-HUVEC-Exo (Fig. 2C). However, the exosome deprivation supernatant derived from high glucose induced HUVEC (HG-HUVEC-Sup^{-exo}) just had a slight effects on the calcification/aging of VSMCs (Fig. 2A–C). The data suggest that exosomes, rather than other contents in the supernatant from HUVEC, promoted VSMCs calcification/aging under high glucose conditions.

3.3. Notch3 is enriched in HG-HUVEC-Exo and involved in regulating VSMCs calcification/aging

To investigate the molecular mechanisms of HG-HUVEC-Exo in VSMCs calcification/aging, proteomic analysis using ITRAQ technology was conducted to detect the protein expression profiles between HG-HUVEC-Exo and NG-HUVEC-Exo. In total, 513 proteins were quantified among the total identified 569 proteins. The identified proteins were classified into three categories including molecular function, cell component and biological process by GO analysis (data not shown). There were 179 proteins differentially expressed between HG-HUVEC-Exo and NG-HUVEC-Exo, among which 74 proteins were significantly higher in HG-HUVEC-Exo and 105 proteins were much lower than NG-HUVEC-Exo. Then, the differentially expressed proteins were searched for in PubMed (https://www.ncbi.nlm.nih.gov/pubmed) and their involved biological processes were explored in UniprotKB (https://www.uniprot.org/). The proteins related to osteoblast differentiation were marked; Notch3 attracted our attention because it was up-regulated in HG-

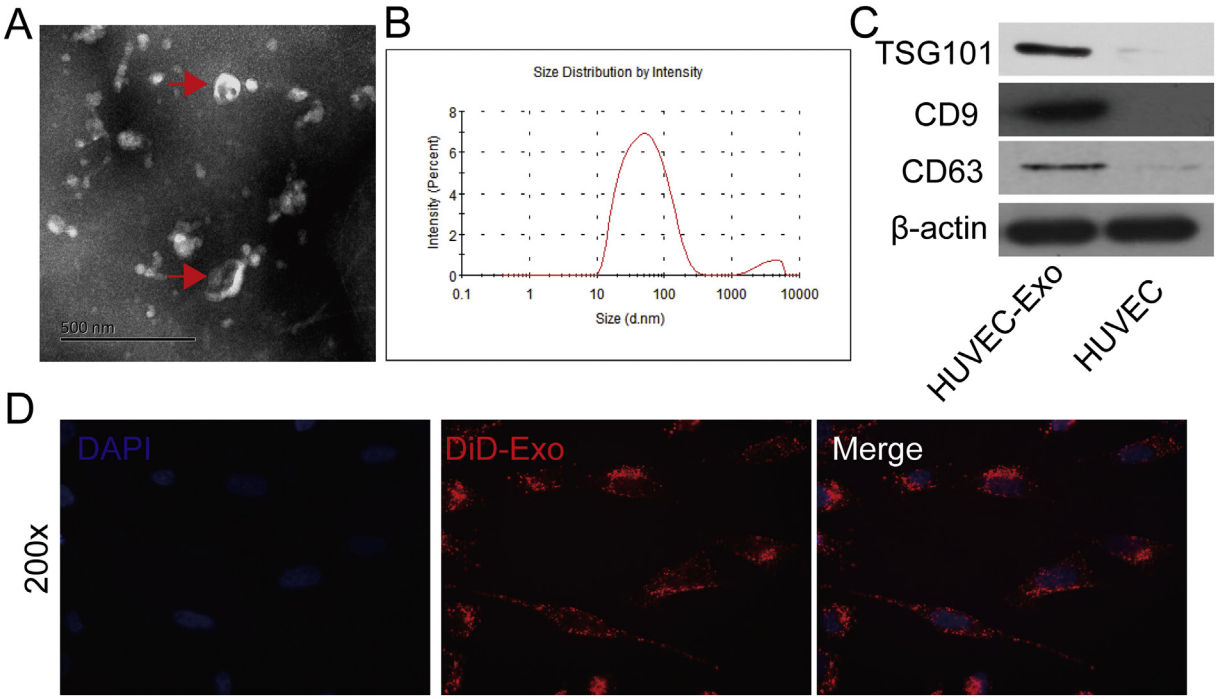

Fig. 1. Exosomes were identified and taken up by VSMCs. (A) Transmission electron microscopy was used to assess the morphology of exosomes. The scale bar is 500 nm. The representative images were shown. (B) The diameter of the exosomes was measured by molecular size analysis. (C) Exosome markers were detected by western blot analysis. (D) Fluorescent microscopy revealed that DiD-labelled exosomes could be taken up and incorporated into VSMCs.

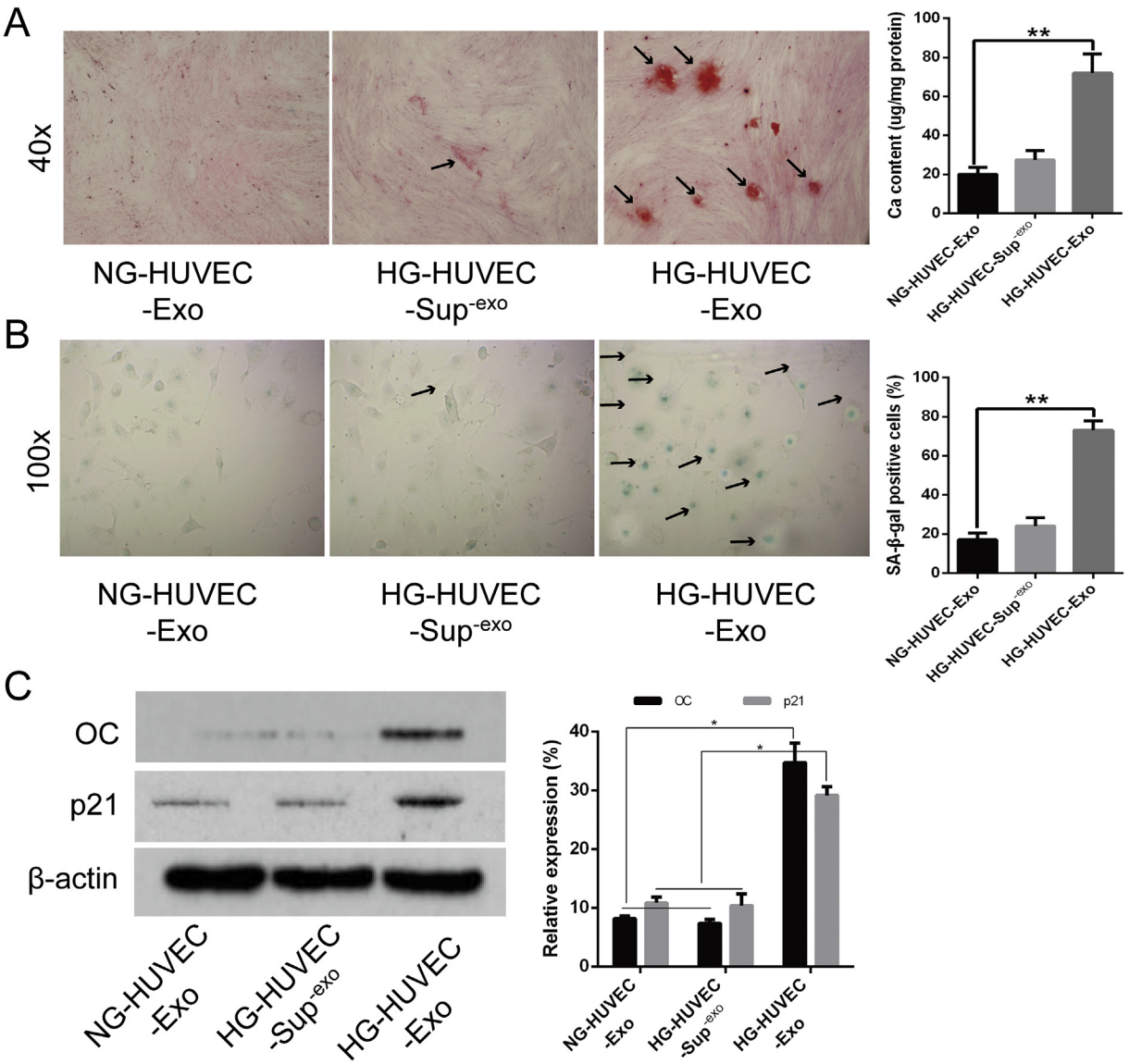

Fig. 2. HG-HUVEC-Exo induce VSMCs calcification/aging. (A) Alizarin Red S staining showed the mineralised nodules and calcium content in VSMCs. The arrow indicate the mineralised nodules. (B) SA-β-gal staining was used to determine senescence VSMCs. The arrow indicate the senescence cells. (C) The expression of OC and p21 were measured by western blot analysis in VSMCs. $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.005$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

HUVEC-Exo compared to NG-HUVEC-Exo (Fig. 3A) and is related to osteoblast differentiation. Further western blot analysis confirmed the enrichment of Notch3 in HG-HUVEC-Exo (Fig. 3B). Additionally, the expression of Notch3 was significantly increased in VSMCs treated with HG-HUVEC-Exo compared with those treated with NG-HUVEC-Exo (Fig. 3C). Importantly, DAPT, an inhibitor of Notch3, was used to inhibit the expression of Notch3 in HUVEC; the results show the down-regulation of Notch3 in exosomes from HUVEC treated with DAPT under high glucose conditions (HG-HUVEC-Exo + DAPT) (Fig. 3C). Interestingly, the ability of HG-HUVEC-Exo to promote the VSMCs calcification/aging was decreased when Notch3 was inhibited in parent HUVEC, which was shown by decreased expression of OC and p21 (Fig. 3C). Taken together, these results demonstrate that Notch3 is enriched in HG-HUVEC-Exo and plays a key role in the process of VSMCs calcification/aging.

3.4. Notch3 is associated with mTOR signalling

In order to explore the mechanism of exosomal Notch3 derived from HUVEC, STRING network interaction analysis (https://string-db.org/cgi/input.pl) was performed on all proteins identified by proteomics. Interestingly, the results show that Notch3 is closely related to the mTOR signalling pathway (Fig. 4A and B). Notch3-associated protein biological enrichment signalling pathways in the GO analysis suggested a close relationship between Notch3 and mTOR. The results are shown in supplemental materials (Table S1–3). Western blot analysis showed that the protein level of Notch3, activated Notch3 (Notch3-ICD), total mTOR and p-mTOR (p-mTOR^{S2448} and p-mTOR^{T2446}) were significantly increased in VSMCs treated with HG-HUVEC-Exo compared with NG-HUVEC-Exo, while the expression of p-mTOR^{S2481} underwent no significant change (Fig. 4B). After inhibiting the expression of Notch3 using DAPT, the expression of mTOR and p-mTOR (p-mTOR^{S2448} and p-mTOR^{T2446}), but not p-mTOR^{S2481}, was also decreased in VSMCs (Fig. 4C). These data suggest that exosomal Notch3 modulates VSMCs calcification/aging through activated p-mTOR^{S2448} and p-mTOR^{T2446}.

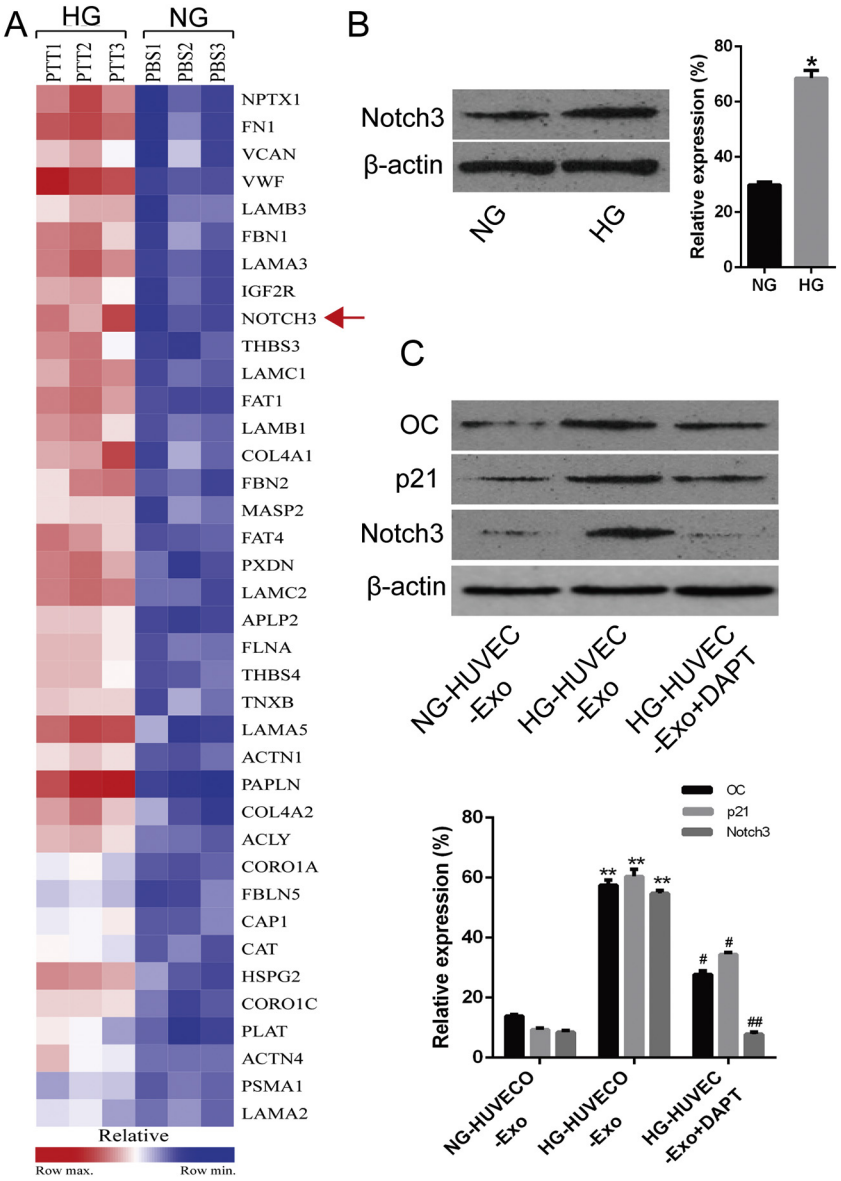

Fig. 3. Notch3 is enriched in HG-HUVEC-Exo and is involved in regulating VSMCs calcification/aging. (A) The differentially expressed proteins (a cutoff of absolute fold change ≥1.3 and $p < 0.05$) between HG-HUVEC-Exo and NG-HUVEC-Exo according to proteomics analysis. (B) The expression of Notch3 in HG-HUVEC-Exo was verified by western blot analysis. (C) Western blot analysis showed that DAPT inhibited the expression of Notch3 in HUVEC, accompanied by decreased expression of OC, p21 and Notch3 in VSMCs. $n = 3$, compared with NG-HUVEC-Exo, $^{*}p < 0.05$, $^{**}p < 0.005$; compared with HG-HUVEC-Exo, $\#p < 0.05$, $\#\#p < 0.005$.

3.5. mTOR signalling is involved in Notch3-mediated VSMCs calcification/aging

To verify whether exosomal Notch3 derived from HUVEC regulates VSMCs calcification/aging via the mTOR signalling pathway, mTOR siRNA (simTOR) was used to knock down mTOR expression. We found that simTOR$^{\#2}$ was the most effective at inhibiting the expression of p-mTOR^{S2448} as well as p-mTOR^{T2446} (Fig. 5A). Therefore, we chose simTOR$^{\#2}$ for further study. When the expression of p-mTOR^{S2448} as well as p-mTOR^{T2446} was knocked down, the effect of HG-HUVEC-Exo on VSMCs calcification/aging was almost abolished, as evidenced by decreased expression of OC and p21 (Fig. 5B). Furthermore, mineralised nodules and SA-β-gal positive cells were also reduced (Fig. 5C and 5D). These results indicate that exosomal Notch3 derived from HG-induced HUVEC regulates VSMCs calcification/aging via the mTOR signalling pathway.

4. Discussion

Diabetes is a severe metabolism- and aging-related disease, but the reasons for diabetic vascular aging-related complications is currently unknown. In the present study, we find that HG induces HUVECto secrete exosomes, which carry Notch3 protein to VSMCs; this promotes VSMCs calcification/aging via the mTOR signalling pathway. These data demonstrate a new role for exosomes in the transmission of information between ECs and VSMCs under hyperglycaemic conditions.

Vascular calcification is one of the most salient features of vascular aging and causes macrovascular complications in patients with diabetes, mainly involving the media of arteries [7,17]. VSMCs are considered to be osteoblast-like cells after undergoing a phenotypic switch, which is manifested by an increase in mineral nodule formation and osteoblast differentiation markers, such as OC [18]. It is widely known that hyperglycaemia can cause diabetic vascular calcification/aging, and that prevention and treatment of the disease is not as simple as

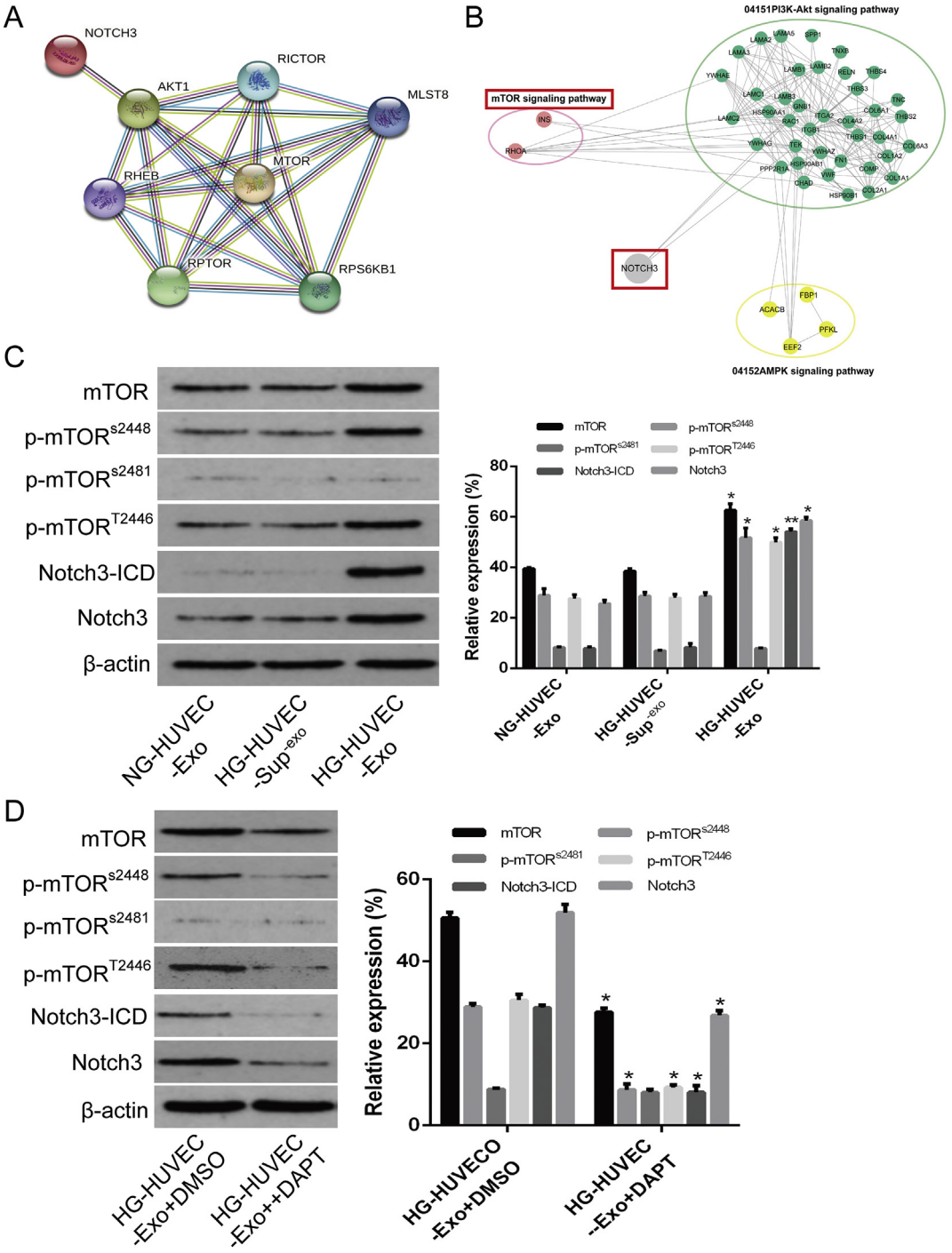

Fig. 4. Notch3 is associated with mTOR signalling. (A) STRING network interaction analysis shows the relationship between Notch3 and mTOR signalling. (B) STRING network interaction analysis shows the relationship between the different upregulated proteins detected in exosomes by proteomic analysis and the mTOR signalling pathway. (C) Western blot analysis shows that protein levels of Notch3, Notch3-ICD, total mTOR and p-mTOR (p-mTOR^{S2448}, p-mTOR^{S2481} and p-mTOR^{T2446}) in VSMCs treated with HG-HUVEC-Exo or NG-HUVEC-Exo. (D) Western blot analysis shows the expression of total mTOR, p-mTOR (p-mTOR^{S2448} and p-mTOR^{T2446} and p-mTOR^{S2481}) in VSMCs treated with HG-HUVEC-Exo with or without DAPT. $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.005$.

lowering blood glucose. In contrast, long-term intensive glucose reduction does not benefit macrovascular disease and fails to reduce the risk of mortality due to cardiovascular events related to vasculopathy [19]. Thus, new therapeutic options are needed to delay diabetic vascular calcification/aging.

Vascular calcification/aging is tightly associated with alterations in the physiological functions and structural properties of the vascular wall, mainly in ECs and VSMCs. Previous studies have focused on the effect of high glucose on ECs rather than VSMCs [6,20]. For example, Song et al. reported that high glucose induces ECs senescence via the PGC-1α/SIRT1 signalling pathway [6]. Another study demonstrated that the speed of endothelial progenitor cell senescence is accelerated in patients with type 2 diabetes mellitus [20]. However, the mechanism by which high glucose influences VSMCs calcification/aging is still unknown. Recently, emerging evidence has suggested that exosomes act as natural vehicles for the delivery of proteins, nucleic acids and signalling molecules in modulating cardiovascular diseases [21–23]. For instance, Lin et al. reported that pulmonary artery EC-derived

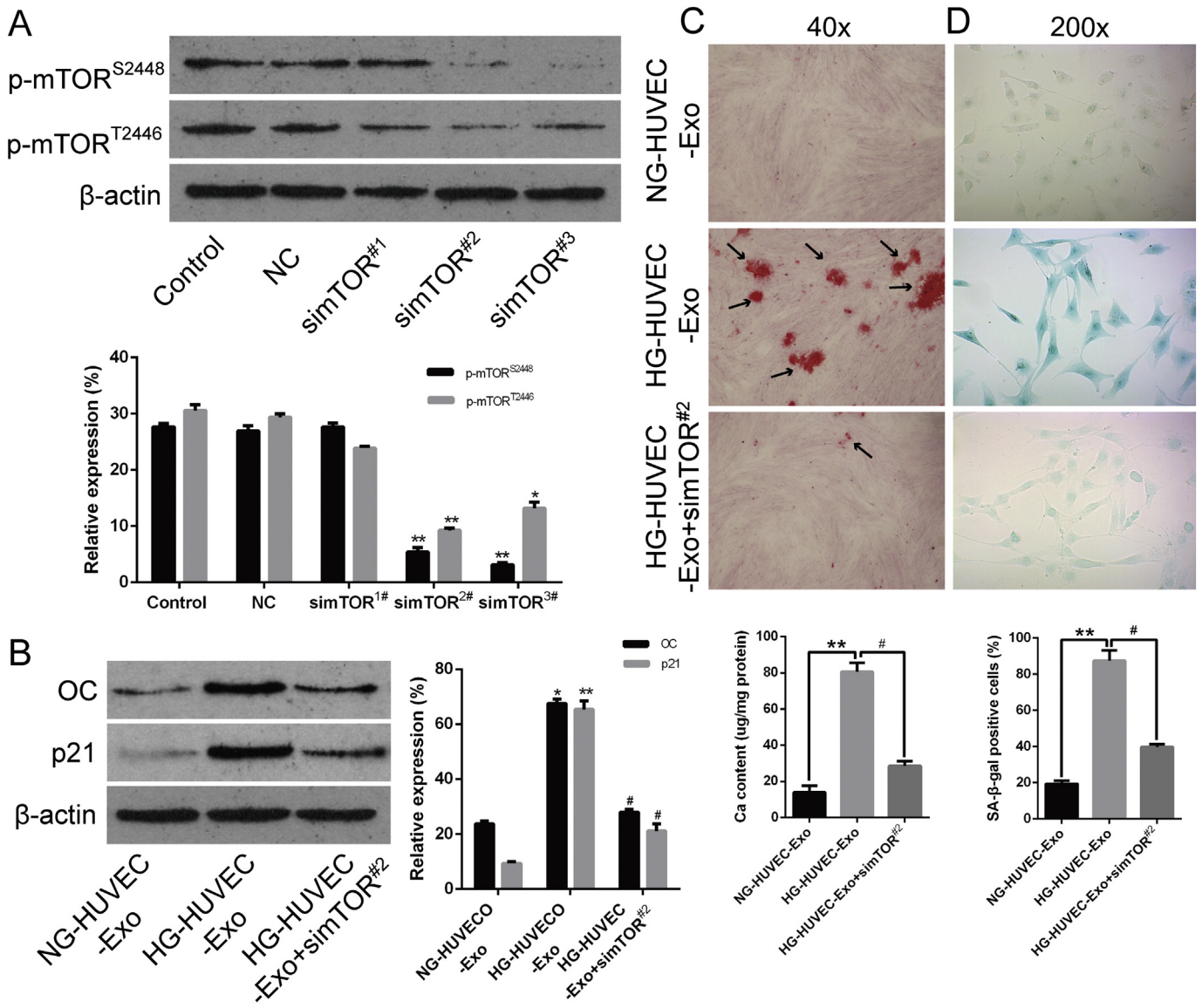

Fig. 5. mTOR signalling is involved in Notch3-mediated VSMCs calcification/aging. (A) The efficiency of simTOR was detected by western blot analysis. (B) The expression of OC and p21 were measured by western blot analysis after knocking down the mTOR signalling pathway. (C) Alizarin Red S staining shows mineralised nodules and the calcium content in VSMCs. (D) SA-β-gal staining indicates SA-β-gal positive VSMCs. $n = 3$, compared with NG-HUVEC-Exo, $^{*}p < 0.05$, $^{**}p < 0.005$; compared with HG-HUVEC-Exo, $\#p < 0.05$, $\#\#p < 0.005$. NC: negative control.

exosomes are involved in overproliferation and apoptosis resistance in pulmonary artery SMC [24]. Another study demonstrated that exosomes secreted by KLF2-transduced HUVEC are enriched in miR-143/145 and induce the atherosclerosis of SMCs [25]. Our previous study demonstrated for the first time that exosomes derived from ECs can regulate VSMCs calcification/senescence [13]. Accordingly, in the present study, we also found that HG-HUVEC-Exo could be transferred to and taken up by VSMCs and then promote VSMCs calcification/aging. However, the specific mechanism by which exosomes interact with target VSMCs needed further study.

The Notch family is an ancient and highly conserved protein that plays an essential role in diabetes and cardiovascular diseases [26,27]. Notch3, a member of the Notch family, is a key regulator of proliferation and phenotypic transformation in VSMCs [28]. The intracellular region of Notch receptors is hydrolysed by metalloproteinases when the extracellular region binds to ligands, releasing the intracellular domain (ICD). The ICD is an activated form of Notch protein, i.e. Notch3-ICD [29]. Previous studies have reported that chromobox protein homolog 3 modulates VSMCs proliferation, migration and apoptosis via a Notch3-mediated pathway [30]. Moreover, Jagged-1/Notch3 signalling is involved in apelin13-induced VSMCs proliferation [31]. Accordingly, in the present study, proteomics analysis showed that Notch3 is enriched in HG-HUVEC-Exo compared with NG-HUVEC-Exo. When the expression of Notch3 was knocked down in HUVEC, the role of HG-HUVEC-Exo in promoting VSMCs calcification/aging was nearly abrogated. These results suggest that HG-HUVEC-Exo regulate VSMCs calcification/aging by carrying Notch3 protein to VSMCs. This finding is consistent with another study demonstrating that Notch3 regulates the human coronary artery SMC contractile phenotype in a three-dimensional co-culture system [32]. However, whether exosomal Notch3 derived from ECs under hyperglycaemic conditions regulates VSMCs calcification/aging *in vivo* needed further study.

mTOR is an atypical serine/threonine protein kinase that acts as an intracellular signal transducer and regulates cellular energy metabolism, aging, and stress in response to changes in the external environment. Recent studies have demonstrated that the inhibition of mTOR signalling can delay aging [33,34], and other reports have focused on studying the effects of mTOR on ECs calcification/senescence [35]. Our

previous studies uncovered a relationship between mTOR signalling and VSMCs calcification/aging and found that inhibition of mTOR signalling can delay vascular calcification/aging [14,17,36]. In the current study, interestingly, STRING network interaction analysis found that Notch3 is closely related to the mTOR signalling pathway. Moreover, the expression of mTOR was increased significantly, which is consistent with an increase in Notch3 in VSMCs treated with HG-HUVEC-Exo. Additionally, the effect of exosomal Notch3 derived from HG-induced HUVEC on VSMCs calcification/aging was significantly attenuated after knocking down the mTOR signalling pathway. These results show that the effect of exosomal Notch3 derived from HG-induced HUVEC on VSMCs calcification/aging is dependent on mTOR signalling.

5. Conclusion

The current study demonstrates that exosomal Notch3 secreted by HUVEC is transmitted to VSMCs and promotes VSMCs calcification/aging through the mTOR signalling pathway. This interesting finding highlights a new way of information exchange between ECs and VSMCs and provides insight into the mechanism of VSMCs calcification/aging in patients with diabetes. Exosomal Notch3 might be a diagnostic and therapeutic target in diabetic vascular calcification/aging.

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Author contributions

YSL and JKZ conceived and designed the experiments. XL performed the experiments, analysed the data and prepared all the figures. SL, YJW, JYZ, JYH, YQN and XJC provided technical support, made substantial contributions to data analysis and revised the manuscript critically for important intellectual content. XL wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116582.

References

[1] X. Lin, J.K. Zhan, Y.J. Wang, P. Tan, Y.Y. Chen, H.Q. Deng, Y.S. Liu, Function, role, and clinical application of microRNAs in vascular aging, Biomed. Res. Int. 2016 (2016) 6021394, https://doi.org/10.1155/2016/6021394.

[2] Y.Q. Peng, D. Xiong, X. Lin, R.R. Cui, F. Xu, J.Y. Zhong, T. Zhu, F. Wu, M.Z. Mao, X.B. Liao, L.Q. Yuan, Oestrogen inhibits arterial calcification by promoting autophagy, Sci. Rep. 7 (1) (2017) 3549, https://doi.org/10.1038/s41598-017-03801-x.

[3] D. Zhang, H. Lu, Z. Chen, Y. Wang, J. Lin, S. Xu, C. Zhang, B. Wang, Z. Yuan, X. Feng, X. Jiang, J. Pan, High glucose induces the aging of mesenchymal stem cells via Akt/mTOR signaling, Mol. Med. Rep. 16 (2) (2017) 1685–1690, https://doi.org/10.3892/mmr.2017.6832.

[4] Q. Zhu, R. Guo, C. Liu, D. Fu, F. Liu, J. Hu, H. Jiang, Endoplasmic reticulum stress-mediated apoptosis contributing to high glucose-induced vascular smooth muscle cell calcification, J. Vasc. Res. 52 (5) (2015) 291–298, https://doi.org/10.1159/000442980.

[5] C.M. Shanahan, Mechanisms of vascular calcification in CKD-evidence for premature ageing? Nat. Rev. Nephrol. 9 (11) (2013) 661–670, https://doi.org/10.1038/nrneph.2013.176.

[6] X. Song, B. Yang, F. Qiu, M. Jia, G. Fu, High glucose and free fatty acids induce endothelial progenitor cell senescence via PGC-1alpha/SIRT1 signaling pathway, Cell Biol. Int. 41 (10) (2017) 1146–1159, https://doi.org/10.1002/cbin.10833.

[7] X. Lin, J.K. Zhan, J.Y. Zhong, Y.J. Wang, Y. Wang, S. Li, J.Y. He, P. Tan, Y.Y. Chen, X.B. Liu, X.J. Cui, Y.S. Liu, lncRNA-ES3/miR-34c-5p/BMF axis is involved in regulating high-glucose-induced calcification/senescence of VSMCs, Aging (Albany NY) 11 (2) (2019) 523–535, https://doi.org/10.18632/aging.101758.

[8] N.X. Chen, D. Duan, K.D. O'Neill, S.M. Moe, High glucose increases the expression of Cbfa1 and BMP-2 and enhances the calcification of vascular smooth muscle cells, Nephrol. Dial. Transplant. 21 (12) (2006) 3435–3442, https://doi.org/10.1093/ndt/gfl429.

[9] Y.B. Zhou, J. Zhang, D.Q. Peng, J.R. Chang, Y. Cai, Y.R. Yu, M.Z. Jia, W. Wu, Y.F. Guan, C.S. Tang, Y.F. Qi, Peroxisome proliferator-activated receptor gamma ligands retard cultured vascular smooth muscle cells calcification induced by high glucose, Cell Biochem. Biophys. 66 (3) (2013) 421–429, https://doi.org/10.1007/s12013-012-9490-7.

[10] J. Wang, X. Sun, J. Zhao, Y. Yang, X. Cai, J. Xu, P. Cao, Exosomes: a novel strategy for treatment and prevention of diseases, Front. Pharmacol. 8 (2017) 300, https://doi.org/10.3389/fphar.2017.00300.

[11] B.W. van Balkom, O.G. de Jong, M. Smits, J. Brummelman, K. den Ouden, P.M. de Bree, M.A. van Eijndhoven, D.M. Pegtel, W. Stoorvogel, T. Wurdinger, M.C. Verhaar, Endothelial cells require miR-214 to secrete exosomes that suppress senescence and induce angiogenesis in human and mouse endothelial cells, Blood 121 (19) (2013) 3997–4006 S1-15 https://doi.org/10.1182/blood-2013-02-478925.

[12] X. Liang, L. Zhang, S. Wang, Q. Han, R.C. Zhao, Exosomes secreted by mesenchymal stem cells promote endothelial cell angiogenesis by transferring miR-125a, J. Cell Sci. 129 (11) (2016) 2182–2189, https://doi.org/10.1242/jcs.170373.

[13] S. Li, J.K. Zhan, Y.J. Wang, X. Lin, J.Y. Zhong, Y. Wang, P. Tan, J.Y. He, X.J. Cui, Y.Y. Chen, W. Huang, Y.S. Liu, Exosomes from hyperglycemia-stimulated vascular endothelial cells contain versican that regulate calcification/senescence in vascular smooth muscle cells, Cell Biosci. 9 (1) (2019), https://doi.org/10.1186/s13578-018-0263-x.

[14] P. Tan, Y.J. Wang, S. Li, Y. Wang, J.Y. He, Y.Y. Chen, H.Q. Deng, W. Huang, J.K. Zhan, Y.S. Liu, The PI3K/Akt/mTOR pathway regulates the replicative senescence of human VSMCs, Mol. Cell. Biochem. 422 (1–2) (2016) 1–10, https://doi.org/10.1007/s11010-016-2796-9.

[15] J.K. Zhan, Y.J. Wang, Y. Wang, Z.Y. Tang, P. Tan, W. Huang, Y.S. Liu, The protective effect of GLP-1 analogue in arterial calcification through attenuating osteoblastic differentiation of human VSMCs, Int. J. Cardiol. 189 (2015) 188–193, https://doi.org/10.1016/j.ijcard.2015.04.086.

[16] C.Y. Chen, S.S. Rao, L. Ren, X.K. Hu, Y.J. Tan, Y. Hu, J. Luo, Y.W. Liu, H. Yin, J. Huang, J. Cao, Z.X. Wang, Z.Z. Liu, H.M. Liu, S.Y. Tang, R. Xu, H. Xie, Exosomal DMBT1 from human urine-derived stem cells facilitates diabetic wound repair by promoting angiogenesis, Theranostics 8 (6) (2018) 1607–1623, https://doi.org/10.7150/thno.22958.

[17] J.K. Zhan, Y.J. Wang, Y. Wang, Z.Y. Tang, P. Tan, W. Huang, Y.S. Liu, Adiponectin attenuates the osteoblastic differentiation of vascular smooth muscle cells through the AMPK/mTOR pathway, Exp. Cell Res. 323 (2) (2014) 352–358, https://doi.org/10.1016/j.yexcr.2014.02.016.

[18] R.R. Cui, S.J. Li, L.J. Liu, L. Yi, Q.H. Liang, X. Zhu, G.Y. Liu, Y. Liu, S.S. Wu, X.B. Liao, L.Q. Yuan, D.A. Mao, E.Y. Liao, MicroRNA-204 regulates vascular smooth muscle cell calcification in vitro and in vivo, Cardiovasc. Res. 96 (2) (2012) 320–329, https://doi.org/10.1093/cvr/cvs258.

[19] S. Zoungas, J. Chalmers, B. Neal, L. Billot, Q. Li, Y. Hirakawa, H. Arima, H. Monaghan, R. Joshi, S. Colagiuri, M.E. Cooper, P. Glasziou, D. Grobbee, P. Hamet, S. Harrap, S. Heller, L. Lisheng, G. Mancia, M. Marre, D.R. Matthews, C.E. Mogensen, V. Perkovic, N. Poulter, A. Rodgers, B. Williams, S. MacMahon, A. Patel, M. Woodward, Follow-up of blood-pressure lowering and glucose control in type 2 diabetes, N. Engl. J. Med. 371 (15) (2014) 1392–1406, https://doi.org/10.1056/NEJMoa1407963.

[20] Q. Yuan, C.P. Hu, Z.C. Gong, Y.P. Bai, S.Y. Liu, Y.J. Li, J.L. Jiang, Accelerated onset of senescence of endothelial progenitor cells in patients with type 2 diabetes mellitus: role of dimethylarginine dimethylaminohydrolase 2 and asymmetric dimethylarginine, Biochem. Biophys. Res. Commun. 458 (4) (2015) 869–876, https://doi.org/10.1016/j.bbrc.2015.02.050.

[21] C. He, S. Zheng, Y. Luo, B. Wang, Exosome theranostics: biology and translational medicine, Theranostics 8 (1) (2018) 237–255, https://doi.org/10.7150/thno.21945.

[22] I.M. Dykes, Exosomes in cardiovascular medicine, Cardiol. Ther. 6 (2) (2017) 225–237, https://doi.org/10.1007/s40119-017-0091-9.

[23] T. Machida, T. Tomofuji, D. Ekuni, T. Maruyama, T. Yoneda, Y. Kawabata, H. Mizuno, H. Miyai, M. Kunitomo, M. Morita, MicroRNAs in salivary exosome as potential biomarkers of aging, Int. J. Mol. Sci. 16 (9) (2015) 21294–21309, https://doi.org/10.3390/ijms160921294.

[24] L. Zhao, H. Luo, X. Li, T. Li, J. He, Q. Qi, Y. Liu, Z. Yu, Exosomes derived from

human pulmonary artery endothelial cells shift the balance between proliferation and apoptosis of smooth muscle cells, Cardiology 137 (1) (2017) 43–53, https://doi.org/10.1159/000453544.
[25] E. Hergenreider, S. Heydt, K. Treguer, T. Boettger, A.J. Horrevoets, A.M. Zeiher, M.P. Scheffer, A.S. Frangakis, X. Yin, M. Mayr, T. Braun, C. Urbich, R.A. Boon, S. Dimmeler, Atheroprotective communication between endothelial cells and smooth muscle cells through miRNAs, Nat. Cell Biol. 14 (3) (2012) 249–256, https://doi.org/10.1038/ncb2441.
[26] G. Luxan, G. D'Amato, D. MacGrogan, J.L. de la Pompa, Endocardial notch signaling in cardiac development and disease, Circ. Res. 118 (1) (2016) e1–e18, https://doi.org/10.1161/circresaha.115.305350.
[27] M.T. Sweetwyne, A. Gruenwald, T. Niranjan, R. Nishinakamura, L.J. Strobl, K. Susztak, Notch1 and Notch2 in podocytes play differential roles during diabetic nephropathy development, Diabetes 64 (12) (2015) 4099–4111, https://doi.org/10.2337/db15-0260.
[28] J.T. Baeten, B. Lilly, Differential regulation of NOTCH2 and NOTCH3 contribute to their unique functions in vascular smooth muscle cells, J. Biol. Chem. 290 (26) (2015) 16226–16237, https://doi.org/10.1074/jbc.M115.655548.
[29] F.P. Lampreia, J.G. Carmelo, F. Anjos-Afonso, Notch signaling in the regulation of hematopoietic stem cell, Curr Stem Cell Rep 3 (3) (2017) 202–209, https://doi.org/10.1007/s40778-017-0090-8.
[30] C. Zhang, D. Chen, E.M. Maguire, S. He, J. Chen, W. An, M. Yang, T.A. Afzal, L.A. Luong, L. Zhang, H. Lei, Q. Wu, Q. Xiao, Cbx3 inhibits vascular smooth muscle cell proliferation, migration, and neointima formation, Cardiovasc. Res. 114 (3) (2018) 443–455, https://doi.org/10.1093/cvr/cvx236.
[31] L. Li, L. Li, F. Xie, Z. Zhang, Y. Guo, G. Tang, D. Lv, Q. Lu, L. Chen, J. Li, Jagged-1/Notch3 signaling transduction pathway is involved in apelin-13-induced vascular smooth muscle cells proliferation, Acta Biochim. Biophys. Sin. Shanghai 45 (10) (2013) 875–881, https://doi.org/10.1093/abbs/gmt085.
[32] A. Bhattacharyya, S. Lin, M. Sandig, K. Mequanint, Regulation of vascular smooth muscle cell phenotype in three-dimensional coculture system by Jagged1-selective Notch3 signaling, Tissue Eng. Part A 20 (7–8) (2014) 1175–1187, https://doi.org/10.1089/ten.TEA.2013.0268.
[33] W.M. Passtoors, M. Beekman, J. Deelen, R. van der Breggen, A.B. Maier, B. Guigas, E. Derhovanessian, D. van Heemst, A.J. de Craen, D.A. Gunn, G. Pawelec, P.E. Slagboom, Gene expression analysis of mTOR pathway: association with human longevity, Aging Cell 12 (1) (2013) 24–31, https://doi.org/10.1111/acel.12015.
[34] S.C. Johnson, P.S. Rabinovitch, M. Kaeberlein, mTOR is a key modulator of ageing and age-related disease, Nature 493 (7432) (2013) 338–345, https://doi.org/10.1038/nature11861.
[35] A.J. Donato, R.G. Morgan, A.E. Walker, L.A. Lesniewski, Cellular and molecular biology of aging endothelial cells, J. Mol. Cell. Cardiol. 89 (Pt B) (2015) 122–135, https://doi.org/10.1016/j.yjmcc.2015.01.021.
[36] J.K. Zhan, Y.J. Wang, Y. Wang, S. Wang, P. Tan, W. Huang, Y.S. Liu, The mammalian target of rapamycin signalling pathway is involved in osteoblastic differentiation of vascular smooth muscle cells, Can. J. Cardiol. 30 (5) (2014) 568–575, https://doi.org/10.1016/j.cjca.2013.11.005.