Biomaterials 232 (2020) 119674

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

NFATc1 + CD31 + CD45 − circulating multipotent stem cells derived from human endocardium and their therapeutic potential

Han-Mo Yang[a,f,g,1], Ju-Young Kim[f,g,1], Hyun-Jai Cho[a,f,g,1], Joo-Eun Lee[f,g], Sooryeonhwa Jin[f,g], Jin Hur[f,g], Yoo-Wook Kwon[f,g], Moon-Woo Seong[b], Eue-Keun Choi[a,g], Hae-Young Lee[a,g], Hyun-Seob Lee[h], Mika Jeon[f,g], Joonoh Kim[f,g], Jaeseok Yang[e], Seil Oh[a,g], Kyung-Suk Suh[c], Sung-Soo Yoon[a], Ki-Bong Kim[d], Byung-Hee Oh[a,g], Young-Bae Park[a,g], Hyo-Soo Kim[a,f,g,i,*]

[a] Department of Internal Medicine, Seoul National University Hospital, Seoul, South Korea
[b] Department of Laboratory Medicine, Seoul National University Hospital, Seoul, South Korea
[c] Department of Surgery, Seoul National University Hospital, Seoul, South Korea
[d] Department of Thoracic and Cardiovascular Surgery, Seoul National University College of Medicine, Seoul, South Korea
[e] Transplantation Center, Seoul National University Hospital, Seoul, South Korea
[f] National Research Laboratory for Stem Cell Niche, Seoul National University Hospital, Seoul, South Korea
[g] Innovative Research Institute for Cell Therapy, Seoul National University Hospital, Seoul, South Korea
[h] Genomics Core Facility, Department of Transdisciplinary Research and Collaboration, Biomedical Research Institute, Seoul National University Hospital, Seoul, South Korea
[i] Molecular Medicine and Biopharmaceutical Sciences, Seoul National University, Seoul, South Korea

ARTICLE INFO

Keywords:
Stem cells
Peripheral blood
Heart
Induced pluripotent stem cells
Endocardium

ABSTRACT

Many studies have shown the existence of cardiac stem cells in the myocardium and epicardial progenitor cells in the epicardium. However, the characteristics of stem cells in the endocardium has not been fully elucidated. In this study, we investigated the origin of newly identified cells in the blood and their therapeutic potential. The new population of cells, identified from human peripheral blood, was quite different from previously reported stem cells. These newly identified cells, which we named Circulating Multipotent Stem (CiMS) cells, were multipotent, and therefore differentiated into multiple lineages *in vitro* and *in vivo*. In order to determine the origin of these cells, we collected peripheral blood from a group of patients who underwent bone marrow, liver, heart, or kidney transplantation. We identified the endocardium as the origin of these cells because the Short Tandem Repeat profile of CiMS cells from the recipient had changed from the recipient's profile to the donor's profile after heart transplantation. CiMS cells significantly increased after stimuli to the endocardium, such as catheter ablation for arrhythmia or acute myocardial infarction. CiMS cells circulate in human peripheral blood and are easily obtainable, suggesting that these cells could be a promising tool for cell therapy.

1. Introduction

The human heart is known to have tissue-resident stem cells such as cardiac stem cells and epicardial progenitor cells [1,2]. These stem or progenitor cells have the capability to regenerate both the myocardium and the epicardium. To characterize and enrich these cardiac stem cells, several markers such as c-kit, Sca-1, and isl1 have been explored [3]. Cardiac stem cells show some advantages over other stem cells because they are believed to differentiate into cardiac lineage cells. Based on this hypothesis, clinical trials, including the SCIPIO and CADUCEUS, have been conducted, showing the potency of cardiac stem cells for cardiac repair [4,5]. In general, surgical or endomyocardial biopsy is carried out to collect these cells. Due to the invasive nature of these procedures, optimal methods to obtain the cells, such as collection from peripheral blood, have been sought to minimize potential harms. Many studies have revealed the presence of stem cells or progenitor cells that are circulating in human peripheral blood [6–11]. In 1997, Asahara et al. first reported the presence of endothelial progenitor cells (EPCs) in peripheral blood [6]. Other studies have demonstrated that there are circulating mesenchymal precursor cells or skeletal stem cells in

* Corresponding author. Molecular Medicine and Biopharmaceutical Sciences, Seoul National University / Cardiovascular Center, Seoul National University Hospital, 28 Yongon-dong Chongno-gu, Seoul, 03080, South Korea.
E-mail address: hyosoo@snu.ac.kr (H.-S. Kim).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.biomaterials.2019.119674
Received 7 October 2019; Accepted 5 December 2019
Available online 10 December 2019
0142-9612/

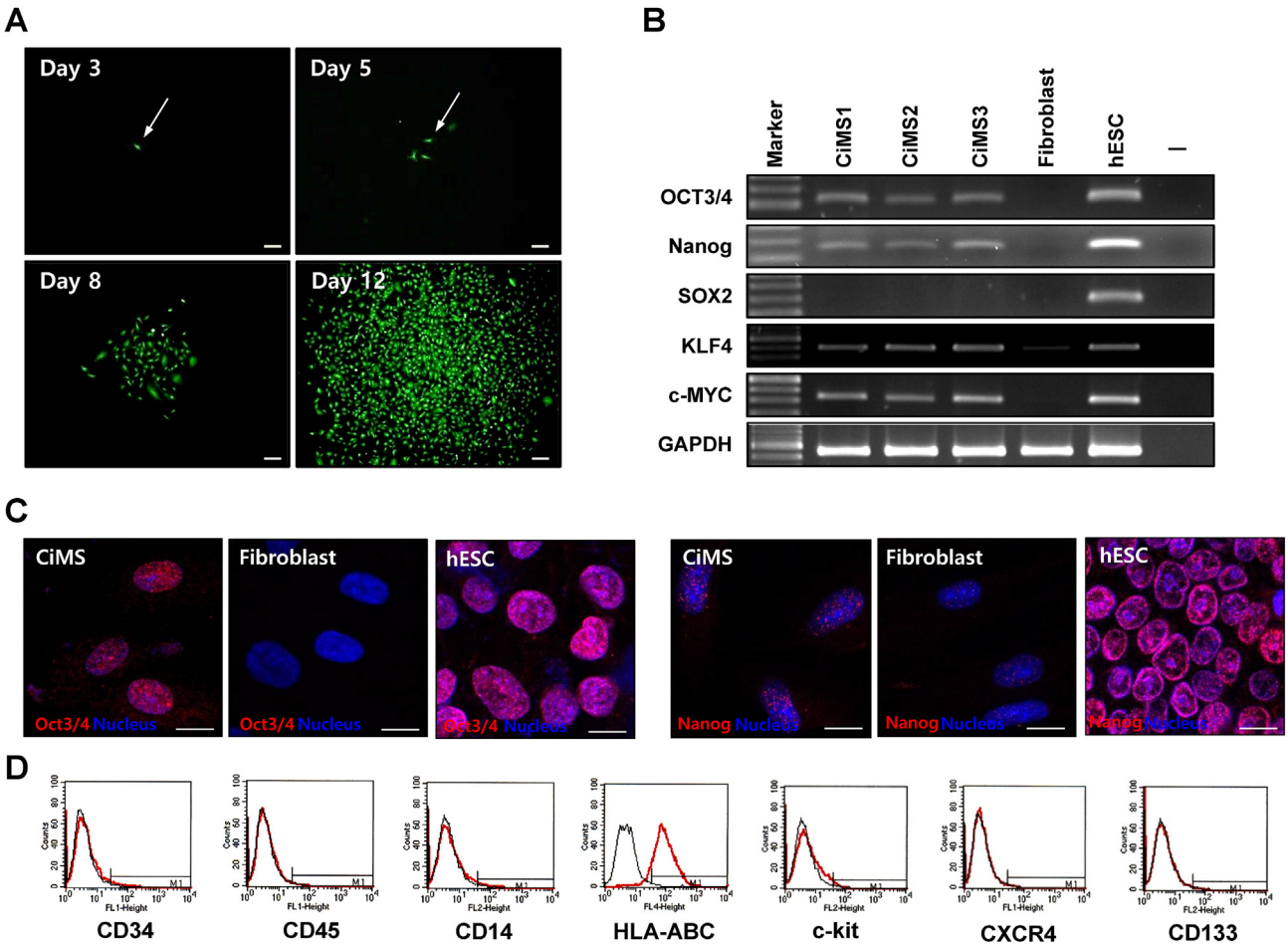

Fig. 1. Clonogenicity and origin of newly identified circulating multipotent stem (CiMS) cells. (A) Clonogenicity of CiMS cells. For the clonal analysis, the newly identified cells were tagged by a lentiviral vector expressing enhanced green fluorescent protein (eGFP) and were seeded at a limiting dilution. After 7–14 days, multicellular clones were obtained. The figures demonstrated sequential changes of the clonal expansion during two weeks. Scale bar is 100 μm. (B) CiMS cells express stemness markers such as Oct3/4, Nanog, and KLF4 that were detected by RT-PCR. hESC = human embryonic stem cells. (C) CiMS cells show positivity for Oct3/4 and Nanog that were detected by Immunofluorescence staining. hESC = human embryonic stem cells. Scale bar is 20 μm. (D) FACS data shows that these cells do not have the characteristics of hematopoietic stem cells or cardiac progenitor cells. (E) The profiles of CiMS cells cultured after transplantation matched to those of the donors only in heart transplantation, suggesting that CiMS cells could be derived from the heart. (F) The STR analysis shows the change in the profiles of CiMS cells from a given patient before and after heart transplantation. The profiles of CiMS cells before transplantation were identical to those of the recipient heart. After transplantation, however, the profiles of CiMS cells changed to those of the donor heart. STR = Short Tandem Repeat, HLA = Human Leukocyte Antigen, CiMS = Circulating Mulpipotent Stem cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

peripheral blood [9,10].

In this study, we identified a novel population of multipotent stem cells circulating in human peripheral blood. These cells, which had the ability to differentiate into cells of all three germ layers, were derived from the endocardium of the human heart. Moreover, we examined their regenerative potential *in vivo* and clinical relevance.

2. Materials and methods

Detailed methods are given in the online Supplemental Data.

2.1. Peripheral blood mononuclear cells (PBMNCs) isolation and circulating multipotent stem (CiMS) cells culture

Human peripheral blood samples (10 mL) were obtained from blood donors. Mononuclear cells were seeded on non-coated six-well plates. The medium was changed every day for up to two weeks after plating.

2.2. Short tandem repeat (STR) analysis

The 16 STR loci investigated in this study were D8S1179, D21S11, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, Amelogenin, D5S818, and FGA.

2.3. Statistics

All data are presented as means ± standard error of means (SEM). Mann-Whitney test, Wilcoxon signed rank test, and Kruskal-Wallis test were used when appropriate. SPSS version 21.0 (SPSS Inc., Chicago, IL) was used for the analysis and P values of < 0.05 were considered to be statistically significant.

2.4. Study approval

All human samples were obtained with written informed consent after the approval by the Institutional Review Board (IRB) of Seoul National University Hospital. All animal experiments were performed after receiving approval from the Institutional Animal Care and Use Committee (IACUC) of Clinical Research Institute in Seoul National University Hospital and complied with the National Research Council (NRC) 'Guidelines for the Care and Use of Laboratory Animals'. In the study determining the origin of CiMS cells, peripheral blood was

E

Transplant Group	Patient	CiMS: Identical to	
		Donor	Recipient
Bone Marrow		The STR profiles	
	#1		O
	#2		O
	#3		O
	#4		O
Liver		The HLA type	
	#5		O
	#6		O
	#7		O
	#8		O
Kidney		The HLA type	
	#9		O
	#10		O
	#11		O
	#12		O
Heart		The STR profiles	
	#13	O	
	#14	O	
	#15	O	
	#16	O	

F

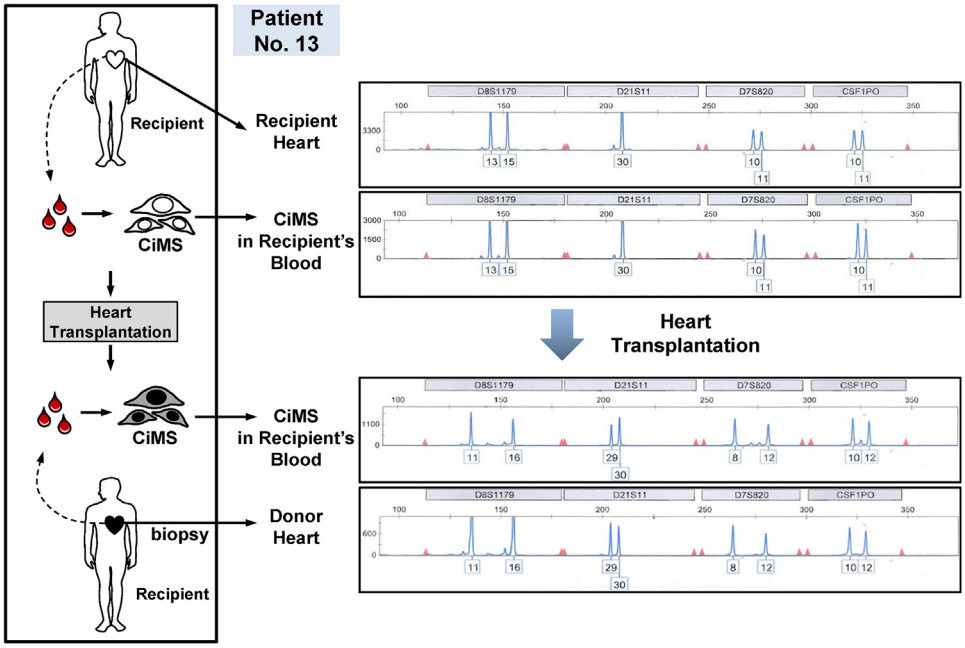

Fig. 1. (*continued*)

collected from patients who underwent transplantation of bone marrow, liver, heart, or kidney (n = 4 per group).

3. Results

3.1. A novel population of stem cells was identified in human peripheral blood

In the previous culture methods for the isolation of circulating stem cells such as EPCs, the culture media were changed every 2 or 3 days in the dishes coated with fibronectin. In contrast, we developed new culture methods using vigorous daily changes of media in non-coated dishes, which enables to remove non-adherent cells such as T cells. With our new culture methods, we identified a new population of cells from peripheral blood mononuclear cells (PBMNCs) after a few days of culture. These cells showed a unique morphology such as dumbbell-like or spindle-shaped (Supplemental Fig. 1A), which was quite different from that of EPCs (Supplemental Figs. 1B and 1C) [11]. One cell was able to form a colony over the two-week period, reflecting the high proliferative activity and clonogenicity (Fig. 1A, Supplemental Fig. 4D). These cells proliferated rapidly and showed an exponential growth curve (Supplemental Fig. 1D). Even after 50 weeks of culture, the cells maintained their proliferative activity and a stable phenotype (Supplemental Fig. 1E). Even after more than 15 passages, they expressed stemness genes such as Oct3/4, Nanog, Kruppel-like factor 4 (KLF4), and c-Myc, which are all well-known reprogramming factors (Fig. 1B and C, Supplemental Figs. 1F and 1G) [12]. They did not, however, express the markers that are specific for hematopoietic or other stem cells by FACS analysis (Fig. 1D). Thus, we called them Circulating Multipotent Stem (CiMS) cells.

3.2. CiMS cells were derived from the human heart, not from the bone marrow

In order to establish the origin of these cells, we collected and examined peripheral blood from patients who had undergone transplantation of bone marrow, liver, heart, or kidney. In the case of the patients with bone marrow transplantation, the Human Leukocyte Antigen (HLA) types of both the donor and the recipient were identical. Therefore, to identify the origin of cells, we utilized a Short Tandem Repeat (STR) analysis. Our hypothesis was that if the STR profile of CiMS cells after bone marrow transplantation matched that of the donor, CiMS cells could have been derived from bone marrow. In contrast, if the profile of CiMS cells was still identical to that of the recipient, CiMS cells could have originated from organs other than bone marrow. Interestingly, the profiles of CiMS cells obtained from the peripheral blood of four patients after bone marrow transplantation were still the same as the profiles of the recipients, but different from those of the donors, suggesting that CiMS cells were not derived from bone marrow (Fig. 1E, Supplemental Fig. 2). To pinpoint the organ from which CiMS cells are derived, we examined peripheral blood from patients who received solid organ transplantations, such as liver, kidney, or heart. After analyzing the HLA types of CiMS cells that were obtained from the peripheral blood of each patient, the HLA types of the recipients' CiMS cells were compared to those of the donor and recipient. In the case of patients with liver and kidney transplantation, the HLA types of CiMS cells after transplantation matched those of the recipients, suggesting that CiMS cells are not derived from the transplanted liver or kidney (Fig. 1E, Supplemental Table 1). In the case of patients with heart transplantation, we collected the peripheral blood and cultured CiMS cells before and after the heart transplantation. In addition, we obtained the heart tissues from both the recipients' removed hearts after surgery and the endocardia of the donor hearts during the endomyocardial biopsy after transplantation. We performed the STR analysis with the CiMS cells and the heart tissues. To our surprise, the STR profile of CiMS cells after transplantation completely changed from that of the recipient to that of the donor heart (Fig. 1E and F, Patient No. 13–16 in Table 1, Supplemental Fig. 3). This indicated that the source of the cultured CiMS cells had changed from the recipient to the donor. Accordingly, this finding strongly suggests that the heart could be the origin of CiMS cells.

Table 1
Change in the STR profiles of patients who underwent heart transplantation.

STR loci	Recipient Heart	Peripheral Blood	CiMS: Pre-TPL	CiMS: Post-TPL	Donor Heart	Recipient Heart	Peripheral Blood	CiMS: Pre-TPL	CiMS: Post-TPL	Donor Heart
	Patient No. 13					Patient No. 14				
D8S1179	13, 15	13, 15	13, 15	11, 16	11, 16	14, 14	14, 14	14, 14	14, 15	14, 15
D21S11	30, 30	30, 30	30, 30	29, 30	29, 30	29, 30	29, 30	29, 30	28, 32.2	28, 32.2
D7S820	10, 11	10, 11	10, 11	8, 12	8, 12	12, 12	12, 12	12, 12	10, 11	10, 11
CSF1PO	10, 11	10, 11	10, 11	10, 12	10, 12	12, 12	12, 12	12, 12	12, 12	12, 12
D3S1358	15,16	15,16	15,16	16, 18	16, 18	15, 15	15, 15	15, 15	15, 17	15, 17
THO1	7, 9	7, 9	7, 9	7, 9	7, 9	7, 9	7, 9	7, 9	6, 9	6, 9
D13S317	9.12	9.12	9.12	10, 13	10, 13	11, 12	11, 12	11, 12	10, 11	10, 11
D16S539	10, 11	10, 11	10, 11	11, 12	11, 12	10, 11	10, 11	10, 11	10, 13	10, 13
D2S1338	19, 23	19, 23	19, 23	21, 25	21, 25	17, 17	17, 17	17, 17	20, 23	20, 23
D19S433	13, 13	13, 13	13, 13	13, 13	13, 13	13, 14.2	13, 14.2	13, 14.2	13, 15.2	13, 15.2
vWA	14, 18	14, 18	14, 18	14, 18	14, 18	16, 19	16, 19	16, 19	17, 18	17, 18
TPOX	11, 12	11, 12	11, 12	9, 11	9, 11	8, 8	8, 8	8, 8	8, 11	8, 11
D18S51	13, 13	13, 13	13, 13	14, 18	14, 18	13, 14	13, 14	13, 14	12, 13	12, 13
D5S818	10, 11	10, 11	10, 11	10, 10	10, 10	9, 13	9, 13	9, 13	11, 13	11, 13
FGA	24, 24	24, 24	24, 24	23, 27	23, 27	19, 22	19, 22	19, 22	23, 23	23, 23
Amelogenin	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y*	X, Y*	X, Y*	X, X†	X, X†
	Patient No. 15					Patient No. 16				
D8S1179	11, 14	11, 14	11, 14	13, 15	13, 15	10, 13	10, 13	10, 13	10, 12	10, 12
D21S11	30, 32.2	30, 32.2	30, 32.2	30, 32.2	30, 32.2	32.2, 32.2	32.2, 32.2	32.2, 32.2	29, 29	29, 29
D7S820	9, 9	9, 9	9, 9	10, 11	10, 11	11, 11	11, 11	11, 11	10, 13	10, 13
CSF1PO	12, 13	12, 13	12, 13	9, 12	9, 12	12, 12	12, 12	12, 12	10, 12	10, 12
D3S1358	16, 18	16, 18	16, 18	15, 15	15, 15	16, 18	16, 18	16, 18	15, 16	15, 16
THO1	6, 9	6, 9	6, 9	7, 9	7, 9	6, 7	6, 7	6, 7	7, 9	7, 9
D13S317	9, 12	9, 12	9, 12	11, 12	11, 12	8, 11	8, 11	8, 11	8, 12	8, 12
D16S539	9, 12	9, 12	9, 12	9, 11	9, 11	11, 12	11, 12	11, 12	11, 11	11, 11
D2S1338	23	23	23	21, 25	21, 25	18, 24	18, 24	18, 24	23, 24	23, 24
D19S433	13, 15.2	13, 15.2	13, 15.2	14, 14	14, 14	13, 16	13, 16	13, 16	13, 15.2	13, 15.2
vWA	17, 17	17, 17	17, 17	15, 17	15, 17	14, 18	14, 18	14, 18	14, 17	14, 17
TPOX	11, 11	11, 11	11, 11	9, 11	9, 11	8, 11	8, 11	8, 11	8, 11	8, 11
D18S51	13, 17	13, 17	13, 17	16, 22	16, 22	14, 15	14, 15	14, 15	16, 16	16, 16
D5S818	9, 12	9, 12	9, 12	12, 12	12, 12	10, 10	10, 10	10, 10	12, 13	12, 13
FGA	24, 24	24, 24	24, 24	21, 24	21, 24	23, 25	23, 25	23, 25	22, 23	22, 23
Amelogenin	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y

Variables in the first column indicate the STR DNA loci. All subjects were male except patient No.14, in which case, the recipient was male* and the donor was female†. The STR analysis showed a change in the profiles of CiMS cells from the recipient patient before and after heart transplantation. The profiles of CiMS cells before transplantation were identical to those of the recipient hearts. After transplantation, however, the profiles of CiMS cells changed to those of the donor hearts. STR = Short Tandem Repeat, TPL = Transplantation.

3.3. CiMS cells in the blood were derived from the endocardial cells that were positive for both CD31 and Nuclear Factor of Activated T cells (NFAT)

To investigate which part of the heart possesses CiMS cells, we cultured the tissues from the endo-, myo-, or epi-cardial layers of the recipients' removed hearts and from the endo- or myo-cardial layers of the biopsied donors' hearts. CiMS cells emerged only from the endocardium. Moreover, these cells cultured from the endocardium after transplantation showed the same STR profiles as the blood-derived CiMS cells, but different profiles from those of the recipients' peripheral blood after transplantation (n = 5, Patient No. 17–21 in Supplemental Table 2 and Supplemental Fig. 4A). Together, these results demonstrate that CiMS cells could be derived from the endocardium of the heart. In the endocardium of the human heart, we identified the cells that were positive for both NFATc1 and CD31 by immunofluorescence staining (Fig. 2A, Supplemental Fig. 4B). In addition, we confirmed that CiMS cells cultured from the peripheral blood or heart tissue expressed NFATc1 at mRNA and protein levels (Fig. 2B–D, Supplemental Figs. 4C and 4D). Besides the expression of NFATc1, specific markers were searched for CiMS cells, which could be unique characteristics when compared to other cell types. Microarrays were performed using CiMS cells, EPCs, gastroepiploic artery endothelial cells (GEAECs), mesenchymal stem cells (MSCs), and Fibroblasts (Supplemental Figs. 4E–H). Among several genes highly expressed in CiMS cells, it was confirmed that several stem cell markers are also highly expressed in the CiMS cells as follows: (1) GATA4 (a marker for cardiac-lineage stem cells), (2) SOX17 (a marker for early mesoendoderm), and (3) ALDH1A2 (a marker for stem cells) (Fig. 2E, Supplemental Fig. 4E–L).

A unique feature of our new method to culture CiMS cells is frequent changes of media in non-coated dishes. We assumed that this method could remove non-adherent cells such as leukocytes or T cells during culture. Based on this assumption, we divided the fresh peripheral blood into CD45-positive and CD45-negative cells by magnetic-activated cell sorting (MACS) or FACS (Supplemental Fig. 4M and 4N). After culture for a few days, CiMS cells appeared only in the CD45-negative fraction (Supplemental Fig. 4M). Thus, we established a method to assess the number of CiMS cells in the peripheral blood. We sorted PBMNCs based on the expression of CD31, NFATc1, and CD45. CiMS cells were cultured only from the fraction that was positive for both CD31 and NFATc1, and negative for CD45 (Fig. 3A, Supplemental Fig. 4N). In addition, in the human heart tissue, CiMS cells were cultured from the same fraction of CD31-positive, NFATc1-positive, and CD45-negative cells (Supplemental Fig. 4O). Since NFATc1 is an intracellular target, it was impossible to culture after the conventional FACS sorting method which needs fixation and makes cells dead. Therefore, we developed a new method of live-cell FACS sorting using transient permeabilization, which enabled us to detect intracellular targets without killing the cells (Supplemental Fig. 5).

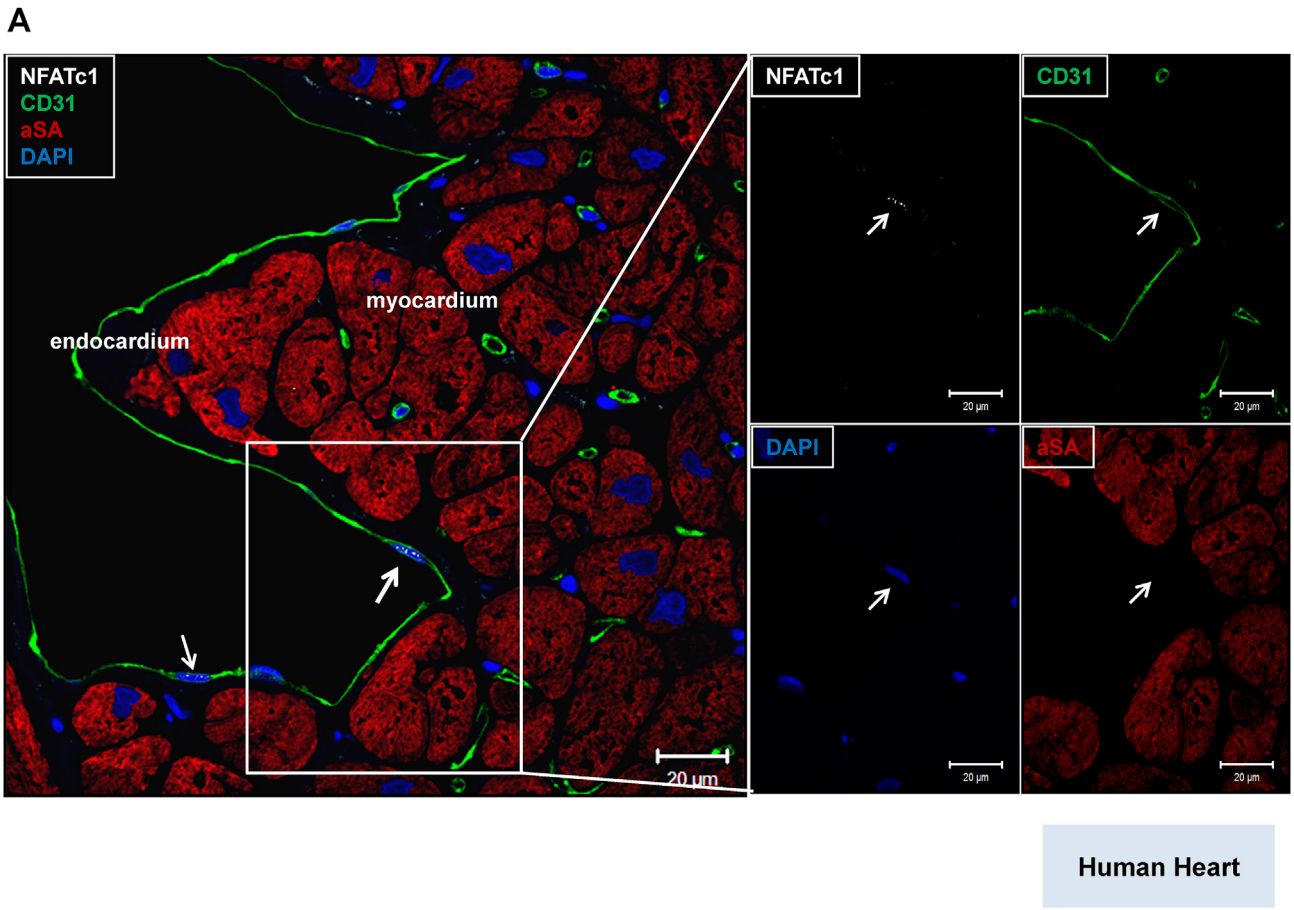

Fig. 2. Origin of CiMS cells: NFATc1 and CD31 double-positive cells in the endocardium. (A) We identified the cells positive for both NFATc1 and CD31 in the human endocardium. NFAT = Nuclear Factor of Activated T cells, a-SA = alpha sarcomeric actinin, DAPI = 4′,6-diamidino-2-phenylindole. (B) RT-PCR shows that CiMS cells cultured from the peripheral blood express NFATc1. hESC = human embryonic stem cells. (C) Representative figure of CiMS cell colony on day 17 after culture, showing positivity for NFATc1 by immunofluorescence staining. This figure was obtained by merging 48 cropped microscopic images individually, because it could not be captured as a single image due to large original size of the colony. Scale bar is 200 μm. (D) Immunofluorescence staining shows that CiMS cells cultured from the heart tissue are also positive for NFATc1. Scale bar is 50 μm. (E) Western blot of several stem cell markers for CIMS cells. After analyzing the microarray data using CiMS cells and other cell types, we confirmed that several stem cell markers are also highly expressed in the CiMS cells: GATA4 (a marker for cardiac-lineage stem cells), SOX17 (a marker for early mesoendoderm), and ALDH1A2 (a marker for stem cells). iPSC-CMC = induced pluripotent stem cells-derived cardiomyocytes, MSC = Mesenchymal stem cells, Fibro = Fibroblasts, EPC = Endothelial progenitor cells, GEAEC = Gastroepiploic artery endothelial cells.

3.4. The number of CiMS cells in blood changed in response to diverse clinical situations

We studied whether the mobilization of CiMS cells from the endocardium to the circulating blood might be affected by clinical situations. When compared to healthy volunteers, the patient group after transplantation under immunosuppressive therapy showed a high proportion of CiMS cells in blood, suggesting the inhibitory effect of T cells on the proliferation of CiMS cells as shown in the culture experiments (Supplemental Figs. 6A–6D) (mean ± SEM, 0.40 ± 0.07% vs. 1.21 ± 0.20%, *P* value = 0.002) (Fig. 3B). In patients with arrhythmias such as paroxysmal supraventricular tachycardia, the level of CiMS cells increased after a catheter ablation therapy which may induce injury in the endocardium (*P* value = 0.031, Wilcoxon signed rank test) (Fig. 3C). In patients with acute myocardial infarction, there was a significant and transient increase in CiMS cells, which could have been derived from the endocardium in the infarcted area (Fig. 3D).

3.5. CiMS cells showed the characteristics of multipotent stem cells and regenerative potential in myocardial infarction and hindlimb ischemia models

To investigate the differentiation potential of CiMS cells, we induced CiMS cells to differentiate into three germ layers. These cells differentiated into mesodermal lineage cells such as adipocyte-like, osteoblast-like, and myoblast-like cells (Fig. 4A, Supplemental Fig. 7A). These cells also differentiated into ectodermal lineage cells such as nestin-positive neural cells and endodermal lineage cells such as hepatocyte-like cells (Fig. 4A, Supplemental Figs. 7A and 7B). These data suggest that CiMS cells are multipotent.

In order to demonstrate regenerative potential *in vivo*, we induced myocardial infarction or the hindlimb ischemia in nude mice. The group injected with CiMS cells showed significant improvement in the systolic function of their hearts (mean ± SEM, 19.35 ± 1.71% vs. 30.92 ± 1.16%, *P* value < 0.001; n = 10 per group) or in the restoration of blood flow to the ischemic limb, when compared to the control group (on day 21, *P* value < 0.001; n = 10 per group) (Fig. 4B–D, Supplemental Figs. 8A–8D). We found that these cells differentiated into cardiomyocytes and vessels in the infarcted heart *in vivo* and *in vitro* (Fig. 4E–H, Supplemental Figs. 9 and 10).

3.6. CiMS cells were obtainable from diverse blood sources and patients

We succeeded in culturing CiMS cells from umbilical cord blood and from patients aged up to 90. We also cultured CiMS cells from patients with coronary artery disease, cardiomyopathy, liver or kidney disease,

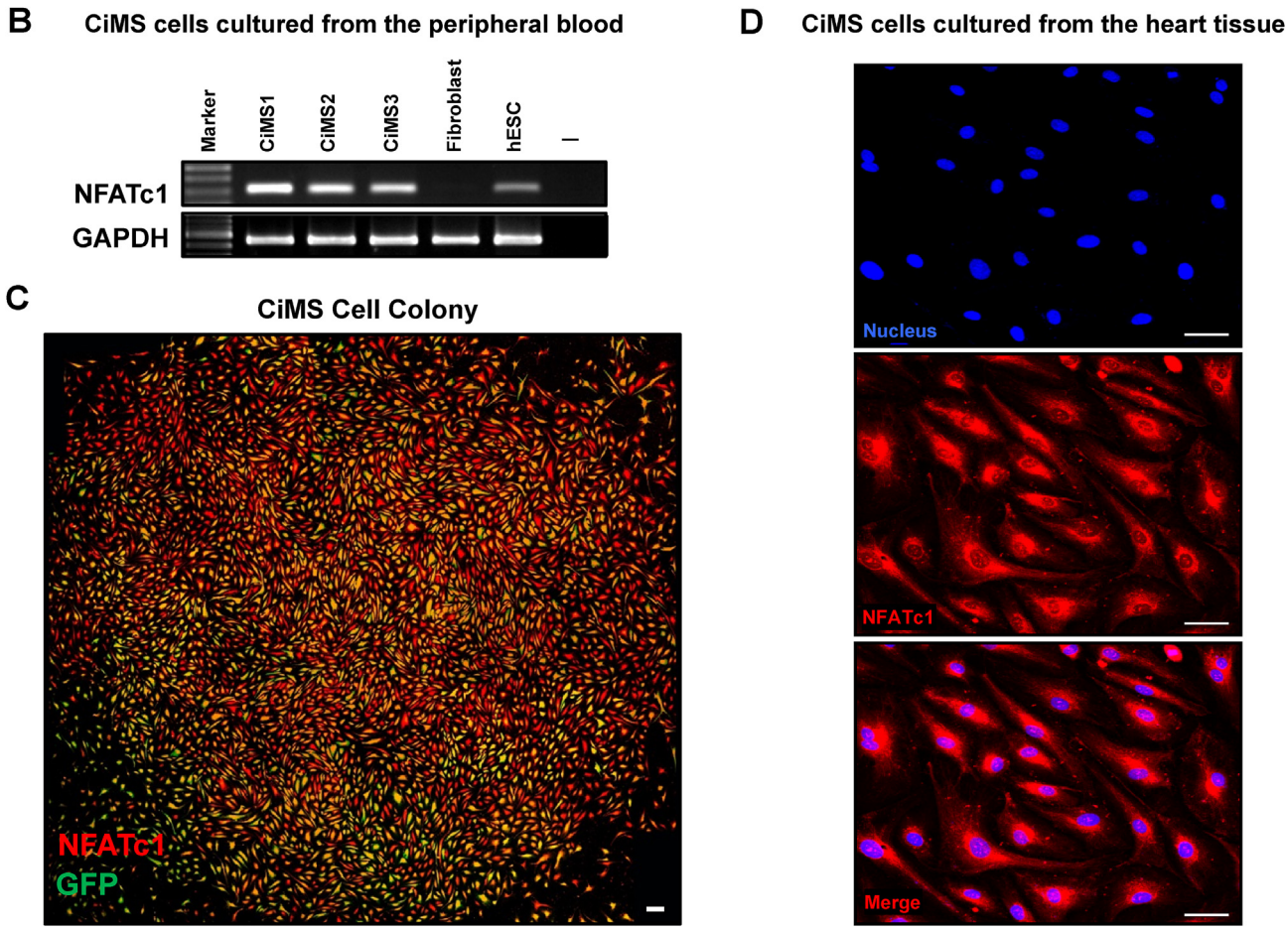

Fig. 2. (*continued*)

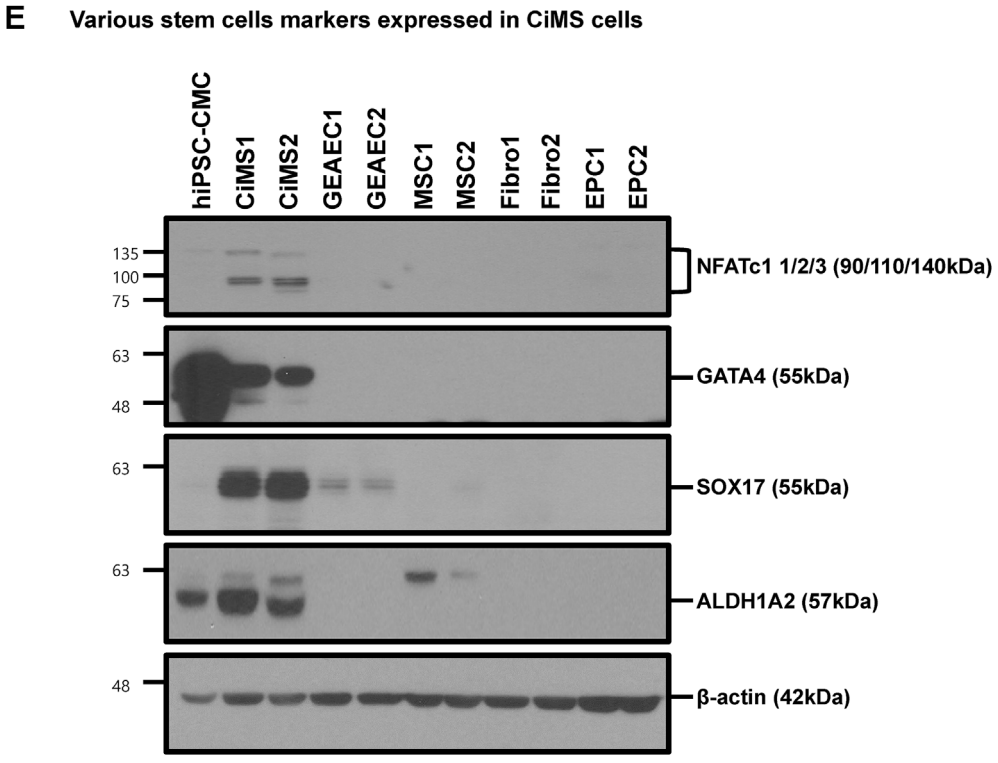

Fig. 2. (*continued*)

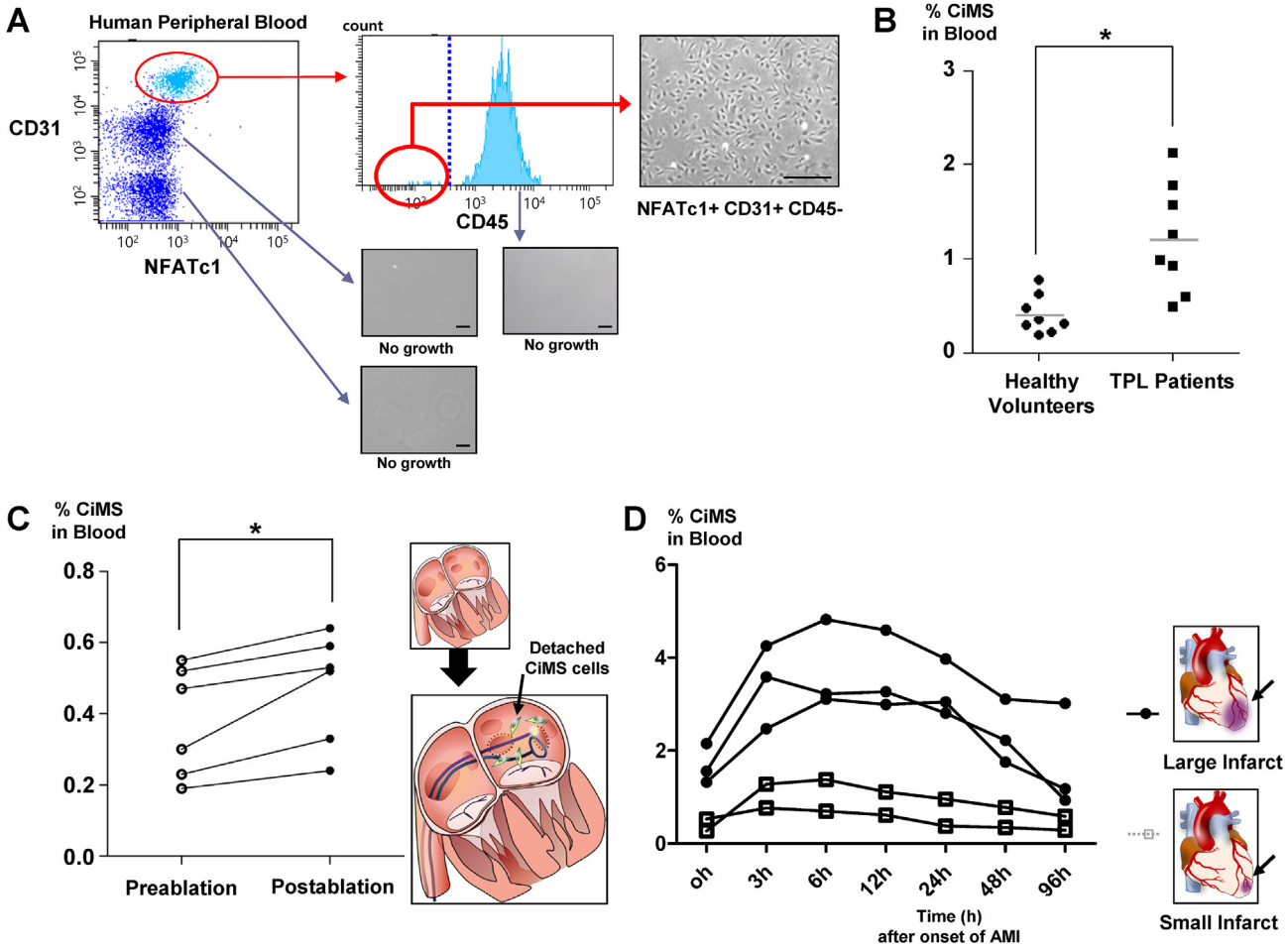

Fig. 3. Proportion of CiMS cells in the peripheral blood and their clinical relevance. (A) CiMS cells could be cultured only from the CD31 + NFATc1 + CD45$^-$ fraction of peripheral blood. Scale bar is 100 μm. (B) Compared to the healthy volunteer group, the patients who underwent transplantation and received immunosuppressive therapy had a high level of CiMS cells in their peripheral blood. TPL = transplantation. % CiMS in Blood indicates the percent of CiMS cells among the total peripheral blood mononuclear cells. (C) The number of CiMS cells increased in peripheral blood after treatment of arrhythmias with catheter ablation. (D) In patients with myocardial infarction, the level of CiMS cells increased significantly at the beginning of the infarction, and then decreased. We noted a difference in this level depending on the size of the infarct (large infarct n = 3, small infarct n = 2).

or hematologic disease. In terms of culture technique, we are able to culture CiMS cells with only 10 mL of peripheral blood, and the success rate of isolation was more than 97%. These findings suggest that we can easily obtain CiMS cells, even in an outpatient department.

4. Discussion

Our extensive research showed that CiMS cells reside in the human endocardium as tissue resident stem cells. Due to their location adjacent to the blood stream, these cells can easily enter the blood after shedding from the endocardium. Therefore, CiMS cells can be easily isolated and cultured from human peripheral blood. In particular, these cells have remarkable regeneration abilities *in vivo* and increase in the blood in response to several clinical situations such as endocardial injury or stimulation. This fact suggests that CiMS cells would be efficient mediators to repair the distant organs under injury. There have been many studies that have shown the substantial benefits of stem cells in regenerating myocardium. However, the methods used in the previous clinical trials were invasive, involving procedures such as bone marrow aspiration, surgical acquisition, and endomyocardial biopsy. On the other hand, our method to isolate stem cells is much less invasive as it involves taking only 10 mL of blood. Compared to other adult stem cells, these cells are multipotent. At this stage, we can draw blood from patients in an outpatient department and stock these cells for potential use in the future as we do with umbilical cord blood from neonates (Fig. 5).

4.1. CiMS cells can be isolated from human peripheral blood

In order to isolate circulating novel stem cells from peripheral blood, we used a culture method which was different from the methods previously used to isolate circulating progenitor cells. The media was frequently changed in non-coated dishes. This method reduced non-adherent cells such as T cells (Supplemental Fig. 6D). Isolation of CiMS cells was easier in the patients of organ transplantation under immunosuppressive therapy and enhanced in CD3-negative fraction or in the absence of supernatant from CD3-positive cells (Fig. 3B, Supplemental Figs. 6A–6C). Based on these observations, we assumed that certain immune cells, such as T cells, could inhibit the proliferation and differentiation of CiMS cells in peripheral blood.

4.2. CiMS cells are derived from the endocardium of the human heart and express NFAT

As described, we collected peripheral blood from patients undergoing heart transplantation. To our surprise, the STR profiles of these cells before transplantation had changed to a completely different profile after the transplantation. We cultured CiMS cells from the

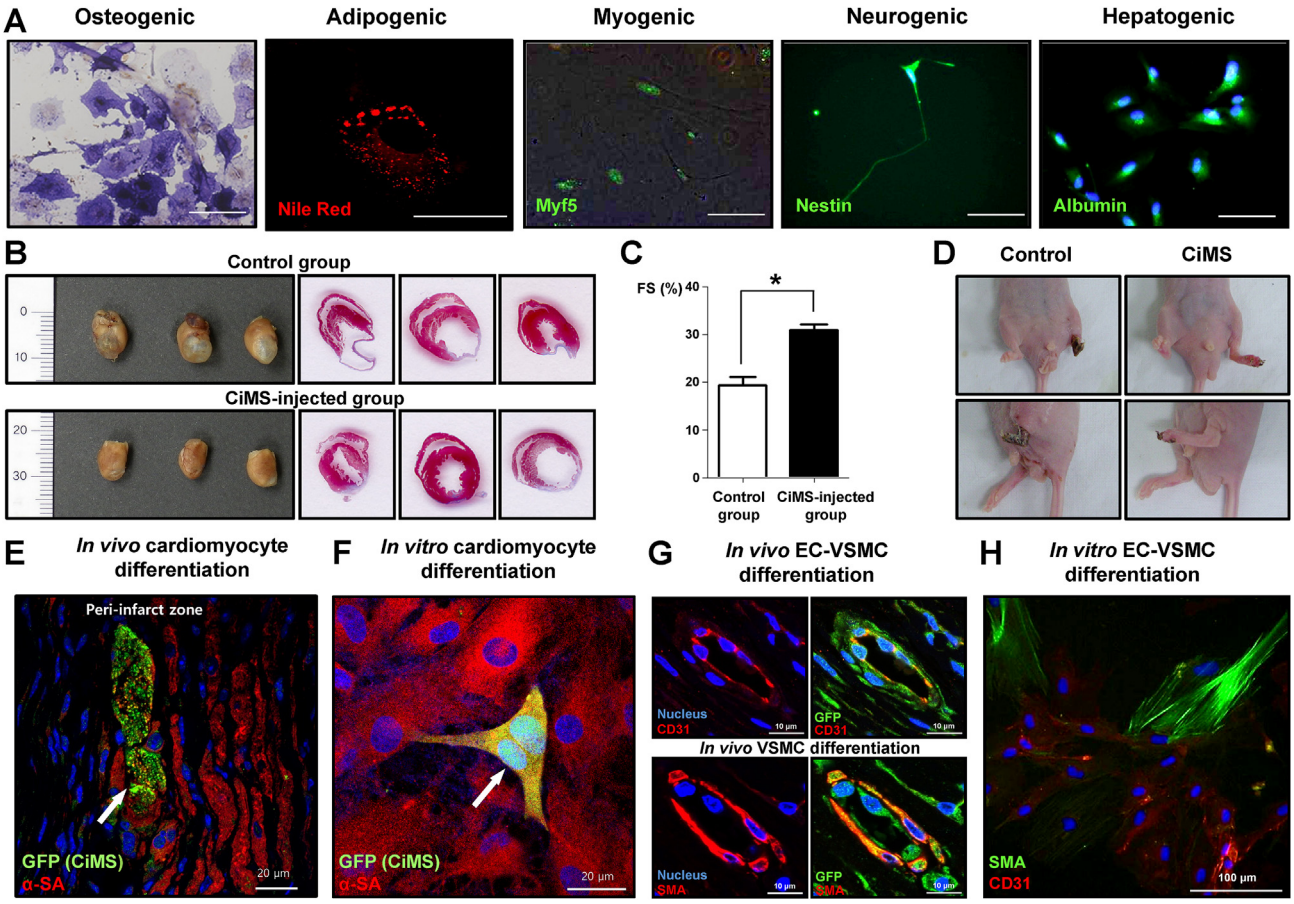

Fig. 4. Multipotency of CiMS cells *in vitro* and *in vivo*. (A) *In vitro* differentiation studies showed that CiMS cells had the ability to differentiate not only into mesodermal lineages, but also into ectodermal or endodermal lineages, suggesting the multipotency of CiMS cells. Scale bar is 100 μm. (B) When injected into a mouse with an infarcted heart *in vivo*, CiMS cells significantly reduced the infarct scar, compared with the media-injected control group (media: phosphate buffered saline, PBS). MT staining = Masson's Trichrome staining. Blue color indicates the area of fibrosis. (C) Compared to the media-injected control group, the CiMS-injected group showed significant improvement in heart function after 2 weeks of myocardial infarction. FS = fractional shortening. The FS of sham-operated group was 51.83 ± 1.92%. (D) In the hindlimb ischemia model, the CiMS cells salvaged the ischemic limb from auto-amputation. (E) The injected CiMS cells differentiated into cardiomyocytes in the infarcted heart *in vivo*. The green color indicates the injected cells that were tagged with GFP lentivirus. a-SA = alpha-sarcomeric actin. (F) The CiMS cells differentiated into cardiomyocytes *in vitro* when cocultured with rat neonatal cardiomyocytes. (G) The injected CiMS cells differentiated into vessels in the infarcted heart *in vivo*. The green color indicates the injected cells that were tagged with GFP lentivirus. EC = endothelial cells, VSMC = vascular smooth muscle cells, SMA = smooth muscle actin. (H) The CiMS cells differentiated into endothelial cells and vascular smooth muscle cells under the *in vitro* culture with EGM-MV2 media. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

endocardial biopsy and peripheral blood after transplantation. The STR profiles of the tissue-derived cells were identical to those of the CiMS cells cultured from blood, but were different from the profiles of recipients' fresh blood that indicated the authentic recipient profiles (Supplemental Fig. 4A, Supplemental Tables 2 and 3, n = 5).

In terms of stem cell markers, c-kit and Wilms tumor 1 (WT-1) are the representative markers for cardiac stem cells and epicardial progenitor cells respectively [1,2]. Since the part of the heart obtained by endomyocardial biopsy is the endocardium, we searched for CiMS-like cells in the endocardium. Studies with mouse embryos or neonates have shown that the endocardium expresses CD31 and NFAT [13–16]. In the developmental period, the endocardial tissue is known to express NFAT and CD31 [13], which we confirmed in mice (Supplemental Fig. 11). Therefore, we examined the human heart to find the cells that were double positive for NFAT and CD31. As expected, we found the double positive cells within the endocardium (Fig. 2A). Although there are controversies regarding the presence of NFAT-positive cells in the coronary vessels in the mouse embryo [17,18], we could not find NFAT-positive cells in the human heart other than the endocardium (Supplemental Figs. 12A–12G). In addition, we found that CiMS cells were produced only from the NFAT and CD31 double-positive fraction of peripheral blood (Fig. 3A). Due to their location, CiMS cells could circulate in the blood, suggesting that it could participate in the healing process of heart after injury as well as of distant organs such as liver, brain, and lower extremities. We are carrying out several experiments to prove this concept.

4.3. Newly developed FACS method using streptolysin O (SLO)-mediated transient permeabilization

With the previously reported cell sorting methods, it has been impossible to sort and culture without killing the cells when the targets are located in the cytosol or nucleus. Since NFATc1, one of the CiMS cell markers, is located in the cytosol, we developed new FACS method to sort and keep NFATc1-positive cells alive using the novel technique of SLO-mediated transient permeabilization (Supplemental Fig. 5).

4.4. Unique features that discriminate CiMS cells from the other types of stem cells

Although CiMS cells seem to share some characteristics with previously reported stem cells such as mesoangioblasts and EPCs, CiMS cells have many distinguishable attributes [19–23]. First, unlike mesoangioblasts, CiMS cells can grow in a non-coated dish, and they show

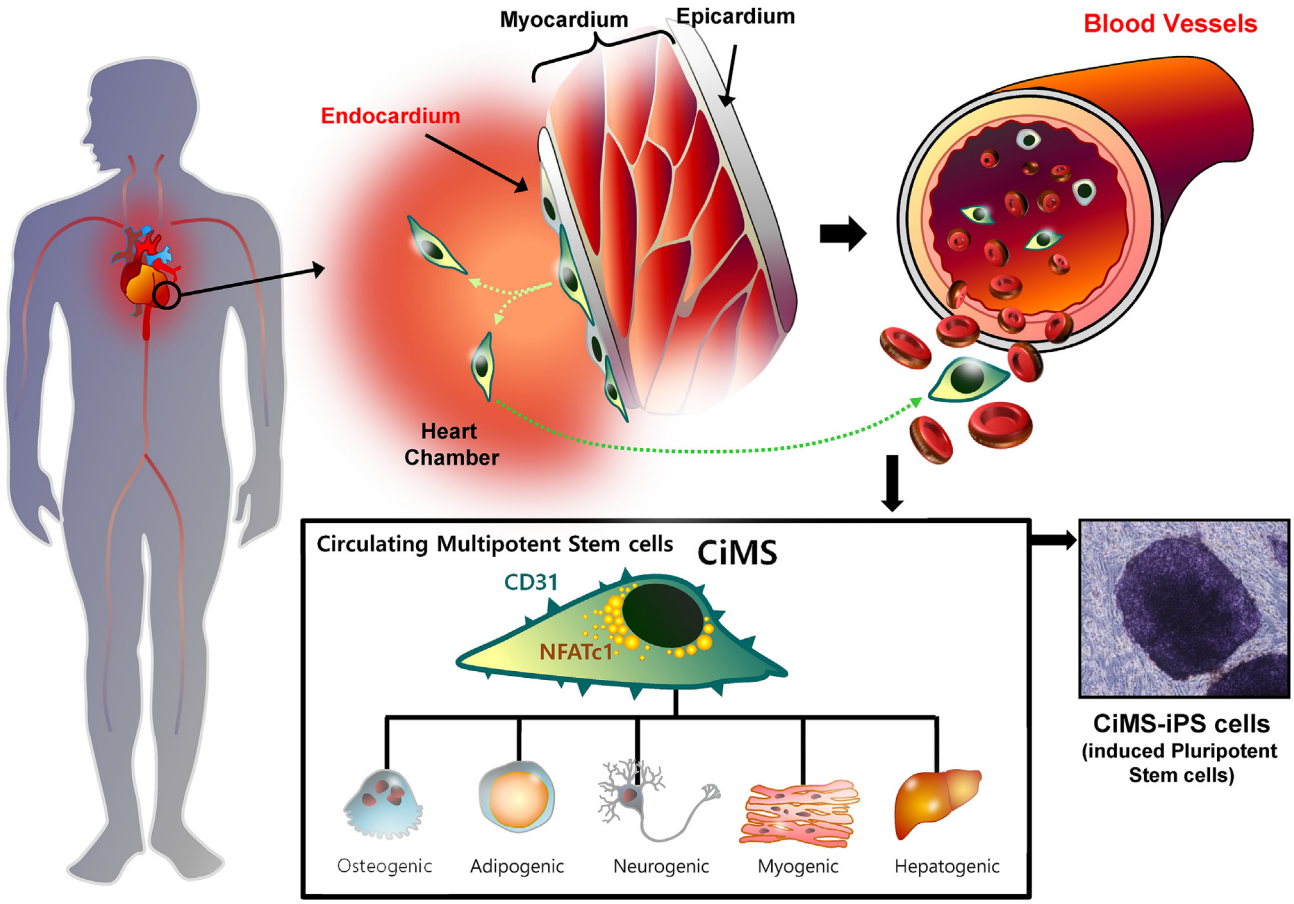

Fig. 5. Schematic figure of CiMS cells. CiMS cells originating from the human endocardium are double positive for NFATc1 and CD31, circulate in peripheral blood, and can differentiate into three germ layers, all of which are ideal characteristics for stem cells to repair the damaged heart as well as the injured distant organs. Due to their anatomical location in endocardium, CiMS cells are easily obtainable from peripheral blood. With CiMS cells, we can easily generate induced pluripotent stem cells, which could be used for cell therapy or stem cell research.

a different morphology. Although CiMS cells do not express CD117, they express MixL1, one of the markers of early mesoderm (Supplemental Figs. 13–15). CiMS cells are totally different from EPCs in terms of origin, morphology, and multi-potentiality. While the origin of EPCs is known to be bone marrow, we suggest that CiMS cells' origin is the human endocardium. CiMS cells have multipotency, but EPCs do not (Supplemental Fig. 15). Although MSCs share some features with our CiMS cells, there are many quite different characteristics in terms of the expression of NFATc1, GATA4, and SOX17 (Fig. 2E) [24]. There have been some studies showing the existence of the progenitor cells called myocardial telocytes in the human endocardial niche. CiMS cells share some characteristics such as the location and the nestin expression [25,26].

4.5. Clinical relevance of CiMS cells

We observed an increase in the level of CiMS cells in patients who had received catheter ablation therapy for arrhythmias. Currently, comprehensive ablation in patients with atrial fibrillation is performed all over the world. We assume that extensive injury in the endocardium can occur, similar to the endothelial injury after angioplasty. Therefore, CiMS cells could possibly participate in the healing process of the extensively injured endocardium.

In the case of patients with myocardial infarction, we also observed a transient increase in these cells in the blood, which could have been shed from the infarcted myocardium. If more data could show the correlation between the infarct size and the increased number of cells, we could get the information regarding the size of the infarcted endocardium by measuring the proportion of CiMS cells in blood. However, the inability of maintaining their level for less than 4 days could explain the limitations of endogenous stem cells to regenerate the damaged heart. Thus, we need additional strategy to augment the potential of stem cells in order to overcome this endogenous limited capability in the regeneration of the damaged heart. Despite this limitation, autologous stem cell-based therapy using CiMS cell could be promising because they could reduce immune rejection, risk of tumorigenesis, and potential arrythmia induced by the long-term survival of the implanted cells.

4.6. Perspective on the use of CiMS cells for stem cell therapy and the generation of induced pluripotent stem cells

Up to this point, various types of adult stem cells have been used as the modality for stem cell therapies of numerous diseases. However, in order to collect these cells from bone marrow, adipose tissue, and muscles, we have had to rely on invasive methods. While we can obtain EPCs from peripheral blood, their capacity to repair tissue is limited [27,28]. Therefore, EPCs from peripheral blood have not been broadly applied in real world practice. CiMS cells can be attained from just 10 mL of peripheral blood. Moreover, as they are multipotent and have the capacity to proliferate rapidly, we can use CiMS cells even for adult stem cell therapy.

Even after a series of successes in the generation of induced pluripotent stem (iPS) cells from various sources of adult somatic cells, several issues have emerged including practical inconvenience of invasive methods to obtain somatic cells or safety concern of genetic

manipulation. To overcome these limitations, some groups demonstrated the generation of iPS cells with T cells in the peripheral blood [29], and we also reported the protein-based generation of iPS cells without genetic manipulation [30]. However, these methods also had some limitations in terms of the cost and complexity of the process. Compared to the previously suggested methods, CiMS-based iPS generation is relatively simple and practical. We have succeeded in the generation of iPS cells from the peripheral blood of patients with various diseases via the culture of CiMS cells (data not shown, Supplemental Fig. 16). Furthermore, since CiMS cells already express some pluripotent genes such as Oct3/4, the generation of iPS cells from CiMS cells seems to be faster than that from other cell types such as fibroblast (Supplemental Fig. 17).

In conclusion, this study demonstrated the presence of circulating multipotent stem cells in human peripheral blood. These cells are derived from the human endocardium, increase in the blood in diverse clinical situations, and have an excellent reparative capability. Furthermore, CiMS cells can be easily obtained from human peripheral blood. These findings suggest their potential for use in cell therapies.

Author's contribution

Y.H., K.J., and C.H. developed the study concept and participated in the experimental work, data analysis, and preparation of the manuscript. L.J. and J.S. performed the experiments and data analysis. H.J., K.Y., S.M., C.E., L.H., Y.J., O.S., S.K., Y.S., and K.K. collected data and participated in data analysis and discussion. O.B. and P.Y. discussed and refined the manuscript. K.H. supervised the project and took an active role in all throughout the processes.

Declaration of competing interest

The authors have declared that no conflict of interest exists.

Acknowledgements

We thank Jaewon Lee for conducting our animal experiments. This study was supported by grants of the Korea Health Technology R&D Project “Korea Research-Driven Hospital (HI14C1277)” and “Strategic Center of Cell & Bio Therapy (HI17C2085)” through the Korea Health Industry Development Institute (KHIDI), funded by the Korea Government (MHW).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biomaterials.2019.119674.

References

[1] A.P. Beltrami, L. Barlucchi, D. Torella, M. Baker, F. Limana, S. Chimenti, H. Kasahara, M. Rota, E. Musso, K. Urbanek, A. Leri, J. Kajstura, B. Nadal-Ginard, P. Anversa, Adult cardiac stem cells are multipotent and support myocardial regeneration, Cell 114 (6) (2003) 763–776.

[2] B. Zhou, Q. Ma, S. Rajagopal, S.M. Wu, I. Domian, J. Rivera-Feliciano, D. Jiang, A. von Gise, S. Ikeda, K.R. Chien, W.T. Pu, Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart, Nature 454 (7200) (2008) 109–113.

[3] C.B. Iancu, D. Iancu, I. Rentea, S. Hostiuc, D. Dermengiu, M.C. Rusu, Molecular signatures of cardiac stem cells, Rom. J. Morphol. Embryol. 56 (4) (2015) 1255–1262.

[4] R. Bolli, A.R. Chugh, D. D'Amario, J.H. Loughran, M.F. Stoddard, S. Ikram, G.M. Beache, S.G. Wagner, A. Leri, T. Hosoda, F. Sanada, J.B. Elmore, P. Goichberg, D. Cappetta, N.K. Solankhi, I. Fahsah, D.G. Rokosh, M.S. Slaughter, J. Kajstura, P. Anversa, Cardiac stem cells in patients with ischaemic cardiomyopathy (SCIPIO): initial results of a randomised phase 1 trial, Lancet 378 (9806) (2011) 1847–1857.

[5] R.R. Makkar, R.R. Smith, K. Cheng, K. Malliaras, L.E. Thomson, D. Berman, L.S. Czer, L. Marban, A. Mendizabal, P.V. Johnston, S.D. Russell, K.H. Schuleri, A.C. Lardo, G. Gerstenblith, E. Marban, Intracoronary cardiosphere-derived cells for heart regeneration after myocardial infarction (CADUCEUS): a prospective, randomised phase 1 trial, Lancet 379 (9819) (2012) 895–904.

[6] T. Asahara, T. Murohara, A. Sullivan, M. Silver, R. van der Zee, T. Li, B. Witzenbichler, G. Schatteman, J.M. Isner, Isolation of putative progenitor endothelial cells for angiogenesis, Science 275 (5302) (1997) 964–967.

[7] Y. Lin, D.J. Weisdorf, A. Solovey, R.P. Hebbel, Origins of circulating endothelial cells and endothelial outgrowth from blood, J Clin Invest 105 (1) (2000) 71–77.

[8] D. Simper, P.G. Stalboerger, C.J. Panetta, S. Wang, N.M. Caplice, Smooth muscle progenitor cells in human blood, Circulation 106 (10) (2002) 1199–1204.

[9] N.J. Zvaifler, L. Marinova-Mutafchieva, G. Adams, C.J. Edwards, J. Moss, J.A. Burger, R.N. Maini, Mesenchymal precursor cells in the blood of normal individuals, Arthritis Res. 2 (6) (2000) 477–488.

[10] S.A. Kuznetsov, M.H. Mankani, S. Gronthos, K. Satomura, P. Bianco, P.G. Robey, Circulating skeletal stem cells, J. Cell Biol. 153 (5) (2001) 1133–1140.

[11] J. Hur, C.H. Yoon, H.S. Kim, J.H. Choi, H.J. Kang, K.K. Hwang, B.H. Oh, M.M. Lee, Y.B. Park, Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis, Arterioscler. Thromb. Vasc. Biol. 24 (2) (2004) 288–293.

[12] K. Takahashi, S. Yamanaka, Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors, Cell 126 (4) (2006) 663–676.

[13] A.M. Misfeldt, S.C. Boyle, K.L. Tompkins, V.L. Bautch, P.A. Labosky, H.S. Baldwin, Endocardial cells are a distinct endothelial lineage derived from Flk1 + multipotent cardiovascular progenitors, Dev. Biol. 333 (1) (2009) 78–89.

[14] J.L. de la Pompa, L.A. Timmerman, H. Takimoto, H. Yoshida, A.J. Elia, E. Samper, J. Potter, A. Wakeham, L. Marengere, B.L. Langille, G.R. Crabtree, T.W. Mak, Role of the NF-ATc transcription factor in morphogenesis of cardiac valves and septum, Nature 392 (6672) (1998) 182–186.

[15] A.M. Ranger, M.J. Grusby, M.R. Hodge, E.M. Gravallese, F.C. de la Brousse, T. Hoey, C. Mickanin, H.S. Baldwin, L.H. Glimcher, The transcription factor NF-ATc is essential for cardiac valve formation, Nature 392 (6672) (1998) 186–190.

[16] A. Rinne, K. Banach, L.A. Blatter, Regulation of nuclear factor of activated T cells (NFAT) in vascular endothelial cells, J. Mol. Cell. Cardiol. 47 (3) (2009) 400–410.

[17] H. Zhang, W. Pu, G. Li, X. Huang, L. He, X. Tian, Q. Liu, L. Zhang, S.M. Wu, H.M. Sucov, B. Zhou, Endocardium minimally contributes to coronary Endothelium in the embryonic ventricular free walls, Circ. Res. 118 (12) (2016) 1880–1893.

[18] B. Wu, Z. Zhang, W. Lui, X. Chen, Y. Wang, A.A. Chamberlain, R.A. Moreno-Rodriguez, R.R. Markwald, B.P. O'Rourke, D.J. Sharp, D. Zheng, J. Lenz, H.S. Baldwin, C.P. Chang, B. Zhou, Endocardial cells form the coronary arteries by angiogenesis through myocardial-endocardial VEGF signaling, Cell 151 (5) (2012) 1083–1096.

[19] M. Sampaolesi, S. Blot, G. D'Antona, N. Granger, R. Tonlorenzi, A. Innocenzi, P. Mognol, J.L. Thibaud, B.G. Galvez, I. Barthelemy, L. Perani, S. Mantero, M. Guttinger, O. Pansarasa, C. Rinaldi, M.G. Cusella De Angelis, Y. Torrente, C. Bordignon, R. Bottinelli, G. Cossu, Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs, Nature 444 (7119) (2006) 574–579.

[20] B.G. Galvez, D. Covarello, R. Tolorenzi, S. Brunelli, A. Dellavalle, S. Crippa, S.A. Mohammed, L. Scialla, I. Cuccovillo, F. Molla, L. Staszewsky, F. Maisano, M. Sampaolesi, R. Latini, G. Cossu, Human cardiac mesoangioblasts isolated from hypertrophic cardiomyopathies are greatly reduced in proliferation and differentiation potency, Cardiovasc. Res. 83 (4) (2009) 707–716.

[21] M. Koyanagi, M. Iwasaki, S. Rupp, F.S. Tedesco, C.H. Yoon, J.N. Boeckel, J. Trauth, C. Schutz, K. Ohtani, R. Goetz, K. Iekushi, P. Bushoven, S. Momma, C. Mummery, R. Passier, R. Henschler, H. Akintuerk, D. Schranz, C. Urbich, B.G. Galvez, G. Cossu, A.M. Zeiher, S. Dimmeler, Sox2 transduction enhances cardiovascular repair capacity of blood-derived mesoangioblasts, Circ. Res. 106 (7) (2010) 1290–1302.

[22] M. Quattrocelli, G. Palazzolo, I. Perini, S. Crippa, M. Cassano, M. Sampaolesi, Mouse and human mesoangioblasts: isolation and characterization from adult skeletal muscles, Methods Mol. Biol. 798 (2012) 65–76.

[23] A. Le Bras, P. Vijayaraj, P. Oettgen, Molecular mechanisms of endothelial differentiation, Vasc. Med. 15 (4) (2010) 321–331.

[24] A.I. Caplan, New MSC: MSCs as pericytes are Sentinels and gatekeepers, J. Orthop. Res. 35 (6) (2017) 1151–1159.

[25] F. Grigoriu, S. Hostiuc, A.D. Vrapciu, M.C. Rusu, Subsets of telocytes: the progenitor cells in the human endocardial niche, Rom. J. Morphol. Embryol. 57 (2 Suppl) (2016) 767–774.

[26] M.C. Rusu, S. Hostiuc, A.D. Vrapciu, L. Mogoanta, V.S. Manoiu, F. Grigoriu, Subsets of telocytes: myocardial telocytes, Ann. Anat. 209 (2017) 37–44.

[27] H.J. Kang, H.S. Kim, S.Y. Zhang, K.W. Park, H.J. Cho, B.K. Koo, Y.J. Kim, D. Soo Lee, D.W. Sohn, K.S. Han, B.H. Oh, M.M. Lee, Y.B. Park, Effects of intracoronary infusion of peripheral blood stem-cells mobilised with granulocyte-colony stimulating factor on left ventricular systolic function and restenosis after coronary stenting in myocardial infarction: the MAGIC cell randomised clinical trial, Lancet 363 (9411) (2004) 751–756.

[28] H.J. Kang, H.Y. Lee, S.H. Na, S.A. Chang, K.W. Park, H.K. Kim, S.Y. Kim, H.J. Chang, W. Lee, W.J. Kang, B.K. Koo, Y.J. Kim, D.S. Lee, D.W. Sohn, K.S. Han, B.H. Oh, Y.B. Park, H.S. Kim, Differential effect of intracoronary infusion of mobilized peripheral blood stem cells by granulocyte colony-stimulating factor on left ventricular function and remodeling in patients with acute myocardial infarction versus old myocardial infarction: the MAGIC Cell-3-DES randomized, controlled trial, Circulation 114 (1 Suppl) (2006) I145–I151.

[29] T. Seki, S. Yuasa, M. Oda, T. Egashira, K. Yae, D. Kusumoto, H. Nakata, S. Tohyama, H. Hashimoto, M. Kodaira, Y. Okada, H. Seimiya, N. Fusaki, M. Hasegawa, K. Fukuda, Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells, Cell Stem Cell 7 (1) (2010) 11–14.

[30] H.J. Cho, C.S. Lee, Y.W. Kwon, J.S. Paek, S.H. Lee, J. Hur, E.J. Lee, T.Y. Roh, I.S. Chu, S.H. Leem, Y. Kim, H.J. Kang, Y.B. Park, H.S. Kim, Induction of pluripotent stem cells from adult somatic cells by protein-based reprogramming without genetic manipulation, Blood 116 (3) (2010) 386–395.