

Original Article

Co-cultures of endothelial cells and smooth muscle cells affect vascular calcification

Wei Xiang^{1*}, Wang Liao^{2*}, Zhuwen Yi³, Xiaojie He³, Yan Ding⁴

¹Department of Pediatrics, Maternal and Child Health Care Hospital of Hainan Province, Haikou 570206, China;

²Department of Cardiology, Hainan General Hospital, Haikou 570102, China; ³Department of Nephropathy, Children's Medical Center, The Second Xiangya Hospital, Central South University, Changsha 410000, China;

⁴Department of Dermatology, Hainan Provincial Dermatology Disease Hospital, Haikou 570206, China. *Equal contributors and co-first authors.

Received November 5, 2016; Accepted April 25, 2017; Epub June 15, 2017; Published June 30, 2017

Abstract: Vascular calcification is common in patients with atherosclerosis, diabetes, renal disease and other aging diseases. In this study, endothelial cells (EC) and smooth muscle cells (SMC) were isolated from apoE-deficient (apoE^{-/-}) and wild type (WT) mice. Co-cultures of EC and SMC were established to study the underlying mechanisms of vascular calcification, based on the changes of vascular calcification-associated markers secreted by two types of cells. The co-cultures were divided into the following four groups: apoE^{-/-}-EC and apoE^{-/-}-SMC (A), WT-EC and apoE^{-/-}-SMC (B), WT-EC and WT-SMC (C), and apoE^{-/-}-EC and WT-SMC (D). The results showed that co-cultures with apoE^{-/-}-EC and apoE^{-/-}-SMC could increase intracellular calcium content and alkaline phosphatase (ALP) activity in EC and SMC, reduce 25-Hydroxyvitamin D₃-1 α -Hydroxylase (CYP27B1) expression in EC, whereas increased in SMC, promote osteopontin (OPN) and bone morphogenetic protein-2 (BMP-2), whereas inhibit osteoprotegerin (OPG) and matrix Gla protein (MGP) protein expression in EC and SMC, accelerate apoptosis of EC and SMC. All above results suggested that co-culture of apoE^{-/-}-EC and apoE^{-/-}-SMC had the highest risk of vascular calcification, and apoE^{-/-}-EC and apoE^{-/-}-SMC could also accelerate calcification of normal WT-SMC and WT-EC, respectively, through interactions between EC and SMC. The interactions between EC and SMC played an important role in the pathogenesis process of vascular calcification, and co-cultures of EC and SMC could be used as an ideal model for investigation of vascular calcification diseases.

Keywords: Vascular calcification, apoE-deficient mice, co-culture, endothelial cells, smooth muscle cells

Introduction

Vascular calcification is common and clinically significant in atherosclerosis, diabetes, hypertension, renal disease and other aging diseases [1-3]. Vascular calcification is no longer regarded as a passive process of calcium phosphate crystals deposition. Recently, more studies confirmed that vascular calcification is a regulated active process similar to that of bone formation, but the exact mechanism remains unclear [4-6]. Thus, the investigation of the underlying mechanisms that might contribute to the progression of vascular calcification is necessary for developing effective preventive and therapeutic strategies.

Some related factors, such as matrix Gla protein (MGP), bone morphogenetic protein-2

(BMP-2) and alkaline phosphatase (ALP), are considered to played an extremely important role in promoting blood vessel wall changes [7, 8]. In some situations, the balance of vascular calcification-related factors is broken and vascular calcification be capable of progress. Vascular endothelial cells (EC) and smooth muscle cells (SMC) are two main types of cells in the blood vessel wall and are closely related in structure and function [9, 10]. Studying EC and SMC structural and metabolic interactions is essential to understand vascular disorders such as vascular calcification and atherosclerosis.

The interactions between EC and SMC are pivotal to blood vessel development and function. EC are in intimate proximity to and communicate with SMC through hetero-cellular junctions

and signaling molecules [10, 11]. SMC metabolism, differentiation, proliferation and vasomotion are regulated by EC, which has an important role in maintaining a stable environment within the vessel wall, and many physiological and pathological processes [12]. The co-culture systems of EC and SMC enables cellular communication via the growth factors, cytokines and other soluble mediators secreted by these two types of cells, and thus becomes a very commonly used method to investigate vascular calcification and atherosclerosis [11, 13, 14].

In this study, EC and SMC were isolated from apoE-deficient (apoE^{-/-}) mice and wild type (WT) mice, and the expression of 25-Hydroxy-vitamin D₃-1 α -Hydroxylase (CYP27B1), osteopontin (OPN), osteoprotegerin (OPG), matrix Gla protein (MGP), bone morphogenetic protein-2 (BMP-2), calcium content and alkaline phosphatase (ALP) activity in both types of cells were examined. *In vitro* model of vascular calcification using the co-culture of EC and SMC was established based on the changes in a series of vascular calcification-associated markers secreted by two types of cells. The ultimate aim is to ascertain role of the interactions between EC and SMC in the pathogenesis of vascular calcification diseases.

Materials and methods

Animals and dissection

Six-week-old male WT mice and apoE^{-/-} mice were purchased from the Animal Center of Beijing University (Beijing, China), and maintained individually in a specific-pathogen-free barrier facility with a 12-hour light cycle. Animal experimental protocols were approved by the Committee on Animal Care of Hainan University and the procedures were performed in accordance with the guidelines for the institutional Animal Care and Use Committee. Mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (20 mg/kg). The thoracic aortas were dissected after sacrifice and immersed in modified Krebs solution of composition (mM): NaCl 115, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, EDTA 0.01 and glucose 11.1.

Laboratory measurements

Approximately 5 mg of thoracic aorta tissue was homogenized in PBS and centrifuged at

8000 g for 10 min at 4°C. The supernatant was used to determine ALP activity by disodium phenyl phosphate method according to the instructions of the manufacturer (Beijing Leadman, Beijing, China). Vascular calcium content was measured by atomic absorption spectrophotometer as previously described [15]. Vascular calcium deposition in thoracic aorta segments from 10-week-old apoE^{-/-} and WT mice was detected using von Kossa staining method. In briefly, aortic rings were embedded in paraffin and cut into 6 μ m thick sections. The sections were immersed with 5% silver nitrate solution for 30 min under the sunlight, washed with water, and treated with 5% sodium thiosulphate solution for 1 min followed by washing with water, and stained with 1% eosin for seconds. The staining results were observed and photographed under a light microscope (Nikon, Tokyo, Japan).

Cell isolation and cultivation

EC and SMC were isolated from the thoracic aortas of 10-week-old apoE^{-/-} mice and WT mice using a collagenase digestion protocol as previously described [16]. The isolated cells were cultured in M199 containing 10% FBS and 1% penicillin/streptomycin for EC and DMEM supplemented with 10% FBS and 1% penicillin/streptomycin for SMC, and incubated at 37°C in a 5% CO₂ atmosphere. The cells were passaged in a split ratio of 1:2. Antibodies to von Willebrand factor and α -smooth muscle actin were used to identify EC and SMC, respectively.

Preparation of EC-SMC co-culture

Cells from apoE^{-/-} mice and WT mice between passages 3 and 6 were used to prepare EC-SMC co-culture systems as described by Li [13]. Briefly, the co-culture systems of EC and SMC were divided into the following four co-culture systems: apoE^{-/-}-EC and apoE^{-/-}-SMC (A), WT-EC and apoE^{-/-}-SMC (B), WT-EC and WT-SMC (C), and apoE^{-/-}-EC and WT-SMC (D). The SMC (1.5×10^5 cells/cm²) were seeded on the upper membrane of Transwell inserts (Corning Incorporated, Corning, NY, USA) and became adherent after incubation for 12 h. The inserts were placed in Transwell inserts, and the EC (1.5×10^5 cells/well) were plated on the inner surface of the membrane. Based on the different requirements for the respective experiments, these plates were co-incubated for 24

Table 1. Mouse aortic vascular calcium content and ALP activity

Measurements	WT mice	apoE ^{-/-} -mice	t value	P value
Vascular calcium content (μg/mg)	0.51±0.08	1.89±0.26***	8.787	0.0009
ALP activity (IU/mg protein)	268.65±21.66	612.30±92.78**	6.247	0.0033

***P<0.001 and **P<0.01 vs WT mice group.

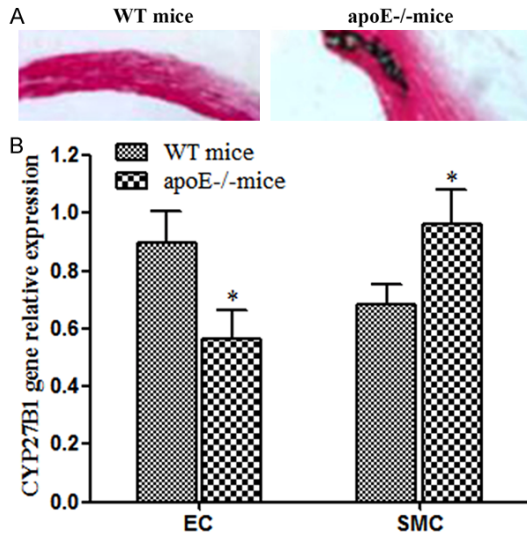


Figure 1. Vascular calcium deposits in aortae from apoE^{-/-} and WT mice (A), and CYP27B1 gene expression in EC and SMC from apoE^{-/-} and WT mice (B). ***P<0.001 and **P<0.01 vs WT mice group.

h, 48 h, and 72 h at 37°C in a 5% CO₂ atmosphere to form four groups of EC-SMC co-culture systems.

Quantitative real-time PCR

Total RNAs were extracted from EC and SMC, and the isolated EC and SMC from co-cultures using the RNeasy mini kit (Qiagen, Hilden, Germany) and the reverse transcription was performed in the reverse transcription kit (Promega, Madison, WI, USA), according to their manufacturer's instructions. The quantitative real-time PCR was carried out using an ABI Prism 7700 instrument (Applied Biosystems, Foster City, CA, USA). The primers were designed by PyroMark Assay Design 2.0 (Qiagen, Hilden, Germany) and synthesized by Beijing Genomics Institute (Beijing, China). The primer sequences for CYP27B1 were forward strand 5-CATCATGGGCAGAGC-ACCGT-3 and reverse strand 5-TCACCATCCGCCGTTAGCAA-3. The PCR reaction was amplified using 40 cycles of denaturing for 40 s at 95°C, annealing for 40 s

at 58°C and extending for 40 s at 72°C with a fluorescence detection system. Relative quantification of mRNA levels was calculated based on a ratio of each target gene concentration to housekeeping gene β-actin concentration.

Western blotting

Thoracic aorta tissue samples were homogenized in ice-cold tissue lysate buffer according to the manufacturer's instructions (Beyotime, Haimen, China). EC and SMC, and the isolated EC and SMC from co-cultures were washed with PBS, gathered and homogenized in ice-cold lysis buffer following the manufacturer's instructions (Beyotime, Haimen, China). The cell and tissue lysates were centrifuged at 48°C and the total protein concentration of the supernatant was determined using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL). Equal amounts of proteins were loaded on SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in TBS-T (Tris-buffered saline containing 0.1% Tween-20) for 2 h at room temperature, and incubated with the designated primary antibodies overnight at 4°C and then with the appropriate secondary antibodies conjugated with HRP for 2 h at room temperature. The protein bands were visualized using the ECL Plus detection reagent (Amersham Biotech, Piscataway, NJ), and band densities were quantified with image analysis software (Bio-Rad, Laboratories, Inc., California).

Cell apoptosis

EC and SMC were separated from 72 h co-culture systems, washed with PBS, and resuspended in the kit reaction buffer containing propidium iodide and annexin V-FITC. After mixing, samples were incubated for 1 h at room temperature and then cell apoptosis was detected using EPICS XL-4 flow cytometry (Beckman, CA, USA). Flow cytometry data were analyzed using the WINMDI software pack-

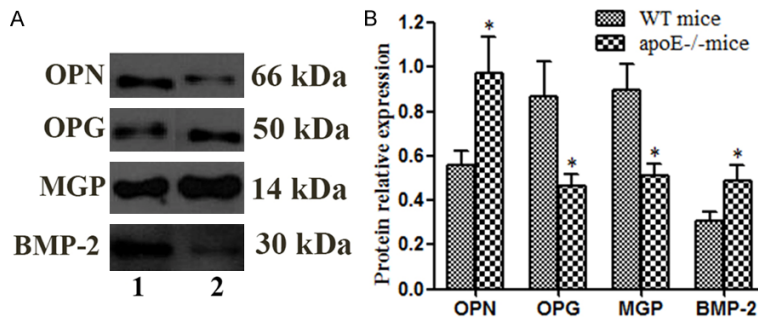


Figure 2. Protein expression levels of OPN, BMP-2, OPG and MGP in thoracic aorta tissue from apoE^{-/-} and WT mice. Lanes 1 and 2 represent protein bands of thoracic aorta tissue from apoE^{-/-} and WT mice, respectively; * $P < 0.05$ vs WT mice group.

age (version 2.9, <http://en.bio-soft.net/other/WinMDI.html>).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.01 (GraphPad software, San Diego, CA). Data were expressed as the mean \pm SD from three repeated experiments. The independent sample *t* test was used to assess the differences of two groups. Differences of EC or SMC isolated from four co-culture systems were analyzed with one-way ANOVA, followed by Newman-Keuls test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

ApoE^{-/-} mice exhibited the presence of significant vascular calcification

Vascular calcium content and ALP activity in 10-week-old apoE^{-/-} mice were significantly increased compared with age-matched WT mice group (**Table 1**). The results of von Kossa staining showed that no calcium deposits were observed in thoracic aorta of WT mice, but massive calcium deposits were frequently found in 10-week-old apoE^{-/-} mice (**Figure 1A** and **1B**). CYP27B1 gene expression in EC from apoE^{-/-} mice was significantly lesser than that in EC from WT mice, whereas CYP27B1 gene in SMC from apoE^{-/-} mice was significantly greater than that in SMC from WT mice (**Figure 1C**). OPN and BMP-2 protein expression in thoracic aorta tissue of apoE^{-/-} mice were significantly higher than those of WT mice, while OPG and MGP protein expression were significantly lower than those of apoE^{-/-} mice (**Figure 2**). The

above data suggested that apoE^{-/-} mice were an ideal model for investigating vascular calcification.

Co-cultures with apoE^{-/-} EC and apoE^{-/-} SMC reduced CYP27B1 expression in EC, whereas increased in SMC

Compared with other co-culture systems, CYP27B1 gene expression in co-culture of apoE^{-/-} EC and apoE^{-/-} SMC (group A) gradually decreased for EC ($P < 0.01$) and increased

for SMC ($P < 0.01$) with the extension of co-culture time. In addition, CYP27B1 gene expression in co-culture systems of group B and group D was gradually decreased for EC ($P < 0.01$) and increased for SMC compared with that of group C, showing that apoE^{-/-} EC and apoE^{-/-} SMC could accelerate calcification of normal SMC and EC, respectively, and finally also increased risk of vascular calcification (**Figure 3**). Trends of CYP27B1 protein expression in EC and SMC, and the isolated EC and SMC from co-cultures were consistent with CYP27B1 gene expression levels (data not show).

Co-cultures with apoE^{-/-} EC and apoE^{-/-} SMC promoted OPN and BMP-2, whereas inhibited OPG and MGP protein expression in EC and SMC

EC and SMC separated from 72 h co-culture systems were used to detect protein expression levels of OPN, BMP-2, OPG and MGP. The results showed that protein expression levels of OPN and BMP-2 were significantly higher, and OPG and MGP protein expression were significantly lower in the isolated apoE^{-/-} EC and apoE^{-/-} SMC from co-culture systems than in other isolated cells (**Figure 4**).

Co-cultures with apoE^{-/-} EC and apoE^{-/-} SMC increased intracellular calcium content and ALP activity in EC and SMC

EC and SMC were separated from 72 h co-culture systems and then used for detection of intracellular calcium content and ALP activity referring to the above method (Laboratory measurements). Both intracellular calcium content and ALP activity were significantly increased in

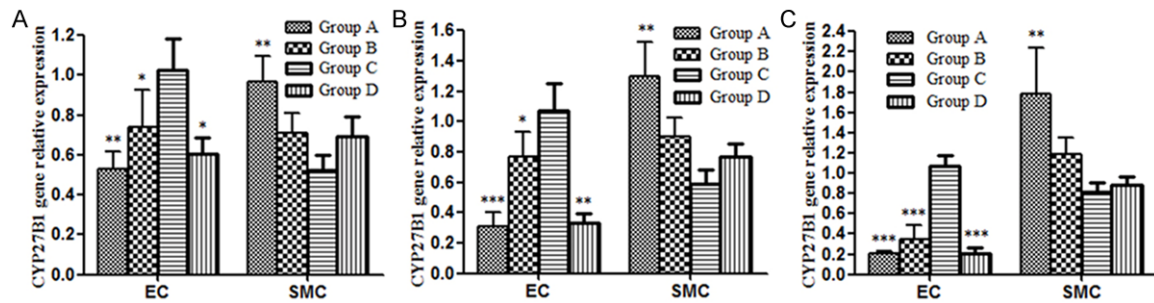


Figure 3. CYP27B1 gene expression in EC and SMC isolated from four co-culture systems for 24 h (A), 48 h (B), and 72 h (C). Group A, Group B, Group C and Group D represent the co-culture system of apoE^{-/-}-EC and apoE^{-/-}-SMC, WT-EC and apoE^{-/-}-SMC, WT-EC and WT-SMC, and apoE^{-/-}-EC and WT-SMC, respectively; ***P<0.001, **P<0.01 and *P<0.05 vs group C.

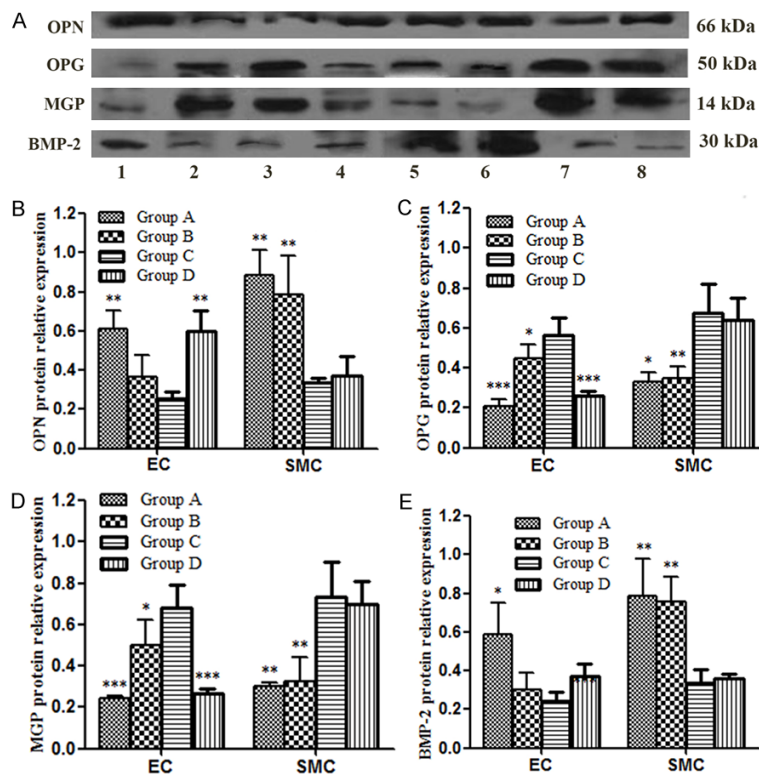


Figure 4. Protein expressions of OPN, BMP-2, OPG and MGP in EC and SMC isolated from each co-culture system at 72 h. Lanes 1, 2, 3 and 4 represent protein bands of EC isolated from Group A, Group B, Group C and Group D, respectively; Lanes 5, 6, 7 and 8 represent protein bands of SMC isolated from Group A, Group B, Group C and Group D, respectively; Group A, Group B, Group C and Group D represent the co-culture system of apoE^{-/-}-EC and apoE^{-/-}-SMC, WT-EC and apoE^{-/-}-SMC, WT-EC and WT-SMC, and apoE^{-/-}-EC and WT-SMC, respectively; ***P<0.001, **P<0.01 and *P<0.05 vs group C.

the isolated apoE^{-/-}-EC and apoE^{-/-}-SMC from co-culture systems compared with other isolated cells. In addition, intracellular calcium content and ALP activity in WT-SMC co-cultured with apoE^{-/-}-EC were also higher than those in

WT-SMC co-cultured with WT-EC, implying that apoE^{-/-}-EC could increase intracellular calcium content and ALP activity in WT-SMC co-cultured with it (Table 2).

Co-cultures with apoE^{-/-}-EC and apoE^{-/-}-SMC accelerated apoptosis of EC and SMC

Cell apoptosis analysis showed that EC and SMC isolated from co-culture of apoE^{-/-}-EC and apoE^{-/-}-SMC had the highest apoptosis rate (group A) compared to those from other three groups. More importantly, the apoptosis rate of EC and SMC in co-culture systems of group B and group D was significantly higher than that of group C (Figure 5), which suggested that apoE^{-/-}-EC and apoE^{-/-}-SMC could accelerate apoptosis of normal SMC and EC co-cultured with them, respectively.

Discussion

In the present study, vascular calcium content and ALP activity were significantly higher in 10-week-old apoE^{-/-}-mice than in age-matched, and massive calcium deposits were observed in thoracic aorta of apoE^{-/-}-mice, which showed an increased vascular calcification in apoE^{-/-}-mice and were consistent with

Table 2. Calcium content and ALP activity in EC and SMC isolated from each co-culture system at 72 h

Groups	EC		SMC	
	Calcium ($\mu\text{g}/\text{mg}$)	ALP (IU/mg protein)	Calcium ($\mu\text{g}/\text{mg}$)	ALP (IU/mg protein)
apoE ^{-/-} -EC and apoE ^{-/-} -SMC	0.65 \pm 0.02***	506.20 \pm 62.65***	0.96 \pm 0.03***	687.36 \pm 112.89***
WT-EC and apoE ^{-/-} -SMC	0.38 \pm 0.02*	213.56 \pm 16.98	0.92 \pm 0.06***	703.66 \pm 110.25***
WT-EC and WT-SMC	0.29 \pm 0.03	198.26 \pm 26.11	0.35 \pm 0.02	222.38 \pm 19.37
apoE ^{-/-} -EC and WT-SMC	0.64 \pm 0.06***	511.33 \pm 88.21***	0.41 \pm 0.03	316.66 \pm 34.60

*** P <0.001 and * P <0.05 vs group C.

previous researches [17, 18]. Therefore, EC and SMC were isolated from apoE^{-/-}-mice and used to develop an in vitro model of vascular calcification. As expected, co-culture of apoE^{-/-}-EC and apoE^{-/-}-SMC had the highest calcium content and ALP activity. But more importantly, we found that apoE^{-/-}-EC could also significantly increase calcium content and ALP activity in WT-SMC co-cultured with it.

The 1 α , 25-Dihydroxyvitamin D₃ as an active metabolite of vitamin D, could exert actions including suppression of vascular calcification and inhibition of vascular smooth muscle proliferation. However, the concentration of active vitamin D was regulated by the vitamin D-activating enzyme CYP27B1 [19-21]. In our study, CYP27B1 mRNA and protein expression levels were significantly decreased in EC and increased in SMC from apoE^{-/-}-mice compared with those from WT mice. In four EC-SMC co-culture systems, CYP27B1 gene and protein expression in co-culture of apoE^{-/-}-EC and apoE^{-/-}-SMC were the lowest for EC and the highest for SMC at each corresponding co-culture time, suggesting the highest risk of vascular calcification. While CYP27B1 gene and protein expression in co-culture systems of group B and group D were also significantly lower for EC and higher for SMC than that of group C, showing that apoE^{-/-}-EC and apoE^{-/-}-SMC could accelerate calcification of normal SMC and EC, respectively, and finally increased risk of vascular calcification.

BMP-2, an important molecule in the regulation of bone formation and in vascular calcification, can promote osteoblast differentiation and is a potent inducer of vascular calcification [22, 23]. OPN exists in calcified vessels and can serve as a vascular calcification marker [24, 25]. Both BMP-2 and OPN protein levels were significantly elevated in thoracic aorta of apoE^{-/-}-mice compared with those of WT mice.

Analysis of in vitro co-cultures of EC and SMC showed that protein expression of OPN and BMP-2 was significantly higher in the isolated apoE^{-/-}-EC and apoE^{-/-}-SMC from co-culture systems than those in isolated WT cells. MGP and OPG were usually considered as calcification inhibitors and protective against vascular calcification [26-28], and exhibited opposite trends with OPN and BMP-2 in thoracic aorta of apoE^{-/-}-mice and in the isolated apoE^{-/-}-EC and apoE^{-/-}-SMC from co-culture systems. These results confirmed that apoE^{-/-}-EC and apoE^{-/-}-SMC in co-culture systems were more likely to lead vascular calcification.

Previous studies suggested that apoptosis resulted in calcification and could be a key regulator of vascular calcification [29, 30]. In the present research, EC and SMC had the highest apoptosis rate in co-culture of apoE^{-/-}-EC and apoE^{-/-}-SMC, which indicated that interactions between apoE^{-/-}-EC and apoE^{-/-}-SMC most promoted apoptosis and finally could cause severe vascular calcification. In addition, apoE^{-/-}-EC and apoE^{-/-}-SMC could also accelerate apoptosis of normal SMC and EC co-culture with them, respectively, and thereby increased risk of vascular calcification.

Taken together, EC-SMC co-cultures were established to investigate the role of interactions between EC and SMC in vascular calcification. By analyzing changes in a series of vascular calcification-associated markers and factors, we revealed that co-culture of apoE^{-/-}-EC and apoE^{-/-}-SMC showed the highest risk of vascular calcification. Noting that apoE^{-/-}-EC and apoE^{-/-}-SMC could also accelerate calcification of normal WT-SMC and WT-EC, respectively, through interactions between cells, and accordingly increased risk of vascular calcification. Thus, the co-culture systems of EC and SMC were capable of not only ascertaining the pathogenesis of vascular calcification, but also

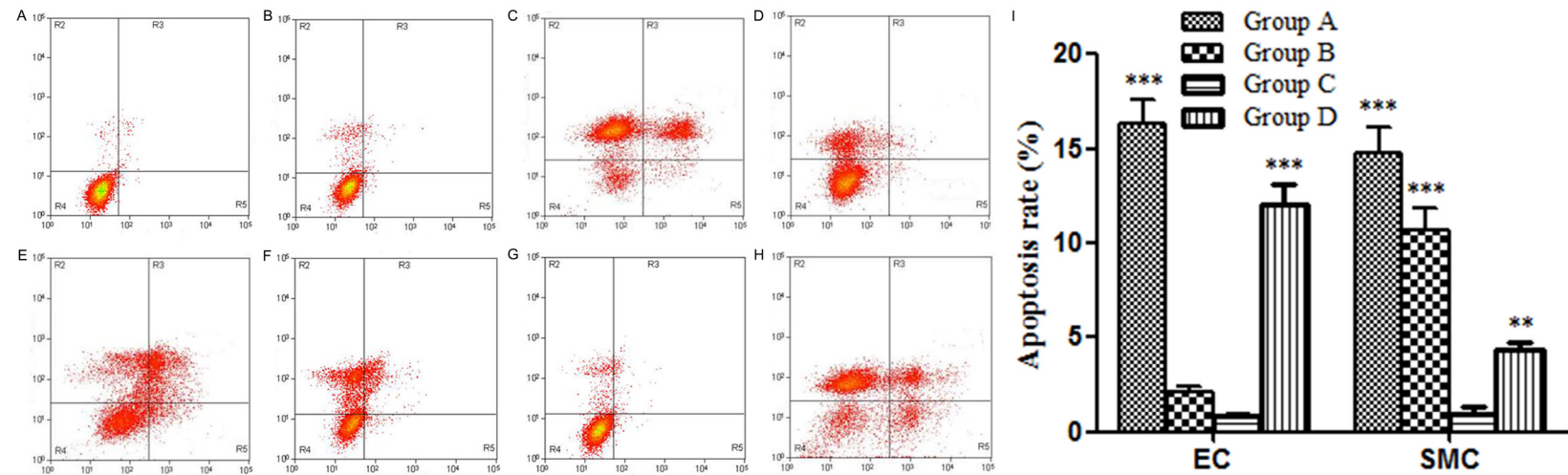


Figure 5. Cell apoptosis analysis in EC and SMC isolated from 72 h co-culture systems by flow cytometry. A, C, E and G: Represent images of flow cytometry analysis in EC isolated from group A, B, C and D, respectively; B, D, F and H: Represent images of flow cytometry analysis in SMC isolated from group A, B, C and D, respectively; I: Represent cell apoptosis results of flow cytometry analysis, in which Group A, Group B, Group C and Group D represent the co-culture system of apoE^{-/-}-EC and apoE^{-/-}-SMC, WT-EC and apoE^{-/-}-SMC, WT-EC and WT-SMC, and apoE^{-/-}-EC and WT-SMC, respectively; *** P <0.001 and ** P <0.01 vs group C.

providing an ideal model of vascular calcification for drug screening.

Acknowledgements

This work was supported by National Natural Science Foundation of China (no. 81260139 and no. 81060073, no. 81560275, no. 6156-2021, no. 30560161), Hainan special projects of Social Development (2015SF39), Hainan Association for academic excellence Youth Science and Technology Innovation Program (201515), Hainan Natural Science Foundation of China (814319), Ministry of Personnel scientific and technological activities subject students project funds (2006).

Disclosure of conflict of interest

None.

Address correspondence to: Xiaojie He, Department of Nephropathy, Children's Medical Center, The Second Xiangya Hospital, Central South University, Changsha 410000, China. Tel: 86-731-85292170; Fax: 86-731-85533525; E-mail: hexiaojiexy2017@163.com; Yan Ding, Department of Dermatology, Hainan Provincial Dermatology Disease Hospital, 49 LongKun-Nan Road, Haikou 570206, China. Tel: 86-898-36689605; Fax: 86-898-36689605; E-mail: yandhk2016@126.com

References

- [1] Parhami E, Tintut Y, Patel JK, Mody N, Hemmat A, Demer LL. Regulation of vascular calcification in atherosclerosis. *Z Kardiol* 2001; 90: 27-30.
- [2] Anand DV, Lim E, Darko D, Bassett P, Hopkins D, Lipkin D, Corder R, Lahiri A. Determinants of progression of coronary artery calcification in type 2 diabetes: role of glycemic control and inflammatory/vascular calcification markers. *J Am Coll Cardiol* 2007; 50: 2218-25.
- [3] Cozzolino M, Dusso AS, Slatopolsky E. Role of calcium-phosphate product and bone-associated proteins on vascular calcification in renal failure. *J Am Soc Nephrol* 2001; 12: 2511-6.
- [4] Towler DA, Shao JS, Cheng SL, Pingsterhaus JM, Loewy AP. Osteogenic regulation of vascular calcification. *Ann N Y Acad Sci* 2006; 1068: 327-33.
- [5] Ndip A, Williams A, Jude EB, Serracino-Inglott F, Richardson S, Smyth JV, Boulton AJ, Alexander MY. The RANKL/RANK/OPG signaling pathway mediates medial arterial calcification in diabetic Charcot neuroarthropathy. *Diabetes* 2011; 60: 2187-96.
- [6] Giachelli CM. Vascular calcification mechanisms. *J Am Soc Nephrol* 2004; 15: 2959-64.
- [7] Evrard S, Delanaye P, Kamel S, Cristol JP, Cavalier E. Vascular calcification: from pathophysiology to biomarkers. *Clin Chim Acta* 2015; 438: 401-14.
- [8] Abedin M, Tintut Y, Demer LL. Vascular calcification mechanisms and clinical ramifications. *Arterioscler Thromb Vasc Biol* 2004; 24: 1161-70.
- [9] Huang H, Ye L, Tang C, Sun T, Wang G. Impacts of low density lipoprotein on adhesive mechanical properties of endothelial cells and collagen IV. *Biomedical Engineering: Applications, Basis and Communications* 2012; 24: 1-7.
- [10] Williams C, Wick TM. Endothelial cell-smooth muscle cell co-culture in a perfusion bioreactor system. *Ann Biomed Eng* 2005; 33: 920-8.
- [11] Davies PF. Vascular cell interactions with special reference to the pathogenesis of atherosclerosis. *Lab Invest* 1986; 55: 5-24.
- [12] Davies PF, Olesen SP, Clapham DE, Morrel EM, Schoen FJ. Endothelial communication. State of the art lecture. *Hypertension* 1988; 11: 563-72.
- [13] Li Y, Guo Y, Chen Y, Wang Y, You Y, Yang Q, Weng X, Li Q, Zhu X, Zhou B, Liu X. Establishment of an interleukin-1 β -induced inflammation-activated endothelial cell-smooth muscle cell-mononuclear cell co-culture model and evaluation of the anti-inflammatory effects of tanshinone IIA on atherosclerosis. *Mol Med Rep* 2015; 12: 1665-76.
- [14] Hergenreider E, Heydt S, Tréguer K, Boettger T, Horrevoets AJ, Zeiher AM, Scheffer MP, Frangakis AS, Yin X, Mayr M, Braun T. Atheroprotective communication between endothelial cells and smooth muscle cells through miRNAs. *Nat Cell Biol* 2012; 14: 249-56.
- [15] Wang QQ, Yan H, An CN, Pu XP. Establishment of a sub-chronic model of vascular calcification induced by hypervitaminosis D3 united with nicotine. *Chinese Pharmacological Bulletin* 2009.
- [16] Liu LH, Paul RJ, Sutliff RL, Miller ML, Lorenz JN, Pun RY, Duffy JJ, Doetschman T, Kimura Y, MacLennan DH, Hoving JB. Defective endothelium-dependent relaxation of vascular smooth muscle and endothelial cell Ca²⁺ signaling in mice lacking sarco (endo) plasmic reticulum Ca²⁺-ATPase isoform 3. *J Biol Chem* 1997; 272: 30538-45.
- [17] Ivanovski O, Nikolov IG, Joki N, Caudrillier A, Phan O, Mentaverri R, Maizel J, Hamada Y, Nguyen-Khoa T, Fukagawa M, Kamel S. The calcimimetic R-568 retards uremia-enhanced vascular calcification and atherosclerosis in apolipoprotein E deficient (apoE^{-/-}) mice. *Atherosclerosis* 2009; 205: 55-62.

- [18] Zeadin M, Butcher M, Werstuck G, Khan M, Yee CK, Shaughnessy SG. Effect of leptin on vascular calcification in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* 2009; 29: 2069-75.
- [19] Muldowney S, Kiely M. Vitamin D and cardio-metabolic health: a review of the evidence. *Nutr Res Rev* 2011; 24: 1-20.
- [20] Zittermann A, Schleithoff SS, Koerfer R. Putting cardiovascular disease and vitamin D insufficiency into perspective. *Br J Nutr* 2005; 94: 483-92.
- [21] Zittermann A, Koerfer R. Vitamin D in the prevention and treatment of coronary heart disease. *Curr Opin Clin Nutr Metab Care* 2008; 11: 752-7.
- [22] Li X, Yang HY, Giachelli CM. BMP-2 promotes phosphate uptake, phenotypic modulation, and calcification of human vascular smooth muscle cells. *Atherosclerosis* 2008; 199: 271-7.
- [23] Derwall M, Malhotra R, Lai CS, Beppu Y, Aikawa E, Seehra JS, Zapol WM, Bloch KD, Paul BY. Inhibition of bone morphogenetic protein signaling reduces vascular calcification and atherosclerosis. *Arterioscler Thromb Vasc Biol* 2012; 32: 613-22.
- [24] Speer MY, McKee MD, Guldberg RE, Liaw L, Yang HY, Tung E, Karsenty G, Giachelli CM. Inactivation of the osteopontin gene enhances vascular calcification of matrix Gla protein-deficient mice evidence for osteopontin as an inducible inhibitor of vascular calcification in vivo. *J Exp Med* 2002; 196: 1047-55.
- [25] Berezin AE, Kremzer AA. Circulating osteopontin as a marker of early coronary vascular calcification in type two diabetes mellitus patients with known asymptomatic coronary artery disease. *Atherosclerosis* 2013; 229: 475-81.
- [26] Khavandgar Z, Roman H, Li J, Lee S, Vali H, Brinckmann J, Davis EC, Murshed M. Elastin haploinsufficiency impedes the progression of arterial calcification in MGP-deficient mice. *J Bone Miner Res* 2014; 29: 327-37.
- [27] Collin-Osdoby P, Rothe L, Bekker S, Anderson F, Huang Y, Osdoby P. Basic fibroblast growth factor stimulates osteoclast recruitment, development, and bone pit resorption in association with angiogenesis in vivo on the chick chorioallantoic membrane and activates isolated avian osteoclast resorption in vitro. *J Bone Miner Res* 2002; 17: 1859-71.
- [28] Bucay N, Sarosi I, Dunstan CR, Morony S, Topley J, Capparelli C, Scully S, Tan HL, Xu W, Lacey DL, Boyle WJ. Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev* 1998; 12: 1260-8.
- [29] Proudfoot D, Skepper JN, Hegyi L, Bennett MR, Shanahan CM, Weissberg PL. Apoptosis regulates human vascular calcification in vitro evidence for initiation of vascular calcification by apoptotic bodies. *Circ Res* 2000; 87: 1055-62.
- [30] Kendrick J, Chonchol M. The role of phosphorus in the development and progression of vascular calcification. *Am J Kidney Dis* 2011; 58: 826-34.