Extracellular vesicles derived from human cardiac mesenchymal stromal cells and their angiogenic potential

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The Department of Medicine,

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Directed by Professor Yangsoo Jang

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This certifies that the Doctoral Dissertation of In Sook Kang is approved.

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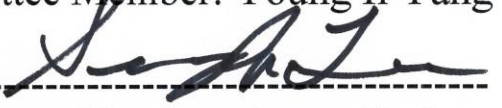

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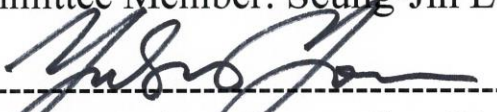

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Despite the recent progress in medical science, patients with terminal heart conditions require heart transplantation. We provided maximal cares including good medications and proper coronary intervention within gold time. However, the heart condition of some patients progressed to irreversible myocardial damage and terminal heart failure due to various causes, such as reperfusion injury. Hence, dream of cardiac regeneration is a great goal to challenge as a cardiologist, and so do I.

I set my doctorate themes with ‘that dream’ and started my research with bulging expectations. However, I was a clinician and had little research experience. Further, the field of stem cells and regenerative medicines were very deep and difficult topics. Hence, I had to work hard and learn through trial and error.

I am grateful for the support and encouragement provided by Professor Yangsoo Jang, Goo Taeg Oh, and Dr. Joowon Suh, which has enabled me to pursue my research career and achieve today’s results. I also want to extend my sincere gratitude to Professor Young Il Yang, who provided the primary human cardiac mesenchymal stromal cells for my research and encouraged me in my research endeavor. I also thank Professor Donghoon Choi, Seung-Jin Lee, and Young-

Sup Yoon for reviewing the manuscript and for taking the time out of their busy schedule to provide valuable suggestions to improve my research.

Although this is a just entry-level research paper, I hope this will form the basis for advanced research in the field of cardiac regeneration just as the LORD told me ‘call to me and I will answer you and tell you great and unsearchable things you do not know.’

Lastly, I would like to thank my family who helped me continue my academic career. I am especially grateful to my brother Dae Jin Kang who always prays for me and the Chief of Agape Choir in Juan Presbyterian Church, Soo Nam Moon for his prayers and encouragement. I would like to dedicate this doctorate degree at Yonsei University to my late mother.

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ABSTRACT

Extracellular vesicles derived from human cardiac mesenchymal stromal cells and their angiogenic potential

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Cardiac regeneration with adult stem cell therapy is one of promising fields to address advanced cardiovascular diseases. In addition, extracellular vesicles (EVs) from stem cells have a role as cellular cargo and as a paracrine factor to improve cardiac function after stem cell therapy. The recent discovery of adult cardiac stem cells has greatly increased the academic interest in cardiovascular regeneration. However, debates regarding c-kit positive cell, putative cardiac stem cell, raise questions about the existence of human adult cardiac stem cells, lately. In our work, we isolated human cardiac mesenchymal stromal cells (CMSCs) through three-dimensional organ culture and compared their

functional efficacy with human bone marrow derived mesenchymal stem cells (BM-MSCs). The isolated CMSCs exhibited $CD90^{low}$, $c\text{-}kit^{negative}$, $CD105^{positive}$ phenotype. Additionally, the CMSCs expressed NANOG, SOX2, and GATA4. To determine the effective type of EVs isolated from different sources of adult stem cells for angiogenesis, we isolated the EVs from CMSCs under normoxic or hypoxia-conditions and EVs from BM-MSCs under normoxic conditions were used as control. Our result demonstrated that EVs isolated from hypoxia-conditioned CMSCs showed the most augmented ability of *in vitro* capillary tube formation than other two groups. This indicated that the EVs secreted from the $CD90^{low}c\text{-}kit^{negative}CD105^{positive}$ CMSCs under hypoxic conditions promote angiogenesis *in vitro*. Our findings provide novel insights into understanding cardiovascular repair.

Key words: cardiac mesenchymal stromal cell; extracellular vesicles; angiogenesis; cardiovascular disease, regeneration

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Ⅰ. INTRODUCTION

Cardiovascular disease is a major global health problem and has remained the leading causes of death, despite of the advances in the current medical technologies.[1] Cardiovascular regeneration with stem cell therapy is one of the promising therapeutic strategies for advanced cardiovascular diseases.[2,3] Adult stem cells, unlike embryonic stem cells, cannot replicate indefinitely and do not have pluripotency. However, many studies have been done with them because

they can avoid ethical issue and are relatively easy to obtain. Although there are diverse origins of adult stem cells, the cells that are mostly studied in the area of cardiovascular regeneration are mesenchymal stem cells (MSCs) and cardiac stem cells.[4] Based on the salutary effect for cardiovascular disease reported in previous studies, there have been numerous preclinical studies as well as clinical trials conducted with the stem cells.[4,5]

The putative 'cardiac stem/progenitor cell (CS/PC)' is also named as cardiac mesenchymal stem cell, cardiac interstitial cell, cardiac mesenchymal stromal cell (CMSC), or cardiosphere derived cell (CDC). These cells are usually obtained from right ventricular septum through cardiac biopsy, or from left atrial sample of cardiac surgery.[6,7] In particular, CDCs indicate the cells derived from cardiosphere, which is a multicellular cluster formed from the primary culture of cardiac specimen.

There are several issues regarding cardiovascular regeneration using different types of CS/PCs. Especially, based on the research that c-kit$^+$ cells were potential CS/PCs rejuvenating heart muscle in mice[8], many researchers had considered these cells as CS/PCs. However, recent studies have proved that c-kit$^+$ cells are only a small proportion of CDCs with low regenerative potential and they are originated from cardiac endothelial cells, rather than CS/PCs.[9,10] Another issue is about cluster of differentiation (CD)90. CD90 was originally discovered as a thymocyte antigen (thy-1) and can be used as a marker of a

variety of stem cells and fibroblasts. Although there is limited knowledge on the role of CD90 in cardiac regeneration, several studies proved that the $CD90^{-}$ CPCs express genes associated with stemness and can differentiate into mature cardiomyocytes with complete sarcomere formation.[9,11,12] Although these cells exhibit a greater regenerative potential in myocardial infarction models, it is still unclear which types of CS/PCs are more important to cardiovascular regeneration.

Recently, the paracrine effect of stem cells is being explored in stem cell therapy. As the successful retention rate of injected stem cells are very low[13,14], many researchers no longer think the stem cells engraft in the heart and differentiate into cardiac cells but they secrete some "paracrine factors" that promote heart tissue growth[15], and extracellular vesicle (EV) is one strong candidate.[16-18] EVs also known as exosome, are nano-sized vesicles composed with lipid bilayer, and have a role as molecular cargo and facilitate intercellular communication[17] and have specificity according to their origin cells.[16]

In this study, we first compared the characterization of human CMSCs and human bone marrow derived MSCs (BM-MSCs), which has been studied for a long time. Although the characteristics of CMSCs and BM-MSCs have been previously studied, very few studies have comparatively evaluated the effects of these cells.[19-21] Particularly, there are limited studies on the $CD90^{low}$ and c-$kit^{negative}$ CS/PCs. To compare the effects of EVs from each cell group, we

evaluated the effects of EVs on angiogenesis *in vitro*. The comparative studies regarding EVs of human BM-MSCs and human CMSCs with normoxic and hypoxic condition can aid in developing novel therapeutic targets.

Ⅱ. MATERIALS AND METHODS

1. Cell culture

The human CMSCs were explanted from cadaveric tissue and expanded as described previously.[22] Briefly, fibrin-supported three-dimensional 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 14 days of the culture, the outgrown cells were collected and suspended in growth culture medium for conventional monolayer culture condition. When the cells reached 80% confluency, the cells were detached and subcultured. The human BM-MSCs and human CMSCs were provided by Young Il Yang M.D (Paik Institute for Clinical Research, Inje University College of Medicine, Busan, Republic of Korea) and approved by the institutional review board of Busan Paik Hospital (No. 2016-11-0006). The human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Walkersville, MD, USA).

For human CMSCs culture, 1:1 mixture of Dulbecco's Modified Eagle's Medium: Nutrient Mixture F12 (DMEN/F12, Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Thermo Scientific, Waltham, MA, USA), 10 ng/mL epidermal growth factor (EGF), 10 ng/mL insulin-growth factor (IGF), 2 ng/mL basic fibroblast growth factor (bFGF) and 10 μg/mL

gentamycin were used. Same culture media was used to culture the BM-MSCs. The HUVECs were cultured in endothelial growth media (EBM)-2 basal medium supplemented with the EGM-2 SingleQuots supplement kit (Cat.No: CC-3162, Lonza, Walkersville, MD, USA) and 2% FBS on gelatin coated dishes. The cells were cultured for 12 passages before use for experiments.

The hypoxia-conditioned human CMSCs were cultured in Hypoxia Incubator Chamber (Cat.No: 27310, STEMCELL^{TM}, Vancouver, Canada) at 2% O_2, 5% CO_2, and 93% N_2 to obtain the hypoxia-conditioned CMSC (CMSC-Hpx) EVs.

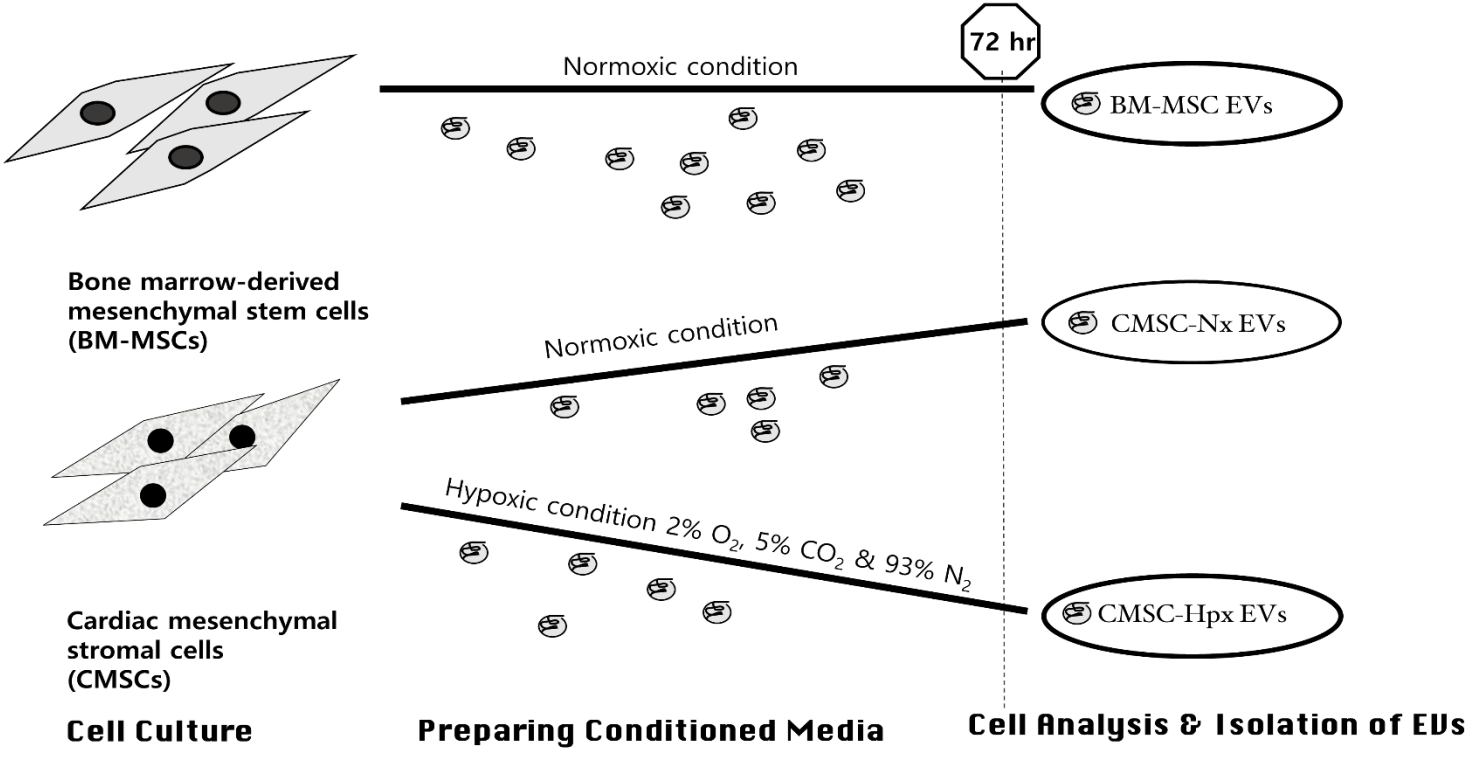


Figure 1. A schematic of preparing conditioned medium. BM-MSC EVs, extracellular vesicles (EVs) from BM-MSCs; CMSC-Nx EVs, EVs from normoxia-conditioned CMSCs; CMSC-Hpx EVs, EVs from hypoxia-conditioned CMSCs.

2. Reverse transcription polymerase chain reaction (RT-PCR) assays

The cells were lysed using TRIzol (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) to extract total ribonucleic acid (RNA). The cell lysate was treated with chloroform and centrifuged to separate the layers. Next, isopropanol was added to the supernatant containing the RNA and incubated at room temperature for 5 min. The samples were then centrifuged to precipitate the nucleic acids. The precipitate was treated with 75% ethanol. After another centrifugation, the pellet was dissolved in diethyl pyrocarbonate-treated water. The purity of the isolated RNA was determined based on OD260/280 using a DeNovix DS-11 spectrophotometer (DeNovix, Wilmington, DE, USA).

The extracted RNA (1 μg) of each group was used as a template for first-strand complementary deoxyribonucleic acid (cDNA) synthesis by reverse transcription with ribonuclease inhibitor and deoxynucleotid solution mix (dNTP mix, Thermo Fisher Scientific, Waltham, MA, USA). The PCR was performed with gene-specific primers (Table 1) and AccuPower PCR premix (Bioneer, Daejeon, Republic of Korea). The PCR conditions were as follows: 50℃ for 2 min and 95℃ for 10 min, followed by 40 cycles of 95℃ for 30 sec, 60℃ for 45 sec and 72℃ for 30 sec. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Table 1. Primer sequences for reverse transcription-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

3. Isolation and characterization of EVs

A. Isolation and quantitation of EVs

The EVs were isolated from 72 hr the medium conditioned either with BM-MSCs, normoxia-conditioned CMSCs (CMSCs-Nx), or hypoxia-conditioned CMSCs (CMSCs-Hpx) using ExoLutE® exosome isolation kits (Rosetta Exosome Inc, Seoul, Republic of Korea). Briefly, the conditioned medium was centrifuged at 500 x g for 10 min, and then 2,000 x g for 15 min. The pre-cleared conditioned medium was mixed with polyethylene glycol 6000 (Sigma-Aldrich, St. Louis, MO, USA) final at 8.3% then incubated at 4℃ for 16 hr to concentrate the EVs as described previously.[23] The sample was centrifuged at 13,000 x g for 10 min and the pellet was resuspended in 8 mL of RPMI1640 medium (Gibco, Waltham, MA, 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 column-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 using the QuantiPro™ BCA assay kit (Sigma-Aldrich, St. Louis, MO, USA). Aliquots of the purified EVs were frozen by liquid nitrogen, and then stored at -80℃ until necessary. Figure 2 shows the detailed process for isolation of EVs.

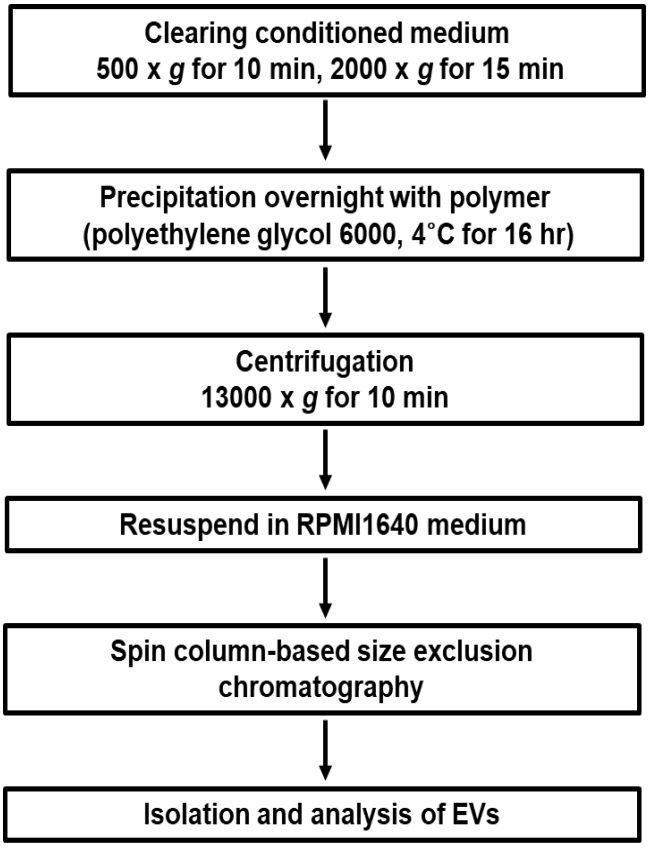


Figure 2. Flow chart for the isolation and purification of the extracellular vesicles (EVs)

B. Transmission electron microcopy

The shape of the purified EVs was determined by transmission electron microscopy. The EVs preparation (5 μl) at $1x10^9$ particles/μl was adsorbed onto glow-discharged carbon-coated copper grids (Electron Microscopy Sciences,

Hatfield, PA, USA) for 5 min. The excess liquid was removed and the grid was washed 10 times with PBS and subsequently stained with 2% uranyl acetate (Ted Pella, Redding, CA, USA). The grid was examined under JEM 1011 microscope (JEOL, Tokyo, Japan) and the images were captured using an ES1000W Erlangshen CCD Camera (Gatan Inc. Pleasanton, CA, USA).

C. Light scattering analysis

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

D. Nanoparticle tracking analysis

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

E. Size-exclusion chromatographic analysis

The chromatograms of the purified EVs were analyzed in a column packed with Sephacryl S500 (GE Healthcare Life Sciences, Chicago, IL, USA) which was connected on a high-pressure liquid chromatography system (UltiMate™ 3000,

Thermo Scientific, Hudson, NH, USA). HEPES-buffered saline (20 mM HEPES, 300 mM NaCl, pH 7.4) was used as a mobile phase with a flow rate at 1.0 ml/min. The purity of the isolated EVs was evaluated at 280 nm wavelength and 1 μg of purified EVs was used for each test.

F. Western blotting

For enrichment test, whole cell lysates (20 μg of total protein) and corresponding purified EVs (2 μg of total protein) were loaded onto the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (4-20%, BIORAD, Hercules, CA, USA). The resolved proteins were transferred onto a polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membrane was blocked by 3% skim milk. Next, the membrane was incubated with the following primary antibodies at 4℃ overnight: mouse anti-human CD9 (BD Biosciences, Cat. No: 555370, 1:1000); mouse anti-human CD63 (BD Biosciences, Cat. No: 556019, 1:1000); mouse anti-human CD81 (Santa Cruz, Cat. No: sc-166029, 1:500); mouse anti-TSG-101 (BD Biosciences, Cat. No: 612697, 1:500). The membrane was then probed with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies.

For de-enrichment test with calnexin antibody (mouse anti-human antibody; BD biosciences, Cat. No: 610523), equal amount of whole cell lysates (2 μg of total protein) and corresponding purified EVs (2 μg of total protein) were analyzed

as described above. The immunoreactive bands were visualized using enhanced chemiluminescence substrate (Thermo Scientific, Hudson, NH, USA).

G. Flow cytometry

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

4. Capillary tube formation assay

The 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, CMSC-Nx and CMSC-Hpx were added to the well containing EGM media at a dose of about 0.4 µg/well (2.1 x 10^9 particles/µg of BM-MSC, 1.9 x 10^9 particles/µg of CMSC-Nx, 1.3 x 10^9 particles/µg of CMSC-Hpx). The HUVECs in the EGM and the HUVECs with the EGM supplemented with vascular

endothelial growth factor (VEGF, 20 ng/ml) were used for negative and positive controls, respectively. All wells were triplicated. Images (5x) were obtained under a light microscope at 4, 6 and 8 hr after the cells with EVs were plated. The total tube length was quantified using Image J (NIH, Bethesda, MD, USA).

5. Statistical analysis

All statistical analyses were performed using GraphPad Prism (La Jolla, San Diego, CA, USA) with the threshold for significance set at level $P < 0.05$. The values are expressed as mean ± standard error. To compare tube length of each groups, we used one-way analysis of variance (ANOVA) followed by Tukey's post hoc test.

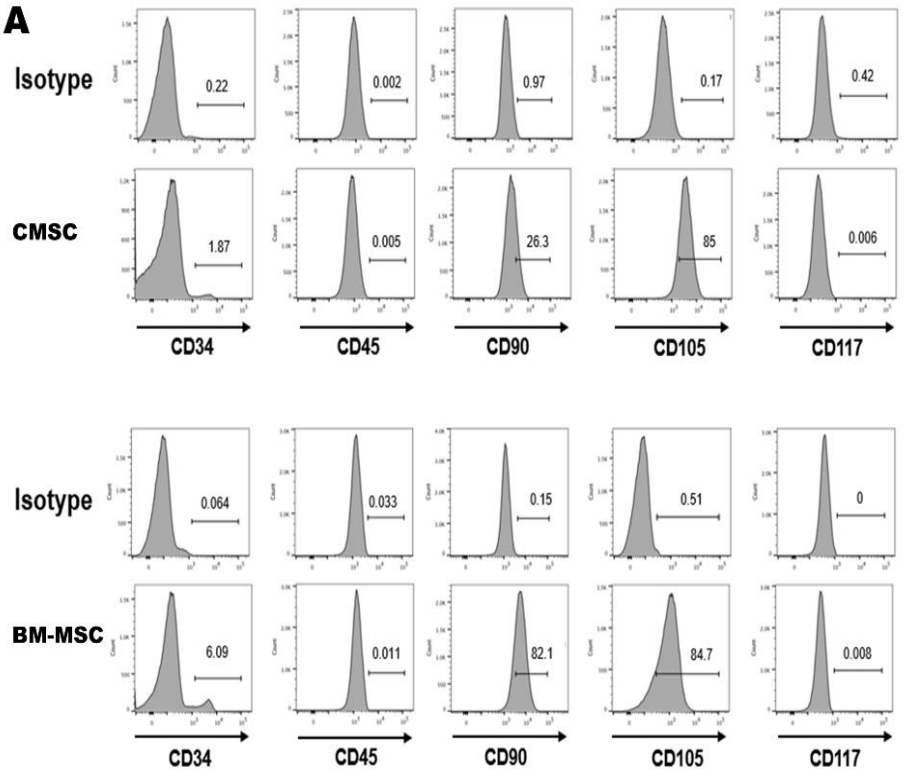
Ⅲ. RESULTS

1. Characteristics of CMSCs and BM-MSCs

The stem cell surface markers were analyzed by flow cytometry (Figure 3A). The expression of CD45, a type I transmembrane protein that is present as various isoforms on the differentiated hematopoietic cells, was detected in less than 1% of CMSCs and BM-MSCs. The expression of CD34, another hematopoietic stem cell maker, was detected in only 1.87% in CMSCs and 6.09% in BM-MSCs. This indicated that these cells are CD45 and CD34 negative cell populations, as previously reported.[24] Importantly, CD90 was differentially expressed in the two cell populations; 82.1% of BM-MSC and 26.3% of CMSCs, which is similar to the expression patterns in the CDCs.[21] The expression of CD105 was detected in more than 80% of CMDSCs and BM-MSCs. CD105, also known as Endoglin, is a membrane glycoprotein that is a part of the transforming growth factor (TGF) β receptor complex and is associated with neoangiogenesis. The expression of CD117 was detected in less than 1% of CMSCs and BM-MSCs. CD117, also known as c-kit, is a stem cell growth factor receptor on the surface of the hematopoietic stem cells and some cancer cells.

To further define characters of the cells, various marker genes was analyzed by RT-PCR (Table 1). NANOG and SOX2 are the markers of embryonic stem

cells that were expressed in all three cell populations (CMSC-Hpx, CMSC-Nx, and BM-MSCs) (Figure 3B). NANOG is a transcriptional factor that helps the embryonic stem cells to maintain pluripotency by suppressing the cell determination factors.[25] SOX2 is a member of the Sox family of transcription factors that have important roles in mammalian development and maintenance of embryonic and neural stem cells. The gene expression levels of NKX2.5, myosin heavy chain, α isoform (MYH6), and GATA4, which are the cardiac marker genes, were analyzed in the three cell populations. The NKX2-5 and MYH6 were not detected in all three groups. The expression of GATA4 was prominent in CMSCs but not in BM-MSCs (Figure 3B). The expression of the homebox protein, NKX2-5 is essential for the development of heart. MYH6 is expressed in human heart during embryonic development and is associated with cardiomyopathy. GATA4 is a critical transcription factor for mammalian cardiac development and is essential for the survival of the embryo. GATA4 and NKX2-5 are early markers of precardiac cells and exhibit synergism as cofactors.[26] GATA4 is expressed in cardiac progenitor cell before the expression of NKX2-5 and ISL1 of cardiac mesoderm.



	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%

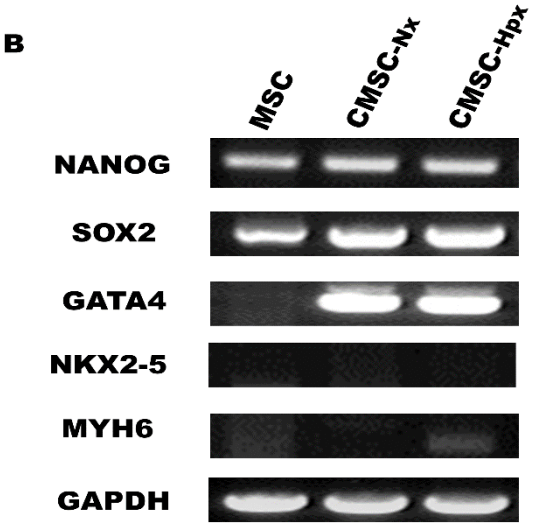


Figure 3. Gene expression of the stem cells at the mRNA and protein level. (A) Flow cytometry result showed negative expression of CD115 (c-kit) and CD45, low expression of CD34, positive expression of CD105, and differential expression of CD90 in the cardiac mesenchymal stromal cells (CMSCs) and bone marrow-derived mesenchymal stem cells (BM-MSCs). (B) The reverse transcription-polymerase chain reaction (RT-PCR) of gene expression in the BM-MSCs, normoxia-conditioned CMSCs (CMSC-Nx), and hypoxia-conditioned CMSCs (CMSC-Hpx). In all the three cell populations, the expression of NANOG and SOX2 was high, while that of NKX2-5 and MYH6 was not evident. GATA4 is expressed only in the CMSCs.

2. Characterization of EVs

The EVs were isolated from the conditioned medium from each cell population. The yields of purified EVs from different cells are summarized in Table 2. The transmission electron microscopy revealed that the EVs exhibited umbilicated round shape EVs (Figure 4A). Measurements by dynamic light scattering analysis demonstrated that the size of EVs was in the range of 95-115 nm (Figure 4B). The CMSC-Hpx EVs were slightly larger than the CMSC-Nx EVs (Figure 4A, B), which concurred with the results of a previous report.[20] The nanoparticle tracking analysis confirmed the size distribution of EVs and revealed sufficient particle concentration, which indicated good purity (Figure 4C). The high performance liquid chromatography analysis revealed that the contaminants proportion in the EV preparation was less than 5% (Figure 5A). The western blotting analysis revealed the expression of tetraspanins, CD9, CD63 and CD81 in all the EV groups (Figure 5B-1). Calnexin, a cellular marker for non-EV particles and is known to be an integral protein of the endoplasmic reticulum. Calnexin expression was not detected in all three EVs types (Figure 5B-2). The expression of TSG 101, which is reported to be expressed in many types of EVs, was not apparent in the EVs used in this study. The expression of TSG 101 is not a requirement to identify EVs.

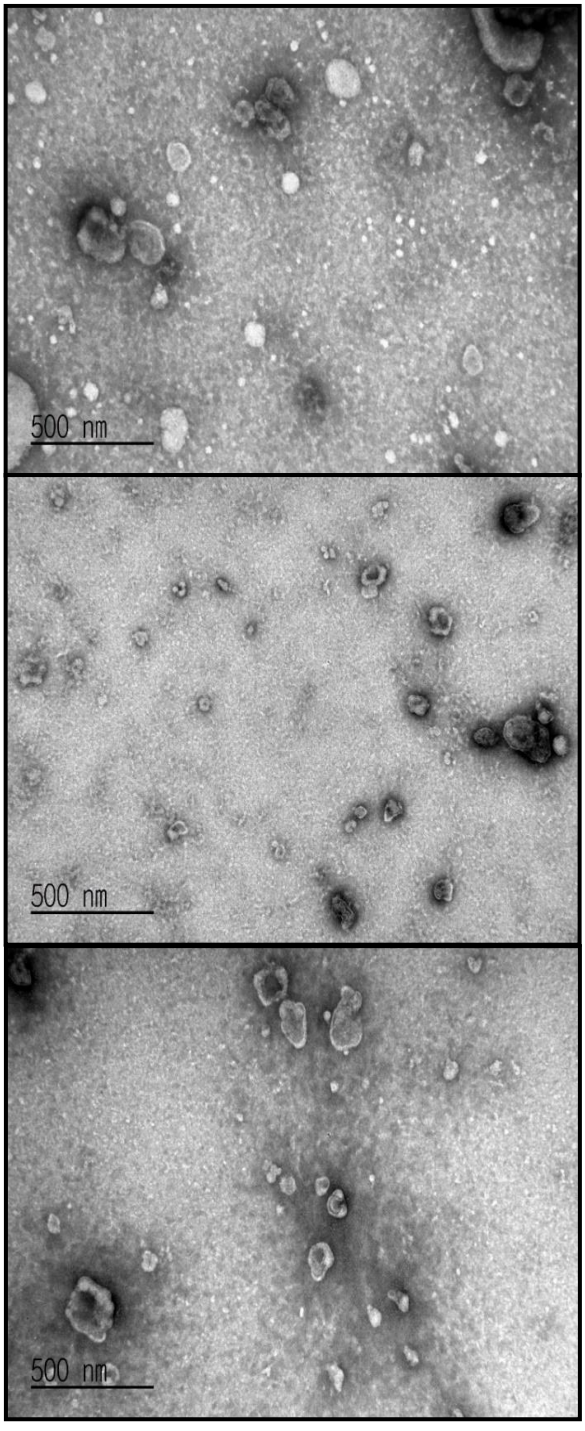
Table 2. Yield of extracellular vesicles (EVs) from cell types

Group	Initial volume of conditioned media (mL)	Final volume after concentration (mL)	Protein Quantification (μg/mL)*	Particles Concentration (particles/mL)†	Purity (particles/μg)
BM-MSC	720	0.72	406.5	8.6×10^{11}	2.1×10^{9}
CMSC-Nx	600	0.12	296.5	5.7×10^{11}	1.9×10^{9}
CMSC-Hpx	450	0.12	276.5	3.7×10^{11}	1.3×10^{9}

BM-MSC, bone marrow-derived mesenchymal stem cell; CMSC-Nx, normoxia-induced cardiac mesenchymal stromal cell; CMSC-Hpx, hypoxia-induced cardiac mesenchymal stromal cells

*Protein quantification by Bicinchoninic acid assay, † Particle quantification by nanoparticle tracking analysis

A

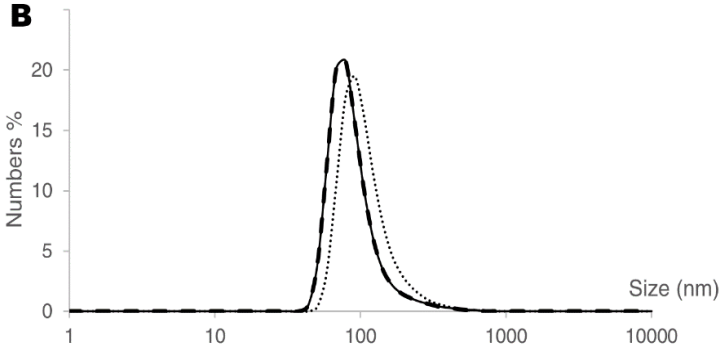


BM-MSC EVs

CMSC-Nx EVs

CMSC-Hpx EVs

B



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

C

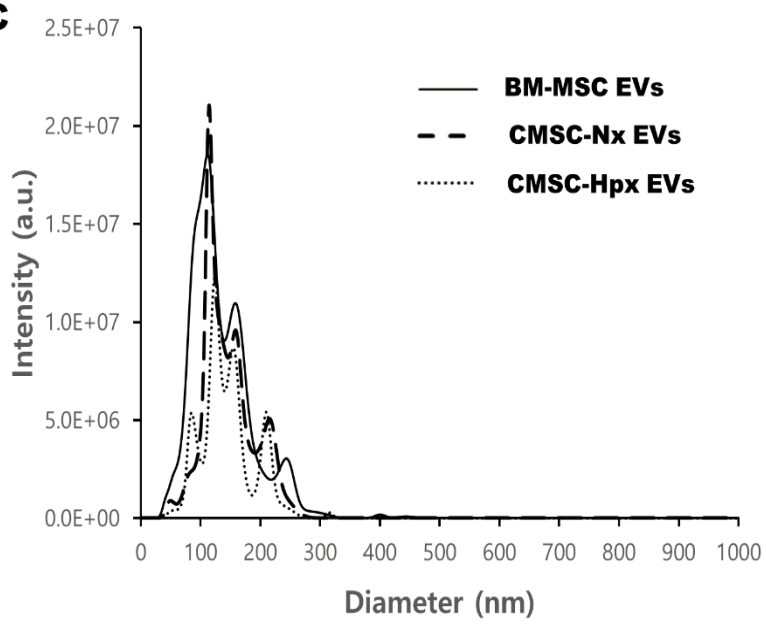
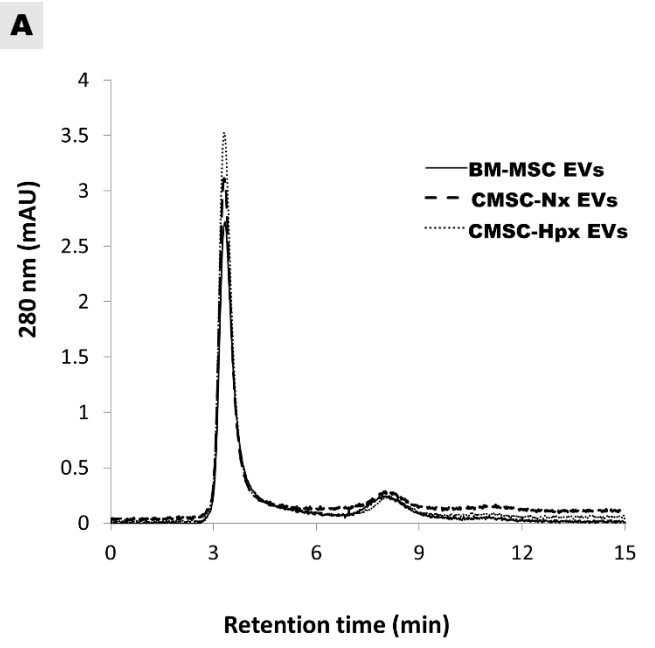


Figure 4. Morphology and size of extracellular vesicles (EVs). The transmission electron microcopy analysis revealed that the EVs exhibited a round cup shape with a size of about 100 nm in all three groups (A). The size distributions of EVs were analyzed by dynamic light scattering analysis (B). The nanoparticle tracking analysis demonstrated particle concentrations corresponding to the vesicle sizes and showed good purity with high particle numbers (C). BM-MSC, bone marrow-derived mesenchymal stem cell; CMSC-Nx, normoxia-induced cardiac mesenchymal stromal cell; CMSC-Hpx, hypoxia-induced cardiac mesenchymal stromal cells



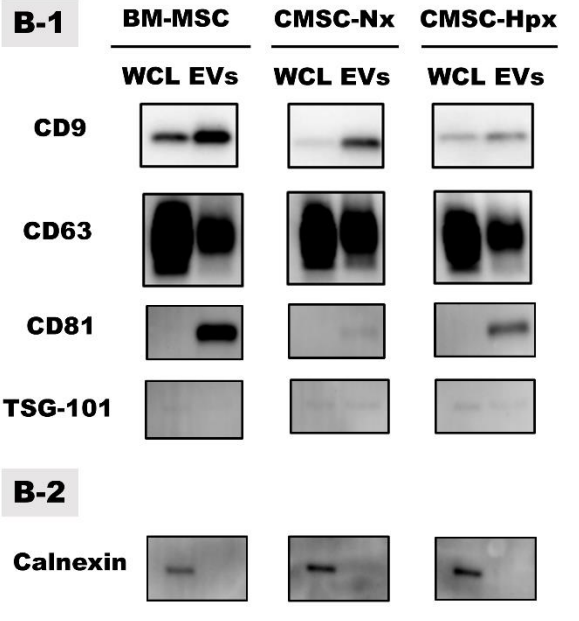
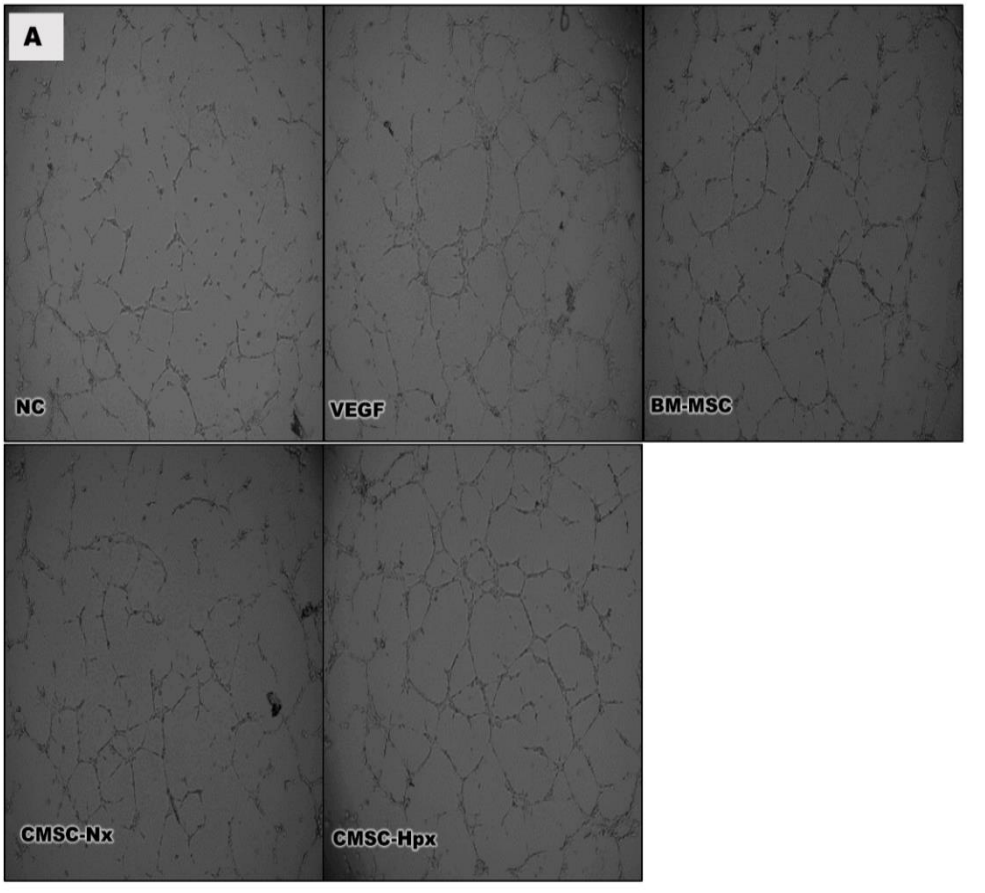


Figure 5. Purity analysis and markers for the extracellular vesicles (EVs). (A) Purity analysis by high performance liquid chromatography showed a sharp peak with an area of 95% before 5 min, which represented EV fractions. A small peak with less than 5% area was detected at about 8 min, which may be related to the contaminated cell debris with small molecular weight. (B-1) Enrichment test. The expression levels of EV proteins (tetraspanins CD9, CD63 and CD81) were detected by western blot analysis in both cell populations. The protein expression levels were compared between the EVs (2 μg of total protein) and whole-cell lysate (WCLs, 20 μg of total protein). The expression levels of CD9 and CD81 slightly varied between the normoxia- and hypoxia-conditioned cardiac mesenchymal stromal cells (CMSCs). (B-2) De-enrichment test using non-EV antibody. The expression of calnexin was not detected in all three type EVs. The same amount of WCLs (2 μg of total protein) and corresponding purified EVs (2 μg of total protein) were used. BM-MSC, bone marrow-derived mesenchymal stem cell; CMSC-Nx, normoxia-induced CMSC; CMSC-Hpx, hypoxia-induced CMSC

3. Effect of EVs on *in vitro* angiogenesis

To evaluate the effects of EVs on angiogenesis, we performed the capillary tube formation assay with HUVECs. HUVECs were cultured in the presence of VEGF, BM-MSC EVs, CMSC-Nx EVs or CMSC-Hpx EVs. The cells cultured only in the medium were used as negative control (NC). The HUVECs treated with BM-MSCs EVs or CMSC-Hpx EVs exhibited significantly improved capillary tube formation when compared to the NC at 6 hr ($P < 0.001$, respectively, Figure 6). The capillary tube formation of HUVECs treated with CMSC-Nx EVs was similar to that of NC. Interestingly, the degree of collapse of tube-like structures formed by HUVECs treated with VEGF or CMSC-Hpx EVs at 8 hr was lesser than that treated with BM-MSC EVs (Figure 6B, C). Treatment with CMSC-Nx EVs did not improve the capillary tube formation. Treatment with CMSC-Hpx EVs enhanced the capillary tube formation and the tube-like structures persisted till 8 hr, which was comparable to the VEGF treatment groups.



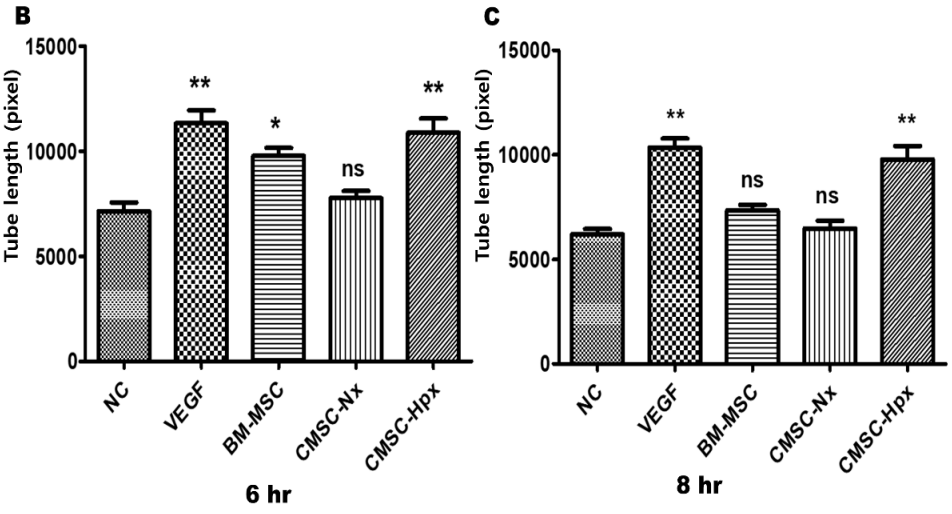


Figure 6. Capillary tube formation assay using human umbilical vein endothelial cells (HUVECs). (A) Microscopic findings of tube formation at 6 hr under different conditions. The HUVECs cultured only in endothelial growth medium (EGM) were used as a negative control (NC), while those cultured in EGM supplemented with vascular endothelial growth factor (VEGF, 20 ng/ml) were used for a positive control. (B) Total tube length at 6 hr revealed augmented tube formation in the groups treated with VEGF, bone marrow-derived mesenchymal stem cell (BM-MSC) extracellular vesicles (EVs), and hypoxia-induced cardiac mesenchymal stromal cell EVs (CMSC-Hpx EVs). (C) Treatment with VEGF or CMSC-Hpx EVs maintained the tube-like structure till 8 hr. CMSC-Nx, normoxia-induced CMSC; * $P < 0.01$, ** $P < 0.001$ vs. NC, ns = no significance; analysis of variance (ANOVA) followed by Turkey post hoc test.

Ⅳ. DISCUSSION

In this study, we have used the CMSCs obtained by ex vivo expansion of human heart tissue using the three-dimensional dynamic organ culture as previously described.[22] These CMSCs did not form cardiosphere, but shared phenotypes similar to the $CD90^{low}$, $c\text{-}kit^{negative}$, $CD105^{positive}$ CDCs. The CMSCs and MSCs can be distinguished based on the expression of CD90.[21]

Recently, an issue concerning the regeneration of CS/PCs, especially $c\text{-}kit^{+}$ cells [6,8,27], was reported to be false.[28] Thirty-one papers from the researcher at the Harvard lab studying the role of $c\text{-}kit^{+}$ cells as potential CS/PCs were retracted due to 'the inclusion of fabricated and/or falsified data' and the ongoing clinical trials involving these cells were stalled.[15,28] Many scientists believe that the studies on putative CS/PS must be reset, although some researchers believe that the $c\text{-}kit^{+}$ cells are still valid for further analysis of regeneration effects based on some animal study results.[15,29] However, the $c\text{-}kit^{+}$ cells are reported to comprise only a minor fraction of the whole CDC population[21] and these cells barely differentiate into cardiomyocytes. Moreover, the remnant $c\text{-}kit^{+}$ cells in the heart are considered to be cardiac endothelial cells rather than CS/PCs.[30] The retrospective analysis of recently completed clinical trial (CADUCEUS) revealed that c-kit expression was not associated with the therapeutic effect of CDCs in human and that the $c\text{-}kit^{+}$ cells were functionally inferior to unsorted

human CDCs.[9] Based on these findings, this study examined the paracrine factors secreted by the CS/PCs, which exhibit low c-kit expression, through EVs to promote angiogenesis. In this study, the expression of c-kit was negligible in both CMSCs and BM-MSCs.

Meanwhile, the CMSCs and BM-MSCs can be distinguished based on CD90 expression. Unlike BM-MSCs, the expression of CD90 was detected in only 26% of CMSCs. Li *et al*. also reported a similar expression pattern in the CDCs. The study reported that the expression of CD90, CD117 and CD105 was detected in 18.4%, 7.0% and 99.9% of CDCs and 99.0%, 5.6% and 99.37% of BM-MSCs, respectively.[21] CD90 is known to be associated with neurite formation and enhanced CD90 expression is associated with poor prognosis in breast cancer and glioma.[31] However, the definite roles of CD90-depleted clones in cardiovascular regeneration are not clear although some researchers published data regarding this topic. They demonstrated difference effects between CD 90^- and $CD90^+$ CDCs, and superiority of $CD90^-$ cells in field of cardiovascular regeneration.[9,11,12] In the chronic infarct rat model, the CD 90^- cardiac stromal cells augmented cardiac function when compared to the CD 90^+ cells. Additionally, the histological analysis revealed that the $CD90^-$ cells increased vascularization in the infarct lesion.[12] Other studies have also reported that the depletion of CD 90^+ cells improved the cardiac function in the murine myocardial infarction model and suggested that the CD 90^- cells secret less inflammatory cytokines and may differentiate into cardiomyocytes.[9]

Interestingly, there was no difference in the secretion levels of VEGF and bFGF between the $CD90^-$ and $CD90^+$ cardiac stromal cells. However, the $CD90^-$ cells exhibited enhanced expression of hepatocyte growth factor (HGF) when compared to the $CD90^+$ cells.[12]

The transcriptional profiles and gene ontology enrichment analysis of adult human cardiosphere-derived $CD90^-$ and $CD90^+$ cells a study showed that CD 90^- clones enriched in expression of gene related to stemness (OCT4, CCNF, PHC1, NR2F6, and ASH 2L), whereas CD 90^+ clones were enriched with fibroblast associated genes (PAK4, SEPT1, MMP2, and FAP) expression.[11] Two separate studies demonstrated that the expression level of CD90/THY-1 changes dynamically during the formation of cardiosphere under different culture conditions.[11,32] Hence, the culture condition may promote stemness and contribute to the acquisition of cardiogenic properties of putative CS/PCs through CD90 depletion. In this study, the CMSCs were isolated as CD 90^{low} CS/PCs under specific culture condition to promote stemness.[22]

Hence, the $CD90^{low}$c-$kit^{negative}CD105^{positive}$ CMSCs isolated in this study are likely to play a significant role in the regeneration of the heart as an active fraction. The majority of researches with CS/PCs have been performed with the heterogeneous population of CDCs or c-kit^+ cells. Therefore, further studies with c-$kit^{negative}CD90^{negative}$ clones are needed to evaluate the more effective therapeutic application of CS/PC and to understand the roles of these cells.

GATA4 is one of the earliest transcription factors and a critical regulator of cardiac development that interacts with many transcriptional factors, such as NKX2-5.[33,34] Although GATA4 is crucial to regulate the expression of NKX2-5, the initial activation of NKX2-5 is reported to require additional signaling pathways.[34,35] In this study, the CMSCs expressed only GATA4 but no NKX2-5. One study using similar rotating tissue culture conditions also reported the expression of GATA4 and limited expression of NKX2-5 in the CS/PCs.[11,36] These cells may be considered to be in early developmental stages before expressing NKX2-5 and further analysis will help to understand the roles of CS/PCs at various developmental stages or marking various stemness during primary culture.

Our results revealed that CMSC-Hpx EVs improved capillary tube formation while CMSC-Nx EVs did not. Previous studies have reported that the EVs from hypoxia-conditioned MSCs and CDCs enhance angiogenesis.[19,20] Hypoxia-conditioned human adipose MSCs are reported to activate the protein kinase A signaling pathway and promote the expression of VEGF.[19] Hypoxia-conditioned CDCs promotes the expression of hypoxia-inducible factor-1α, miR-210, 130a, and 126 in exosome secreted from these cells, which subsequently enhances the capillary tube formation.[20] One study revealed that the treatment with neonate (0-1 month) exosome from normoxia- and hypoxia-conditioned CPCs improve the rat cardiac function with reducing

fibrosis and improved of hypertrophy. However, at an older age, only the exosome from the hypoxia-conditioned CPCs were reported to improve cardiac function.[37] One important difference is that the study conducted with c-kit^{+} CPCs which is only minor portion in this study. Nonetheless, it suggests that the EVs from hypoxia-conditioned CPCs are more advantageous for cardiac regeneration than the EVs form normoxia-conditioned CPCs and that the clinical potential of EVs varies greatly depending on the cell culture environment. A comparative study of the MSCs and CDCs revealed that the production of VEGF *in vitro* was similar in both cell groups, but the production of HGF in the CDCs was reported to be higher than that in the MSCs. Another study demonstrated that CD 90^{-} CDCs secreted higher HGF levels than the CD 90^{+} CDCs.[12,21] Therefore, HGF secreted from CMSCs may plays an important role in angiogenesis. Further researches are needed to verify these results using proteomics or mircoRNAs approaches.

Future studies on EVs from CMSCs must address several questions. First, the yield of EVs from CMSCs was relatively low when compared to that of BM-MSC EVs (Figure 7). The EVs from CMSCs tend to be more attached to each other than the EVs from BM-MSCs, which may due to the different properties of EVs in the liquid. This may have caused unpredictable frequent loss during the EV isolation.

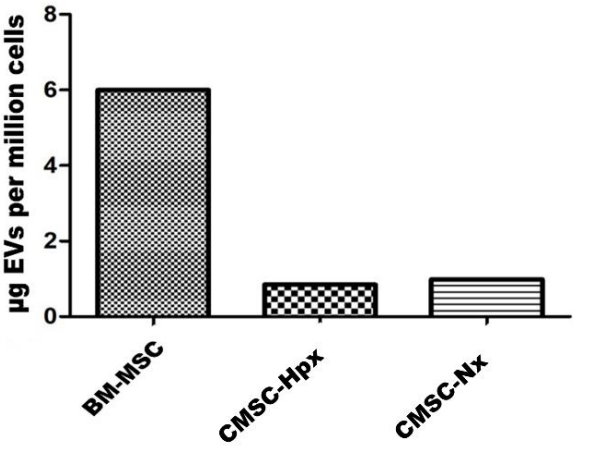


Figure 7. Yields of extracellular vesicles (EVs) based on the cell types. The highest EV yield was obtained from bone marrow-derived mesenchymal stem cell (BM-MSC), which was almost 7 times the yield of EVs from cardiac mesenchymal stromal cells (CMSCs). CMSC-Hpx, hypoxia-induced CMSC; CMSC-Nx, normoxia-induced CMSC.

Therefore, it was difficult to obtain sufficient EVs from CMSCs in the limited amount of human samples. These technical problems should be solved in future experiments. Secondly, the capillary tube formations did not increase in proportion to the concentration of EVs. The highest capillary tube formation was observed at EV dose of 2 μg/ml (0.4 μg/well) in all three groups, which decreased at higher concentration of EVs (Figure 8). Severe studies have reported the biphasic results of tube augmentation with increasing concentration of EVs, although others demonstrated that angiogenesis increases in a dose dependently with EVs treatment.[20,38] Additionally, they used 10 times more EV

concentration than the amounts used in this works (25 μg/ml versus 2 μg/ml). These variations among different laboratories could be caused by various technical reasons, such as different isolation methods, the purity of EVs, and different cell culture environment. Controlling the detailed cell culture condition and the standardized isolation methods of EVs are needed to solve the quality and the quantity variation of EVs.

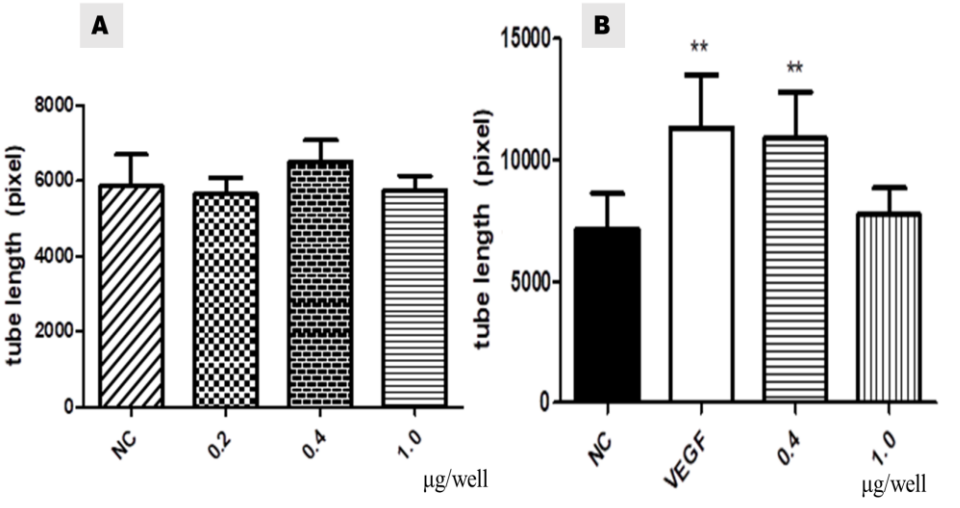


Figure 8. Capillary tube formation after treatment with different concentrations of extracellular vesicles (EVs). (A) EVs from normoxia-conditioned cardiac mesenchymal stromal cell (CMSC-Nx EVs) dose-dependently enhanced tube formation at low dose but decreased the tube formation at a higher dose (1.0 μg/well). Similar findings were observed after treatment with hypoxia-conditioned cardiac mesenchymal stromal cells (CMSC-Hpx EVs) (B). NC, negative control; ** $P < 0.01$ vs. NC

Ⅴ. CONCLUSION

The results of this study demonstrated that less than 1% CMSCs were c-kit positive cells and that only one quarter cells were CD90 positive in contrast to BM-MSCs. The character of the CMSCs can be summarized as CD 90^{low}, c-$kit^{negative}$, CD $105^{positive}$, GATA4 positive and negative expression of NKX2-5. Previous studies have reported that this phenotype is associated with improved cardiac function after myocardial damage. This study also demonstrated that the CMSC-Hpx EVs promoted better capillary tube augmentation in the HUVECs than BM-MSC EVs, which was comparable to the VEGF group. Future studies should focus on elucidating the mechanism underlying these effects and further functional characterization of the EVs from CMSC-Hpx.

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ABSTRACT (IN KOREAN)

사람 심장 중간엽 기질세포로부터 유래한 세포외 소포체 및 이들의 혈관 생성 유도 능력

<지도교수 장 양 수>

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강 인 숙

줄기세포에 의한 심장 재생은 가까운 미래에 말기 심혈관 질환을 해결할 수 있는 치료제로 많은 기대를 모으고 있는 분야 중의 하나이다. 또한, 줄기세포로부터 유래된 세포외 소포체 (extracellular vesicles, EVs)는 세포의 운반체로서의 역할을 하며 줄기 세포 치료 후에 나타나는 심기능 개선에 측분비인자 (paracrine factor)로 역할을 하는 것으로 알려져 있다. 최근 연구를 통해 심장 성체 줄기세포의 존재가 알려지면서 심장 재생 연구는 더욱 활발하게 진행되었다. 그러나 잠재적 심장 줄기세포로 여겨졌던 c-kit 양성

세포에 대한 논란이 발생하면서 사람에게 심장 성체 줄기세포가 과연 존재하는 가에 대한 의문까지 제기 되고 있는 상황이다. 이에 본 연구에서는 사람 심장 조직의 생체외 (*ex vivo*) 증식 및 3 차원 동적 조직배양을 통해 심장 중간엽 기질세포 (cardiac mesenchymal stromal cell; CMSC)를 분리 및 동정하여, 가장 많이 연구된 성체 줄기세포의 하나인 사람 골수 유래 중간엽 줄기세포 (human bone marrow derived mesenchymal stem cells)와 특징 및 성능을 비교 하였다. 이를 통해 CMSCs 가 $CD90^{low}$, $c\text{-}kit^{negative}$, $CD105^{positive}$ 의 표현형을 나타내며 NANOG, SOX2 및 GATA4 를 발현함을 확인 할 수 있었다.

또한 각각의 세포로부터 EVs 를 추출하여 혈관 형성에 더욱 효과적인 EVs 가 무엇인지 평가 하였다. 이를 위해 CMSCs 의 EVs 는 정상 산소와 저 산소 조건에서 세포를 배양하여 추출 및 실험을 하였다. 사람 제대 정맥 세포를 이용한 모세 관 형성 (capillary tube formation) 실험에서 저 산소 조건에서 배양된 CMSC 로부터 얻은 EVs 가 더 효과적으로 모세 관 형성을 하는 것이 관찰되었다.

본 연구를 통해 저 산소 조건의 $CD90^{low}$, $c\text{-}kit^{negative}$, $CD105^{positive}$ CMSCs 가 체외 미세 관 형성 실험에서 다른 군에 비해 좀 더 효과적인 EVs 를 분비한다는 것을 알 수 있었으며 이러한 결과는

심혈관 복구에 대한 이해에 더 많은 통찰력을 주며 향후 연구 설계에 도움이 될 것으로 기대한다.

핵심되는 말: 심장 중간엽 기질세포, 세포외 소포체, 혈관 신생, 심혈관 재생