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Characterization of human cardiac mesenchymal stromal cells and their extracellular vesicles comparing with human bone marrow derived mesenchymal stem cells

Running title; human cardiac mesenchymal stromal cells and their EVs

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ABSTRACT

Cardiac regeneration with adult stem-cell (ASC) therapy is a promising field to address advanced cardiovascular diseases. In addition, extracellular vesicles (EVs) from ASCs have been implicated in acting as paracrine factors to improve cardiac functions in ASC therapy. In our work, we isolated human cardiac mesenchymal stromal cells (h-CMSCs) by means of three-dimensional organ culture (3D culture) during *ex vivo* expansion of cardiac tissue, to compare the functional efficacy with human bone-marrow derived mesenchymal stem cells (h-BM-MSCs), one of the actively studied ASCs. We characterized the h-CMSCs as $CD90^{low}$, c-$kit^{negative}$, $CD105^{positive}$ phenotype and these cells express NANOG, SOX2, and GATA4. To identify the more effective type of EVs for angiogenesis among the different sources of ASCs, we isolated EVs which were derived from CMSCs with either normoxic or hypoxic condition and BM-MSCs. Our *in vitro* tube-formation results demonstrated that the angiogenic effects of EVs from hypoxia-treated CMSCs (CMSC-Hpx EVs) were greater than the well-known effects of EVs from BM-MSCs (BM-MSC EVs), and these were even comparable to human vascular endothelial growth factor (hVEGF), a potent angiogenic factor. Therefore, we present here that $CD90^{low}$c-$kit^{negative}CD105^{positive}$ CMSCs under hypoxic conditions secrete functionally superior EVs for *in vitro* angiogenesis. Our findings will allow more insights on understanding myocardial repair.

Keywords: mesenchymal stem cell; extracellular vesicles; cardiovascular disease; regeneration

INTRODUCTION

Cardiovascular disease (CVD) is a global major health problem and has remained a leading cause of death, despite the advances in the current medical technologies (1). Cardiovascular regeneration (CVR) with adult stem-cell (ASC) therapy is a promising field for addressing advanced CVD (2, 3). Although there are diverse origins of ASCs, the cells that are mostly studied for CVR are mesenchymal stem cells (MSCs) and cardiac stem cells (4). Given the salutary effects of the ASCs for CVD reported by previous studies, there have been many preclinical studies as well as clinical trials conducted with the ASCs (4, 5).

The putative 'cardiac stem/progenitor cell (CS/CPC)' is also called a cardiac mesenchymal stem cell, cardiac interstitial cell, cardiac mesenchymal stromal cell (CMSC), or cardiosphere-derived cell (CDC). These cells are usually obtained from the right ventricular septum through cardiac biopsy, or from a left atrial sample of cardiac surgery (6, 7). In particular, CDCs indicate the cells derived from cardiospheres; multicellular clusters formed from the primary culture of cardiac specimen. There are several issues about CVR using different types of CS/CPCs. Especially, based on the research that c-kit$^+$ cells were potential CS/CPCs for rejuvenating heart muscles in mice (8), many researchers had considered these cells to be CS/CPCs. However, recent studies have proved that c-kit$^+$ cells are only a small proportion of CDCs, have low regenerative potential, and originate from cardiac endothelial cells, rather than from CS/CPCs (9, 10). Another issue is about CD90, which was originally discovered as a thymocyte antigen (thy-1) and can be used as a marker for a variety of stem cells and fibroblasts. Despite limited information regarding CD90 in CVR, several studies proved that CD90$^-$ CPCs expressed the genes related to stemness and could differentiate into mature cardiomyocytes with complete sarcomere formation (9, 11, 12). Although these cells also showed a greater regenerative potential in myocardial infarction (MI), it is still unclear which types of CS/CPCs are more important to the CVR.

Recently, another important concept has been receiving attention in ASC therapy; the paracrine effect. Many studies showed that the cardiac functional advances were not the direct effects of the transplanted CS/CPCs themselves. Instead, such improvement seemed to be induced by means of paracrine factors (13, 14); the extracellular vesicle (EV) is one strong candidate (15-17). EVs, also known as exosomes, are nanosized vesicles encompassed by lipid bilayer with diameters from 30~130 nm. EVs exist in almost all biological fluids, including blood, urine, and cell culture medium. They are released from the cells when multivesicular bodies fuse with the cell membrane. They participate as cellular cargos by carrying genetic materials and proteins, facilitate intercellular communication (16), and have specificity that depends on their originating cells (15). EVs from CDCs could inhibit apoptosis, promote proliferation of cardiomyocytes, and improve cardiac functions in MI mouse models by means of some miRNAs (18, 19).

In this study, we first compared the characterization of h-CMSCs and h-BM-MSCs, and identified EVs from the media of the cells. Relatively few studies have compared the effects of these cells directly (20-22), and especially $CD90^{low}$, $c\text{-}kit^{negative}$ CS/CPCs need to be further investigated. To compare the effects of EVs from each cell group, we evaluated *in vitro* effects of EVs on angiogenesis. Therefore, the comparative studies regarding EVs of h-BM-MSCs and h-CMSCs with normoxic and hypoxic conditions would help decide on future effective therapeutic target.

RESULTS

Characterization of h-BM-MSCs and h-CMSCs

To characterize the cultured h-BM-MSCs and h-CMSCs, we analyzed various cell surface markers for hematopoietic or cardiac cells by flow cytometry (Fig. 1A). Expression of a cluster of differentiation (CD)45, a type I transmembrane protein that is present in various isoforms

on differentiated hematopoietic cells, is less than 1% in both cell populations. Another hematopoietic stem-cell marker, CD34, is expressed only 1.87% in CMSCs and 6.09% in BM-MSCs, which meant that these cells are a CD45 and CD34 negative cell population, as previously reported (23). Importantly, CD90 is differently expressed in each cell population; 82.1% of BM-MSCs vs. 26.3% of CMSCs, which is similar to CDCs described (22). Both BM-MSCs and CMSCs express CD105 in about 80% of the cell population. CD105 (Endoglin), a membrane glycoprotein and a part of the TGF β receptor complex, plays a critical role in neoangiogenesis. CD117, known also as c-kit, is a stem-cell growth factor receptor on the surface of hematopoietic stem cells and some cancer cells. Expression levels of CD117 are less than 1% in both cell groups.

To further define the characteristics of the cells, we did RT-PCR assays for various marker genes. NANOG and SOX2, the transcriptional factors of embryonic stem cells, were expressed in all three cell populations (Fig. 1B). For cardiac markers, mRNAs of NKX2-5 and myosin heavy chain, the α isoform (MYH6), were not noticeable in all groups, but the expression of GATA4, a cardiac-specific transcription factor for proper mammalian cardiac development, was prominent in CMSCs but not in BM-MSCs (Fig. 1B). GATA4 and NKX2-5 are early markers of precardiac cells and present synergism as cofactors (24). GATA4 is expressed in CS/CPCs followed by the expression of NKX2-5 and ISL1 of the cardiac mesoderm.

Characterization of EVs isolated from CMSCs and BM-MSCs

EVs were isolated from conditioned media from each cell population. Transmission electron microscopy showed that EVs exhibited umbilicated round morphologies (Fig. 2A). Size measurements by dynamic light-scattering (DLS) analysis found a size of 95~115 nm for EVs (Fig. 2B). CMSC-Hpx EVs are slightly larger than the EVs from normoxia-conditioned CMSCs (CMSC-Nx) (Fig. 2A, B), which was similar to a previous report (21). Nanoparticle

tracking analysis (NTA) confirmed the size distribution of EVs and showed sufficient particle concentration (Fig. 2C). The purities of isolated EVs were analyzed by a size exclusion chromatography with high-performance liquid chromatography (HPLC) system, which exhibited levels of contaminating substances smaller than EVs were less than 5% in all EV preparations (Fig. 3A). Immunoblotting analysis have shown the enrichment of EV surface markers tetraspanins (CD9, CD63, and CD81) with minor differences at expression levels (Fig. 3B), while H2B histone, one of common contaminants in EV preparation was not detected in all isolated EVs used in this study (Fig. 3B).

CMSC-Hpx EVs exhibit functional superiority on *in vitro* angiogenesis

To evaluate the effects of EVs on angiogenesis, we did a tube-formation assay with HUVECs (human umbilical vein endothelial cells). HUVECs were cultured with hVEGF (positive control), BM-MSC EVs, CMSC-Nx EVs, or CMSC-Hpx EVs, and these were compared with the cells with media only (negative control; NC). HUVECs treated with BM-MSC EVs and CMSC-Hpx EVs showed tube formation significantly better than did HUVECs with NC at 6 h (Fig. 4), whereas CMSC-Nx EVs failed to demonstrate superiority over NC in tube formation. The formation of tube-like structures was increased with time up to 6 h in all groups of experiments. Interestingly, the tube-like structures lasted for 8 h in the cells treated with VEGF or CMSC-Hpx, whereas these structures started to collapse around 8 h in the group of BM-MSC EVs (Fig. 4B, C). With our results, CMSC-Nx EVs did not show improvement in tube formation, and only CMSC-Hpx EVs were able to improve the tube formation, creating tube-like structures that persisted for 8 h, all of which were comparable to the effect of VEGF.

DISCUSSION

We used the CMSCs obtained by *ex vivo* expansion of human heart tissue using 3D culture as previously described (25). These CMSCs did not form cardiospheres, but shared similar phenotypes with CDCs of $CD90^{low}$, c-$kit^{negative}$, $CD105^{positive}$. The CMSCs were distinguished from MSCs in the expression of CD90 (22).

Recently, a decision has been made on an issue concerning the regeneration of CS/CPCs, especially c-kit^+ cells (6, 8, 26): 31 papers from a researcher at the Harvard lab studying the role of c-kit^+ cells as potential CS/CPCs were retracted because of 'the inclusion of fabricated and/or falsified data' and the ongoing clinical trials involving these cells were paused (27, 28). Many scientists say that it is time to reset the putative CS/CPC research, although there are some researchers saying that c-kit^+ cells are still valid for further analysis of regeneration effects based on some animal study results (28, 29). However, it was known that c-kit^+ cells are only a minor fraction of the whole CDC from previous reports (22), and these cells barely differentiate into cardiomyocytes. Moreover, the remnant c-kit^+ cells in the heart are regarded as cardiac endothelial cells rather than as CS/CPCs (30). Retrospective analysis of a recently completed clinical trial (CADUCEUS) demonstrated that c-kit expression was not related to the therapeutic effect of CDCs in humans, and that c-kit^+ cells were functionally inferior to unsorted human CDCs (9). Given these previous studies, our study tested the idea that CS/CPCs with low c-kit expression may secrete paracrine factors by means of EVs to promote angiogenesis. In our study, both BM-MSCs and CMSCs hardly expressed c-kit.

Meanwhile, CMSCs and BM-MSCs are distinguished by CD90 expression, and unlike BM-MSCs, CMSCs expressed only 26% of CD90. Li *et al*. also reported a similar profile of gene expression: the expression of CD90 as 18.4% in CDCs, compared with an expression of 99.0% in BM-MSCs (22). Thy-1 knock-out mice are viable and grossly normal (31), and the definite roles of $CD90^-$ clones in CVR are not clear, but some researchers reported data regarding this

topic. They demonstrated the superiority of $CD90^-$ CDCs over $CD90^+$ CDCs (9, 11, 12). In the chronic MI rat model, injection of $CD90^-$ CMSCs augmented cardiac function outperforming $CD90^+$ cells, and histological analysis of $CD90^-$ cells revealed an increase in vascularization within the infarct lesion (12). Other studies reported similar salutary effects, since $CD90^-$ cells secret less inflammatory cytokines and may differentiate into cardiomyocytes (9). Interestingly, $CD90^-$ cells expressed significantly more hepatocyte growth factor (HGF) than did $CD90^+$ cells, and both $CD90^-$ and $CD90^+$ CMSCs secrete VEGF or basic fibroblast growth factor (bFGF) comparably (12).

By means of the transcriptional profiles and gene ontology enrichment analysis of adult human CDCs subdivided by the expression of CD90, $CD90^-$ clones were enriched with the genes related to stemness, whereas $CD90^+$ clones were enriched with fibroblast-associated genes (11). Two separate studies found that the expression level of CD90/THY-1 changes dynamically at the formation of cardiospheres under different culture conditions (11, 32). Our CMSCs were isolated as $CD90^{low}$ CS/CPCs under specific culture condition to promote stemness (25).

Hence, our $CD90^{low}$c-$kit^{negative}CD105^{positive}$ CMSCs are likely to play a significant role in the regeneration of the heart as an active fraction. Most research with CS/CPCs has been done with CDCs of heterogeneous populations or with c-kit^+ cells, so far. Therefore, further studies with c-$kit^{negative}$ and $CD90^{negative}$ clones are needed to reveal the more effective therapeutic application of CS/CPC and would be helpful for understanding the roles of these cells.

GATA4 is one of the earliest transcription factors and a critical regulator of cardiac development, and interacts with many transcriptional factors, such as NKX2-5 (33, 34). Although GATA4 is crucial for regulating the expression of NKX2-5, it has been proposed that additional signaling pathways are needed for the initial activation of NKX2-5 (34, 35). In our results, CMSCs expressed only GATA4, but not NKX2-5. Other studies using CS/CPCs from

rotating tissue-culture conditions similar to our work, have also reported that GATA4 is expressed with limited expression of NKX2-5, which proposed these cells in early developmental stages (11, 36). Therefore, our cells may also be considered to be in early developmental stages before expressing NKX2-5, and further analysis will help us understand the roles of CS/CPCs at various developmental stages or marking various kinds of stemness during primary culture.

Our results showed that CMSC-Hpx EVs improved tube formation, but CMSC-Nx EVs did not. EV release from hypoxic MSCs was associated with an increased hypoxia-inducible factor 1-alpha activation, which could increase angiogenesis through Jagged-1 (38, 39). Hypoxia-treated EVs from human adipose-derived MSCs were reported to activate the protein kinase A signaling pathway and promote the expression of VEGF (20). EVs from hypoxic CDCs ($CD90^{high}$ cells) were reported to contain the increased level of EV-assocoated miRNAs and improved the tube formation of HUVECs (21). A study using EVs from CPC-Hpx ($c\text{-}kit^{+}$) reported the improvement of rat cardiac function and hypertrophy (14). These imply that tube formation of $CD90^{low}c\text{-}kit^{negative}$ CMSC EVs under hypoxia was increased by triggering such signaling pathways. Further studies with our $CD90^{low}c\text{-}kit^{negative}$ CMSCs will add valuable insights on understanding the extracellular vesicular influence of these cells in hypoxic conditions. The clinical potential of EVs could greatly depend on the cell-culture environment. Further research is needed to examine how CMSCs under hypoxic conditions could produce more effective EVs. In earlier works, growth factors including HGF, VEGF, and bFGF were reported to be related to these functional differences between CDCs and MSCs (22) and between the $CD90^{-}$ and $CD90^{+}$ CDCs (12). Since EVs have been reported to contain various growth factors, it is very likely that the more effective function of CMSC-Hpx EVs in our study resulted from the secretion of EVs with growth factors like HGF.

There are several questions to be answered by future research with EVs from CMSCs. First, the yield of EVs from CMSCs was lower than the yield of BM-MSC EVs (Supplementary Fig. 1). CMSC-EV tended to be more clingy, possibly because of the different nature of EVs, than were BM-MSC EVs, which may have caused unpredictable frequent loss during the EV isolation. Therefore, it was difficult to obtain sufficient EVs from CMSCs of our limited human samples. These technical problems should be solved for further experiments.

Second, the degree of tube formation was not increased in proportion to the concentration of EVs. Instead, the effect was best at the dose of 2 μg/ml (0.4 μg/well) of EVs in all three groups, but was decreased with a higher concentration of EVs (Supplementary Fig. 2). There have been reports of biphasic results of tube augmentation with increasing concentration of EVs, although others demonstrated that angiogenesis increases dose-dependently with EVs treatment (21, 37). Additionally, they used ten times more EV concentration than were used in our work (25 μg/ml versus 2 μg/ml). These differences between different laboratories could have resulted for various technical reasons, such as different isolation methods, the purity of EVs, and different cell-culture environments. Controlling the detailed cell-culture condition and the standardized isolation methods of EVs are needed to solve the quality and quantity differences of EVs.

Our study showed that the hypoxic condition of $CD90^{low}$c-$kit^{negative}$ CMSCs could increase the secretion of EVs with active components for angiogenesis. These imply the functional importance of $CD90^{low}$c-$kit^{negative}$ CMSCs, which has often been underestimated in the CVD. Therefore, our results would help us understand the role of $CD90^{low}$c-$kit^{negative}$ cardiac cells and apply their EVs clinically to cardiac regeneration after CVD. Further investigations are needed to define the mechanism and the functional characterization of CMSC-Hpx EVs.

MATERIALS AND METHODS

Details are provided in the Supplementary materials.

Human CMSCs preparation

CMSCs were explanted from cadaveric tissue and expanded as described previously (25). Briefly, after 14 d of dynamic tissue culture, outgrown cells were collected and suspended in growth-culture media for a conventional monolayer culture condition. When cells reached 80% confluence, they were detached and subcultured. Experiments were approved by the institutional review board of Busan Paik Hospital (IRB No. 2016-11-0006).

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CONFLICT OF INTEREST

The authors have no conflicting interests.

Figure Legends

Figure 1. Characterization of the cells

(A) Flow cytometry results showed negative expression of CD115 and CD45, low expression of CD34, positive expression of CD105, and different expression of CD90 in the BM-MSCs and CMSCs.

(B) RT-PCR analysis showed that the mRNA expression levels of NANOG, SOX2 in BM-MSCs, CMSC-Nx, and CMSC-Hpx were high, whereas the levels were hardly detectable for NKX2-4 and MYH6. The expression of GATA4 was detected only in CMSCs.

Figure 2. Morphology and size of EVs

(A) Transmission electron microcopy showed about 100 nm of round EVs with a cup shape in each group. DLS analysis measured the size distributions (B) and the NTA demonstrated the high particle concentrations corresponding vesicle sizes, and the good purity (C).

Figure 3. Purity analysis and markers for EVs

(A) Purity analysis by HPLC showed about 95% of elutes were within 5 min, which represented EV fractions in BMSCs and CMSCs. The small peak at 8 min contains substances with small molecular weight. (B) Immunoblotting analysis against EV surface markers, CD9, CD63, and CD81 on EV groups and whole cell lysates. H2B, histone, one of common contaminating substance in EV preparation, was not detected in all isolated EVs.

Figure 4. Tube-formation assay of HUVECs treated with three types of EVs

(A) Microscopy images were taken for the tube-formation assay of HUVECs at 6 h. Endothelial growth media was used for a negative control (NC) and VEGF (20 ng/ml) was used for positive control; Cells were treated with media including EVs from BM-MSCs,

CMSC-Nx, or CMSC-Hpx for experiments. Detailed methods are described in the supplemental materials.

(B) Total tube length at 6 h showed the augmented tube formation in the groups treated with VEGF, BM-MSC EVs, or CMSC-Hpx EVs; * $P < 0.01$, ** $P < 0.001$ vs. NC, ns = no significance; ANOVA followed by Turkey post hoc analysis.

(C) The tube-like structure was sustained until 8 h in VEGF and CMSC-Hpx EVs; * $P < 0.05$, ** $P < 0.01$; student *t*-test within group at 6 h and 8 h.

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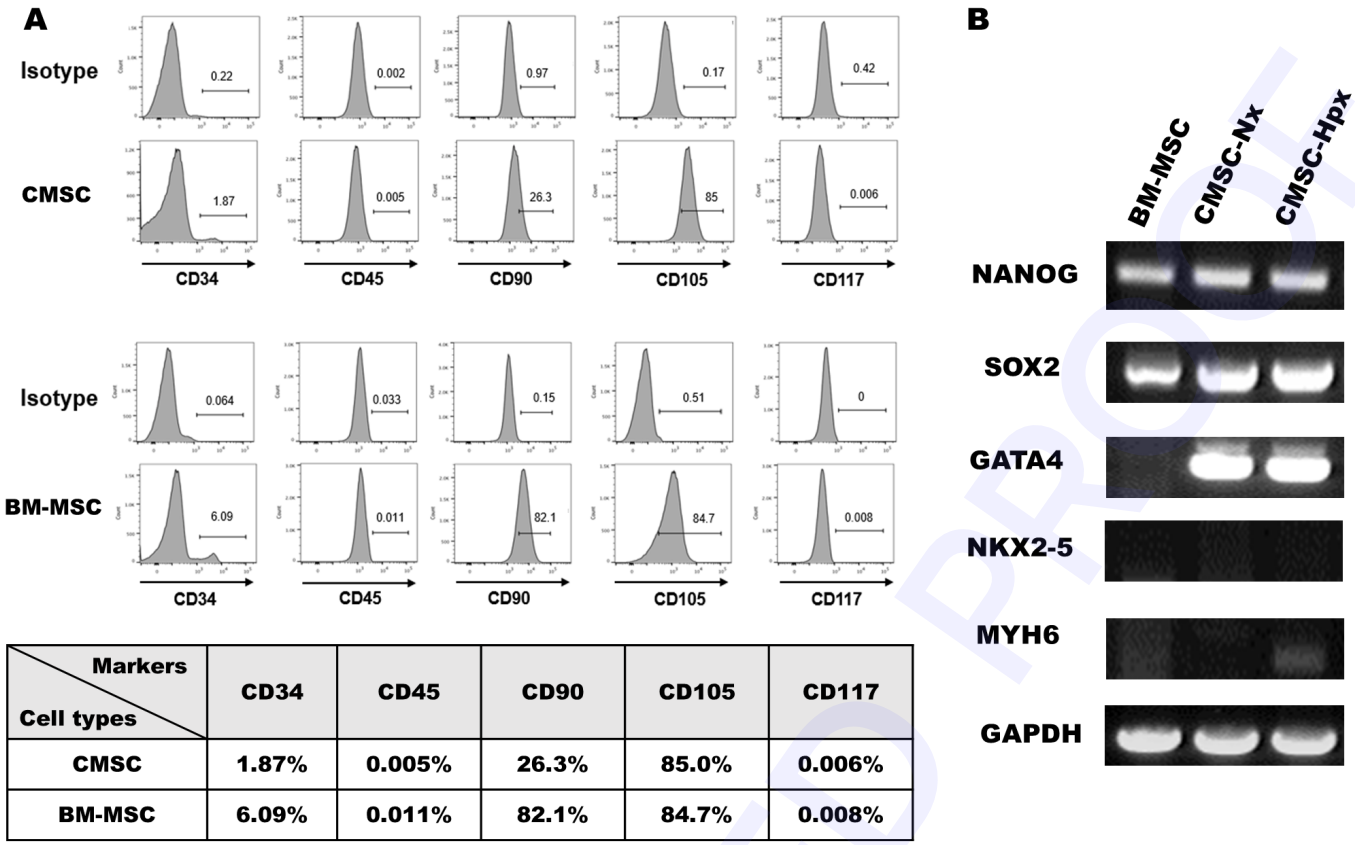
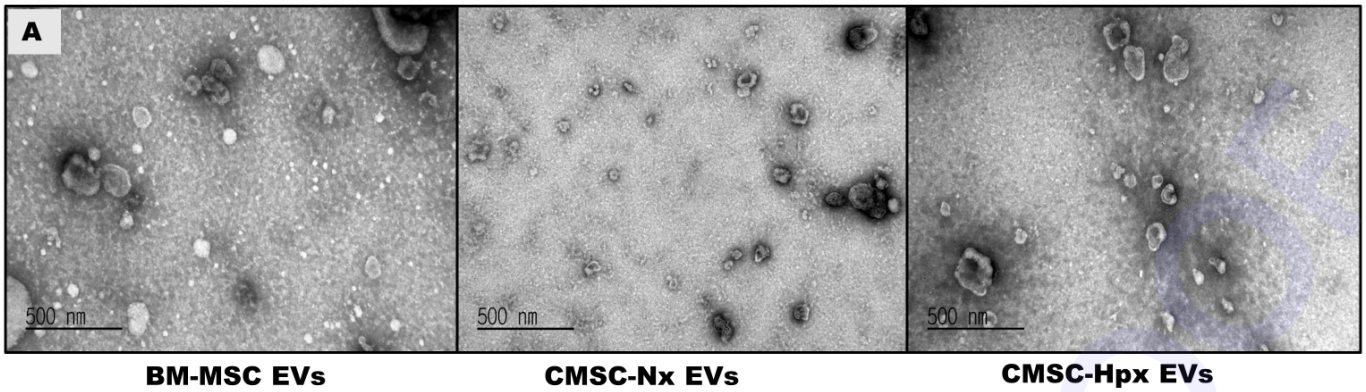
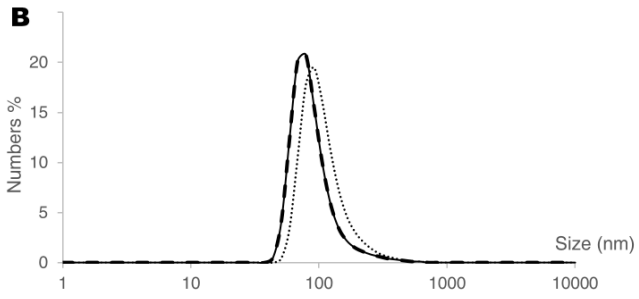


Markers / Cell types	CD34	CD45	CD90	CD105	CD117
CMSC	1.87%	0.005%	26.3%	85.0%	0.006%
BM-MSC	6.09%	0.011%	82.1%	84.7%	0.008%

Fig. 1. Figure 1





	Mean ± SD (nm)
BM-MSC EVs	108.3 ± 41.4
CMSC-Nx EVs	95.8 ± 35.0
CMSC-Hpx EVs	114.0 ± 41.5

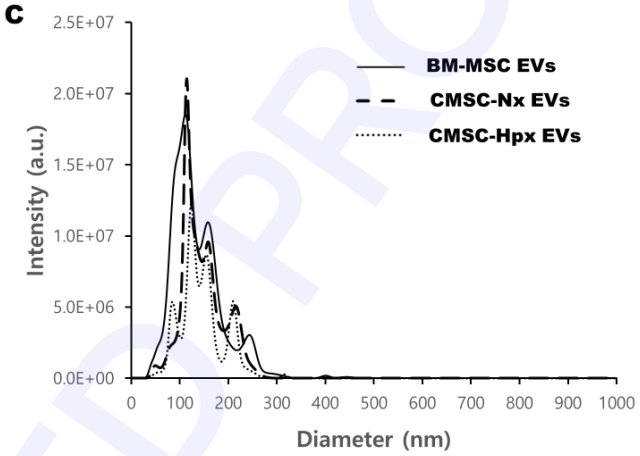


Fig. 2. Figure 2

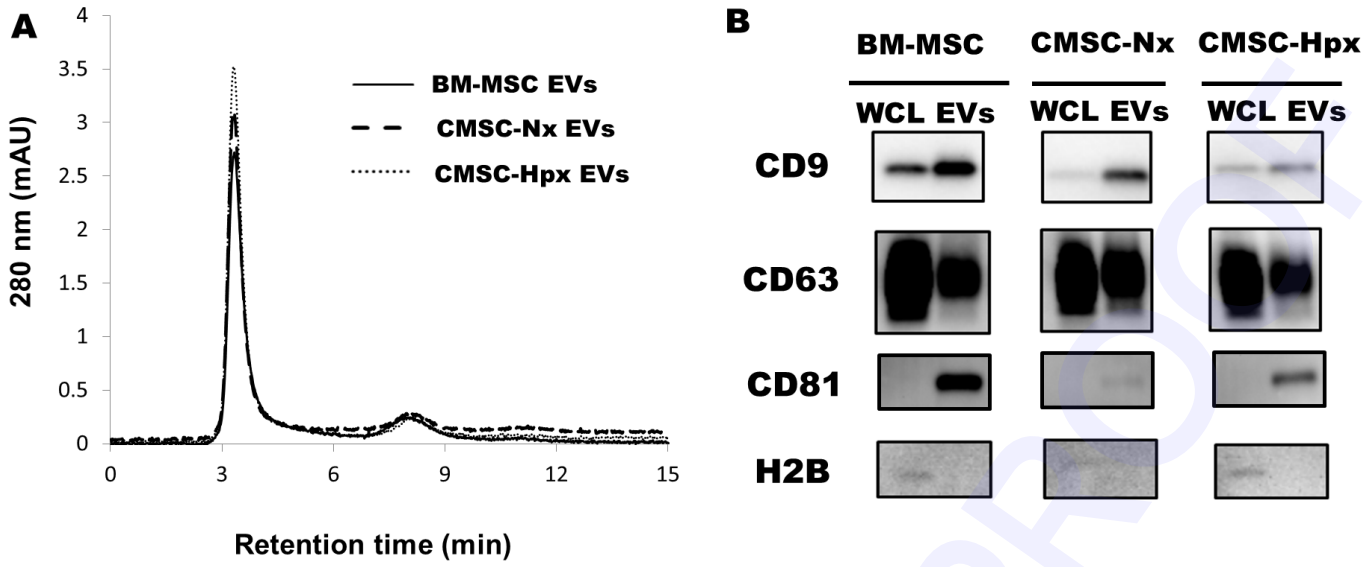


Fig. 3. Figure 3

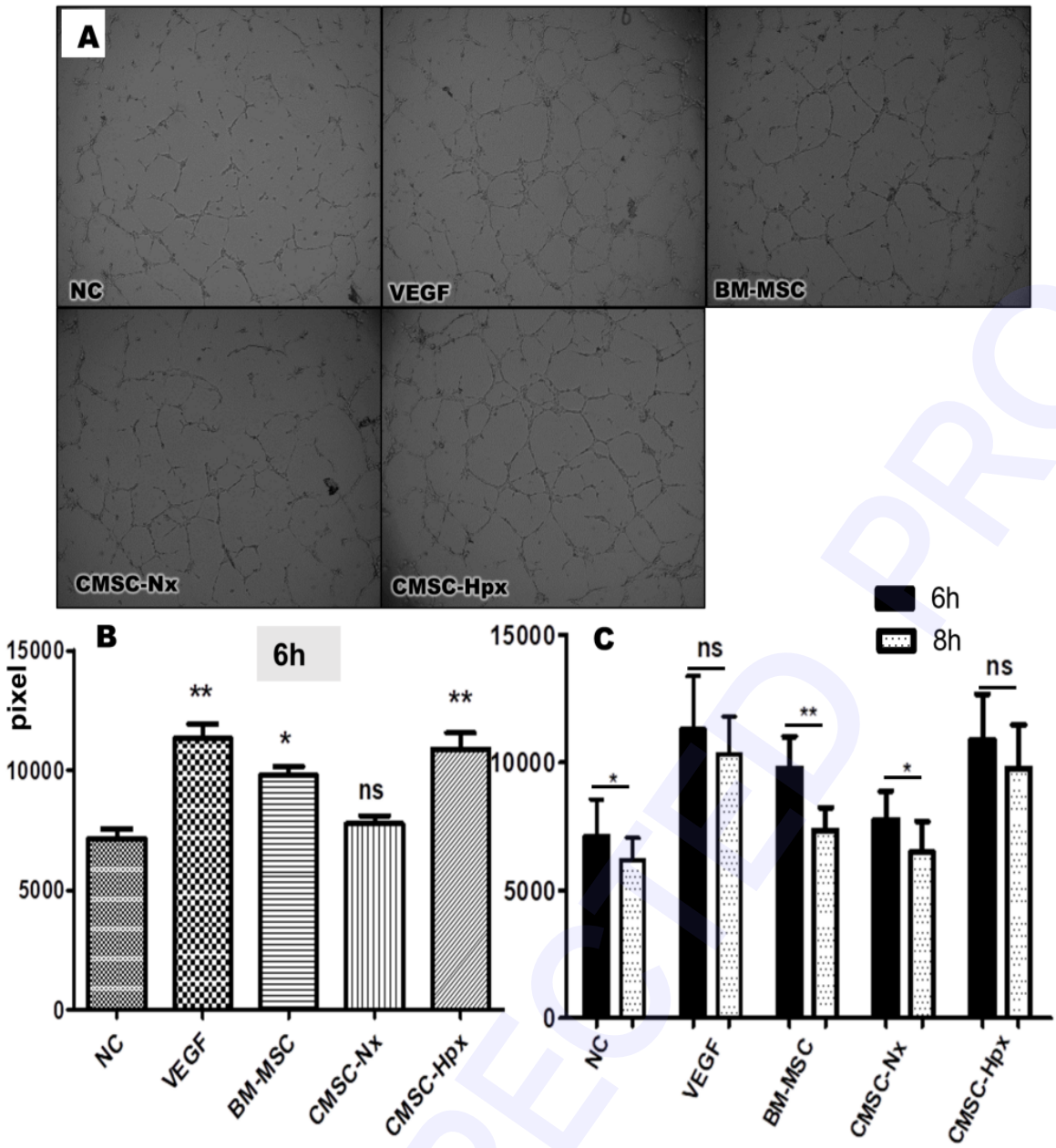


Fig. 4. Figure 4

Characterization of human cardiac mesenchymal stromal cells and their extracellular vesicles comparing with human bone marrow derived mesenchymal stem cells

Running title; human cardiac mesenchymal stromal cells and their EVs

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SUPPLEMENTARY INFORMATION

MATERIALS AND METHODS

Human cells

Human cardiac mesenchymal stromal cells (h-CMSCs) were explanted from cadaveric tissue and expanded which were as described previously (1). Briefly, fibrin-supported 3D myocardial organ cultures were performed under dynamic conditions at 15 rpm after removing epicardium and endocardium. The myocardium was minced into 2-3 mm^3 fragments and washed with phosphate-buffered saline (PBS). After 7 d of the culture, outgrown cells were collected and suspended in growth culture media for a conventional monolayer culture condition. When cells reached 80% confluence, the cells were detached and subcultured. Human bone marrow derived mesenchymal stem cells (h-BM-MSCs) and h-CMSCs were provided by Young Il Yang M.D (Paik Institute for Clinical Research, Inje University College of Medicine, Busan, Republic of Korea). Human umbilical cord blood cell (HUVEC)s were purchased from Lonza (MD, USA).

For h-CMSC culture, 1:1 mixture of Dulbecco's Modified Eagle's Medium: Nutrient Mixture F12 (DMEN/F12) (Gibco, USA) with epidermal growth factor (EGF) 10 ng/mL, insulin-growth factor (IGF) 10 ng/mL, basic fibroblast growth factor (bFGF) 2 ng/mL and gentamycin 10 μg/mL were used. One to one mixture of DMEN/F12 with 10% fetal bovine serum (FBS) (Thermo Scientific, USA) and gentamycin 10 μg/mL were used for MSC culture. For HUVEC culture, EBM-2 basal medium supplemented with the EGM-2 SingleQuots supplement kit (Cat.No.: CC-3162, Lonza, MD, USA) and 2% FBS were used on the gelatin coated dishes. At passage 12 were used for the experiments.

Hypoxic treatment was performed with human CMSCs for hypoxic CMSC EVs by using Hypoxia Incubator Chamber (Cat.No.: 27310, STEMCELL^{TM} , Canada) under the condition of 2% O_2, 5% CO_2 and 93% N_2.

Reverse transcriptase polymerase chain reaction (RT-PCR) assays

For total RNA extraction, the cells were lysed in TRIzol (*Invitrogen,* Thermo Fisher Scientific, NH, USA). After adding chloroform and centrifuging and add isopropanol to the supernatant containing the RNA. Centrifuging after standing at room temperature for 5 min, add 75% ethanol to the pellet. Add diethyl pyrocarbonated treated water to the pellet after another centrifugation. The purity of isolated RNA was determined by OD260/280 using a DeNovix DS-11 spectrophotometer (DeNovix, DE, USA).

1 μg of extracted RNA of each group was used as a template for first-strand cDNA synthesis by reverse transcription (RT) with RNase inhibitor and dNTP mix (Thermo Fisher Scientific, NH, USA). PCR was performed for 40 cycles with gene-specific primer sequences (Table) and AccuPower PCR premix (Bioneer, Daejeon, Republic of Korea). The following PCR conditions were used: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles for 30 s at 95°C, 45 s at 60°C and 72°C for 30 s. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Extracellular vesicles isolation and characterization

Isolation of EVs

EVs from 72 h the medium conditioned either with BM-MSCs, normoxia or hypoxia-treated CMSCs were isolated using ExoLutE® exosome isolation kit (Rosetta Exosome Inc, Seoul, Republic of Korea). Briefly, the medium harvested from the culture was differentially centrifuged at 500 x g for 10 min and 2,000 x g for 15 min, respectively. To enrich EVs from

the pre-cleared conditioned medium as described previously (2), the medium was treated with polyethylene glycol 6000 (Sigma-Aldrich, MO, USA) final at 8.3% then incubated at 4°C for 16 h. After centrifugation at 13,000 x g for 10 min, the pellet was resuspended in 8 mL of RPMI1640 medium (Gibco, NH, USA) to purify EVs further. According to the manufacturer's instruction of ExoLutE® exosome isolation kit, the highly pure EVs were finally isolated by spin-based size-exclusion chromatography which was pre-equilibrated with HEPES-buffered saline (22 mM HEPES, 150 mM NaCl, pH7.4). Protein quantity in the EV preparation was measured by QuantiProTM BCA assay kit (Sigma-Aldrich, MO, USA) and aliquots of the purified EVs were frozen by liquid nitrogen then stored at -80°C until necessary.

Transmission electron microcopy

Purified EVs were subjected on transmission electron microscopy to determine their shapes. 5 µl of EVs preparation at $1x10^9$ particles ul^{-1} was adsorbed onto glow-discharged carbon-coated copper grids (Electron Microscopy Sciences, PA, USA) for 5 min. Following the removal of excess liquid, the grid was washed 10 times with PBS and subsequently stained with 2% uranyl acetate (Ted Pella, Redding, CA, USA). The grid was examined in JEM 1011 microscope (JEOL, Tokyo, Japan) and images were recorded with an ES1000W Erlangshen CCD Camera (Gatan Inc. Pleasanton, CA, USA).

Measurement of EV size distribution

The size distributions of purified EVs were measured with Zetasizer Nano ZS (Malvern Instrument Ltd., Malvern, UK). The size distribution was determined by the light scattering against infra-red light (wavelength = 633 nm) with Dynamic V6 software. Results are mean values from five measurements.

Nanoparticle tracking analysis

The particle concentrations of purified EVs were determined using a Nanosight LM10-HS system (Nanosight Ltd., Amesbury, UK). Appropriately diluted purified EVs were injected into the chamber and visualized using a 405 nm laser. The recorded images with a camera level at 12 were analyzed using the nanoparticle tracking analysis (NTA) software (version 2.3) with a threshold at 5.

Size-exclusion chromatographic analysis

The chromatograms of purified EVs were analyzed by a column packed with Sephacryl S500 (GE Healthcare Life Sciences, USA) which was connected on a high-pressure liquid chromatography system (Thermo Scientific, UltiMate™ 3000, USA). HEPES-buffered saline (20 mM HEPES, 300 mM NaCl, pH7.4) was used as a mobile phase with a flow rate at 1.0 ml/min. 280 nm wavelength was recorded to analyze the purity of isolated EVs and 1 μg of purified EVs was used for each test.

Western blotting

Whole cell lysates (20 μg of total protein) and corresponding purified EVs (2 μg of total protein) were loaded onto SDS-PAGE gels (4-20%, Bio-Rad, CA, USA), and then transferred to polyvinylidene difluoride membranes (Millipore, MA, USA). The membrane was blocked by 3% skim milk and incubated at 4℃ for overnight either with primary antibodies (mouse anti-human CD9; BD Biosciences, Cat. No.: 555370, 1:1000, mouse anti-human CD63; BD Biosciences, Cat. No.: 556019, 1:1000, and mouse anti-human CD81; Santa Cruz, Cat. No.: sc-166029, 1:500). After incubation, the membrane was followed by the incubation with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies. The immunoreactive bands were visualized using enhanced chemiluminescence substrate (Thermo Scientific, NH, USA).

Flow cytometry

Cells were incubated with an anti-CD16/32 monoclonal Ab (BioLegend, CA, USA) at 4°C for 30 min (to block the Fc receptors). Cells were stained with specific antibodies against the CD34, CD45, CD90, CD105 and CD117 (Cat. No.: 343514, 368505, 328107, 323217 and 313203, respectively, BioLegend. CA, USA) surface markers in FACS buffer (PBS containing 2% FBS) at 4°C for 30 min, and washed with FACS buffer. Stained cells were acquired using a BD LSRFortessa flow cytometer and analyzed with the FlowJo software (Tree Star, CA, USA).

Tube formation assay

HUVECs were plated on Matrigel coated (75 µl per well) 96-well plates at a density of 1.25 x 10^4 cells per well. Three types of EVs from BM-MSC, normoxia and hypoxia-treated CMSCs were added to the well with EGM media at a dose of about 0.4 µg/well (2 µg/ml; 2.1 x 10^9 particles/µg BM-MSC, 1.9 x 10^9 particles/µg normoxic CMSC, 1.3 x 10^9 particles/µg hypoxic CMSC). HUVECs with EGM media only (negative control: NC) and HUVECs with EGM plus vascular endothelial growth factor (VEGF, 20 ng/ml) were used for negative and positive control. All wells were triplicated. Images (x 5) were obtained under light microscopy 4, 6 and 8 h after cells with EVs plating. Quantification of total tube length was performed using Image J (NIH, MD, USA).

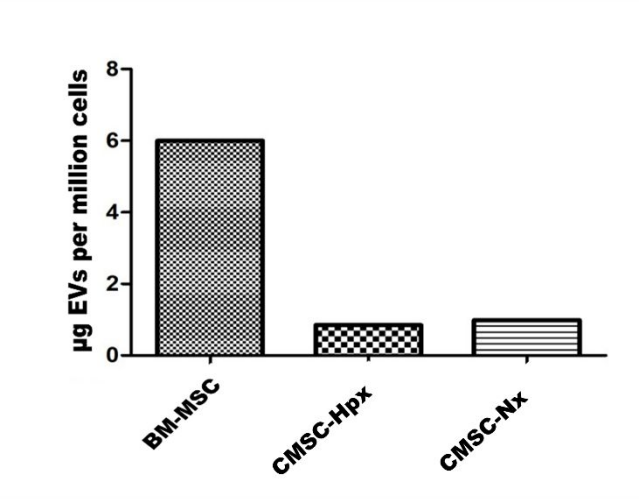
Statistical analysis

Statistical analyses were performed using GraphPad Prism (La Jolla, CA, USA) with the threshold for significance set at level $P < 0.05$. Values are expressed as mean ± standard error. To compare tube length of each groups, we used the one way ANOVA followed by the Tukey's post hoc analysis for all pairwise comparisons.

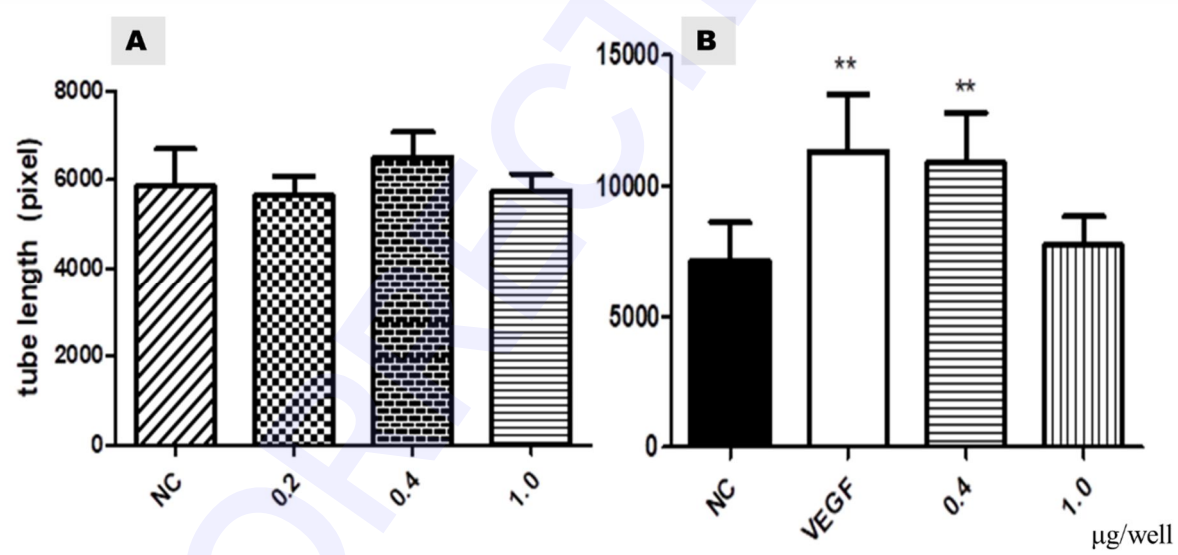
Table. Primers for reverse transcriptase polymerase chain reaction

Gene	Forward primer (5′ to 3′)	Reverse primer (3′ to 5′)
GATA4	GACGGGTCACTATCTGTGCAAC	AGACATCGCACTGACTGAGAAC
NKX 2-5	CGCCCTTCTCAGTCAAAGAC	AGATCTTGACCTGCGTGGAC
MYH6	GTCATTGCTGAAACCGAGAATG	GCAAAGTACTGGATGACACGCT
NANOG	AGTCCCAAAGGCAAACAACCCACTTC	TGCTGGAGGCTGAGGTATTTCTGTCTC
SOX2	ATGCACCGCTACGACGTGA	CTTTTGCACCCCTCCCATTT
GAPDH	AAGTGGATATTGTTGCCATC	ACTGTGGTCATGAGTCCTTC

Supplementary Figures



Supplementary figure 1. Yields of extracellular vesicles per million cells; yields of extracellular vesicles per million cells were highly variable according to the cell types.



Supplementary figure 2. Tube formation according the concentration of EVs

Normoxic CMSCs EVs showed increasing tube formation according to the concentration of EVs in low dose but decreased at higher dose 1.0 μg/well (A) and similar findings were observed in hypoxic CMSCs EVs (B). NC, negative control; ** $P < 0.01$ vs. NC

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1. Kim JT, Chung HJ, Seo JY et al (2015) A fibrin-supported myocardial organ culture for isolation of cardiac stem cells via the recapitulation of cardiac homeostasis. Biomaterials 48, 66-83

2. Lee C, Mitsialis SA, Aslam M et al (2012) Exosomes mediate the cytoprotective action of mesenchymal stromal cells on hypoxia-induced pulmonary hypertension. Circulation 126, 2601-2611