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Mark Andrade April 9, 2019

Generation and functional characterization of PSC-CX3CR1^{+} cells

by

Mark Andrade

Young-sup Yoon
Adviser

Biology

Young-sup Yoon

Adviser

Victor Corces

Committee Member

Alessandro Veneziani

Committee Member

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Mark Andrade

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Abstract

Generation and functional characterization of PSC-CX3CR1^{+} cells

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Cardiomyocytes (CM) in the adult mammalian heart have limited proliferative capabilities which result in the inability of the heart to regenerate. The field of stem/progenitor cell therapies had turned to cardiac progenitor cells as an essential entity to overcome the boundaries of mammalian heart regeneration. Modern biology has been able to identify many embryonic and postnatal stem/progenitor populations demonstrating cardiogenic properties. However, to date only embryonic cardiac progenitor cells remain non-controversial about their roles in cardiogenesis. Furthermore, studies revolving around these populations have many limitations and display shortcomings in clinical trials when compare to their preclinical results. Hence, it is evident that further functional characterization, including further identification of cardiovascular progenitor cells, for embryonic progenitor cells needs to be established before clinical trials results live up to their expectations. A recent study has revealed that macrophage precursors positive to CX3CR1 arise in the yolk sack and migrate to fetal organs during embryonic development.[1] Additionally, in our lab, a cell lineage study has demonstrated that these cx3cr1 cell lines contribute not only to organogenesis but also to the cardiovascular system. Hence, here we establish a system by which, CX3CR1^{+} cells are derived from mouse embryonic stem cells and can contribute to the cardiovascular system. *In vitro*, under cardiac promoting media, they demonstrated direct differentiation into both cardiomyocytes (CMs) and endothelial cells (ECs). *Ex vivo*, co-culturing these cells with

a fetal mouse heart demonstrated their ability to migrate toward the ex vivo fetal mouse heart and incorporate themselves into the CM population. In vivo, using a myocardial infarction (MI) mouse model, injection of these cells with biomaterial recapitulated both the *in vitro* and *ex vivo* results. This study adds insights to our understanding of multipotent cell lines and add to the advancement of stem cell therapy for heart regeneration.

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I. Introduction

Heart failure is among one of the most prominent causes of mortality in first world countries.[2] Generally, the loss of myocardial contractility and the limited regenerative ability of the adult mammalian heart truncate the capacity to recover from this detrimental phenomenon through regeneration.[3] However, since the beginning of the 21st century, we have been able to identify stem/progenitor populations capable of contributing to the cardiovascular system.[4]

To date, extensive investigations have been made to use stem/progenitor populations from postnatal organs for heart regeneration, but the results are controversial.[4] For example, genetic lineage tracing revealed that two postnatal populations known as c-kit^{+} and SCA1^{+} cells have no or minimal contribution to cardiomyocyte (CMs) generation *in vivo*.[5,6] Furthermore, clinical trials using c-kit+ cells for heart regeneration showed modest or minimal improvement which is disappointing compared to what the animal studies had shown. Additionally, studies such as Janssens *et al*., PROMETHEUS, and REGENERATE AMI, used different stem cell populations in clinical trials ranging from Bone marrow-derived cells to Mesenchymal stem cells, all of which resulted as inconclusive or no improvement in heart failure patients.[3] The culmination of these data led to the termination of the use of postnatal stem/progenitor populations for cell therapy treatment.

On the other hand, lineage tracing studies revealed several embryonic CPC populations such as Isl1^{+} and FLK-1^{+} cells that proved to be contributing to cardiogenesis during development. Ema *et al*., demonstrated a population of FLK-1^{+} (gene encoding for VEGF receptor) cells served as an intermediate cell population that could differentiate

into CMs and other vascular lineages during embryonic development.[7] Furthermore, Bu *et al.* demonstrated, that human fetal ISL1^{+} cells could self-expand and give rise to intermediate progenitors to generate different cardiac lineages *in vitro*.[8] Even though these developmental insights into embryonic CPC were made, they remain controversial of their role in the postnatal stage.[9]

To compensate for this limitation, studies turned to pluripotent stem cells (PSCs) to generate CPCs exogenously. Kattman *et al*. proved that FLK-1^{+} cells could be derived from embryonic stem cells and cultured with cardiac promoting media to produce colonies displaying CM, endothelial cells (ECs), and vascular smooth muscle cell properties.[10] Menasche *et al.* generated and isolated human ESC-derived CPCs by using Isl-1 and SSEA-1, a cardiac transcription factor and a stem cell marker, which were then implanted on a patch and resulted in minimal heart functionality increase in heart failure patients.[11] With concrete evidence that we could exogenously produce embryonic CPCs, cell therapy with these populations has become more prevalently usef throughout the years. However, their results are limited by the fact that their cell population was selected via a nonspecific stem cell marker instead of a cardiac progenitor cell marker, indicating that their cell population could have been a heterogeneous mixture of many cell types. Taking all of this into consideration, there exists a knowledge gap that needs to be filled regarding cell therapy using multipotent progenitor cells, with embryonic CPCs predominantly being used for heart failure therapy than stem/progenitor cells from postnatal organs. However, the idea of using PSCs for generation and therapeutic use of multipotent progenitor cells has not been extensively explored.

Our previous data demonstrated via lineage tracing, that an embryonic population of cells, known as CX3CR1^{+} cells, contribute to cardiovascular lineages during development but not adulthood. Thus, we make use of the surface marker CX3CR1 to obtain an enriched novel progenitor cell population. With this in consideration, this study aims to generate a population of CX3CR1^{+} cells from mouse ESCs and demonstrate their ability to differentiate into cardiovascular lineages *in vitro*, *ex vivo,* and *in vivo*. The findings of this study will expand and advance the field of multipotent progenitor cells and provide a new putative therapeutic progenitor cell population capable of regenerating the mammalian heart.

II. Results

CX3CR1^{+} cells were generated from mouse embryonic stem cells (mESCs)

By using mESCs, a new protocol was devised to differentiate them and use CX3CR1 as a sorting marker. The devised method consisted of two phases, a hematopoietic induction phase and a macrophage induction phase. By implementing this protocol, the expression kinetics of CX3CR1 was determined, via flowcytometry, to occur during the hematopoietic phase (Figure 1A). The expression of CX3CR1 was highest on day 4 and rapidly declined after (Figure 1B).

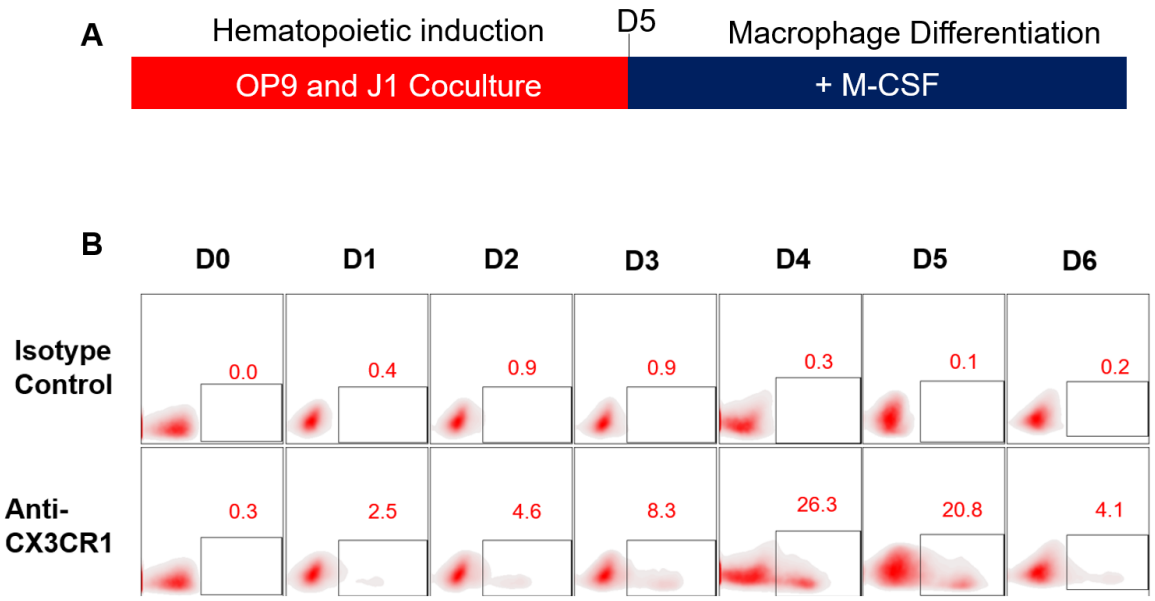
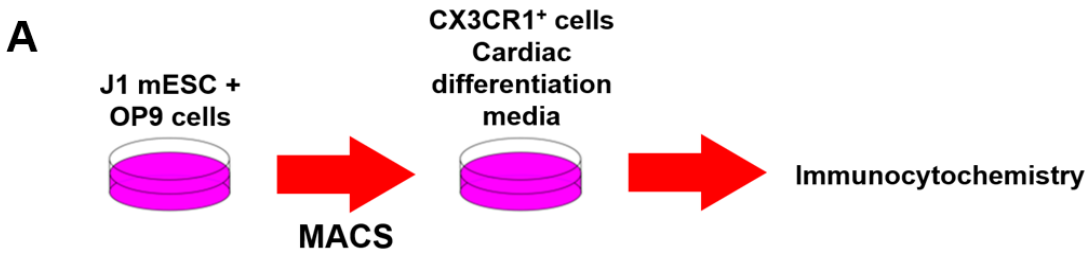


Figure 1. CX3CR1 expression kinetics on differentiating mESCs. A. Visual schematic of the culturing conditions used to differentiate J1 mESCs into hematopoietic (0-5 days) and macrophage lineages (5-8 days). B. At each day of OP9 and J1 coculture, cells were dissociated and subjected to flow cytometry to quantify the percentage of CX3CR1^{+} cells.

mESC-derived CX3CR1^{+} cells retain capacity to differentiate into cardiovascular cell lineages *in vitro*

After establishing the expression kinetics of CX3CR1 cells, their potential to give rise to CMs was tested. MESCs were subjected to hematopoietic induction and were sorted on day 4 using magnetic activated cell sorting (MACS). After sorting, they were further subjected to cardiac differentiation conditions (Figure 1A). After five days of culturing, immunocytochemistry on the mESC-derived CX3CR1^{+} cells demonstrated the expression of TNNT2 and MYH6, two CM markers (Figures 2B and 2C). In addition to this, EC markers, BSL1 and CDH5 also positively marked our derived cells (Figure 2). A

visual analysis of the morphology of the CM marker positive cells were renditions of polygonal shapes. Additionally, EC marker positive cells displayed tubular-like structures (Figures 2D and 2E). The immunostaining analysis suggests that the CX3CR1^{+} cells do indeed have cardiovascular differentiation capacity.



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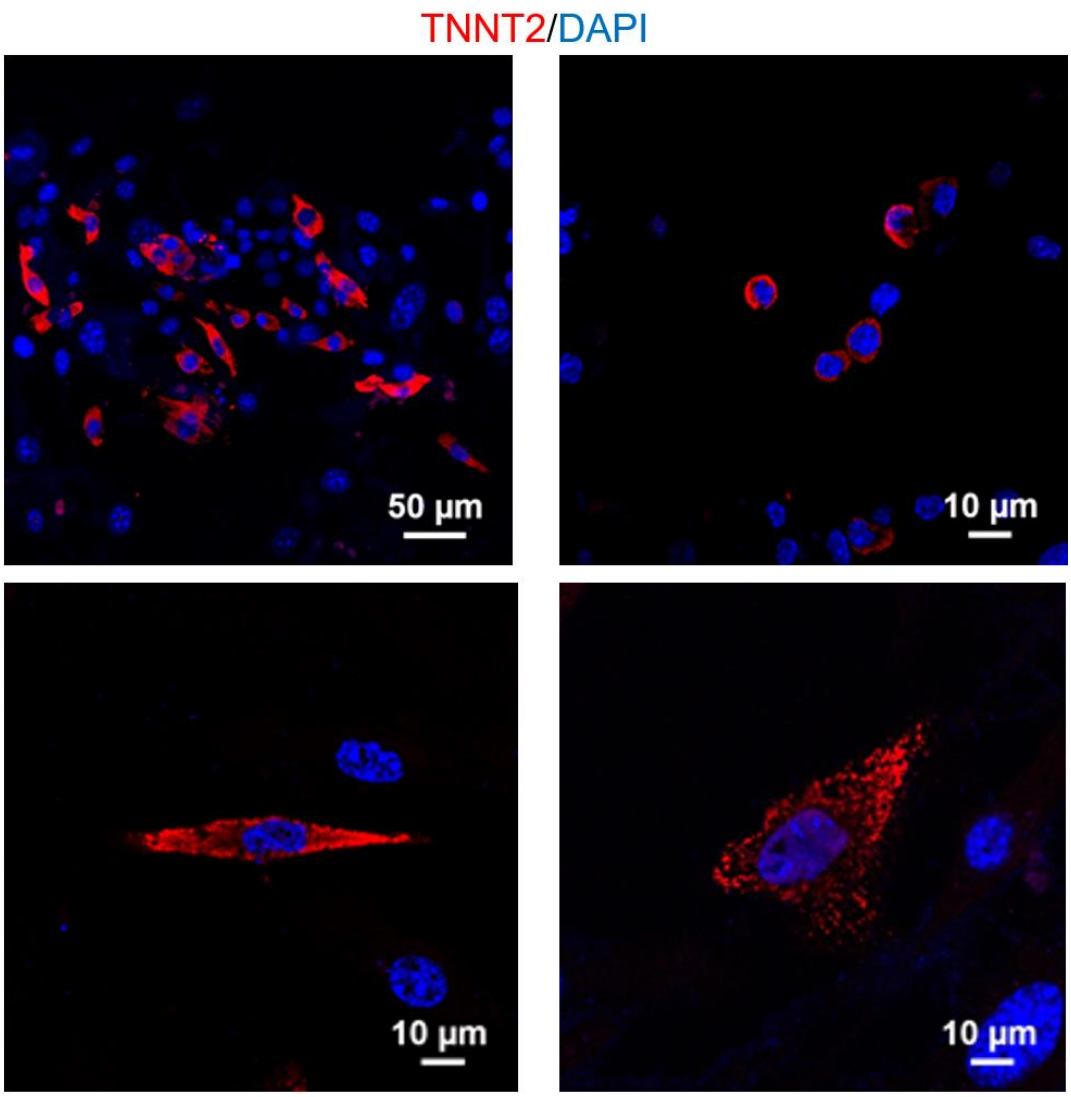


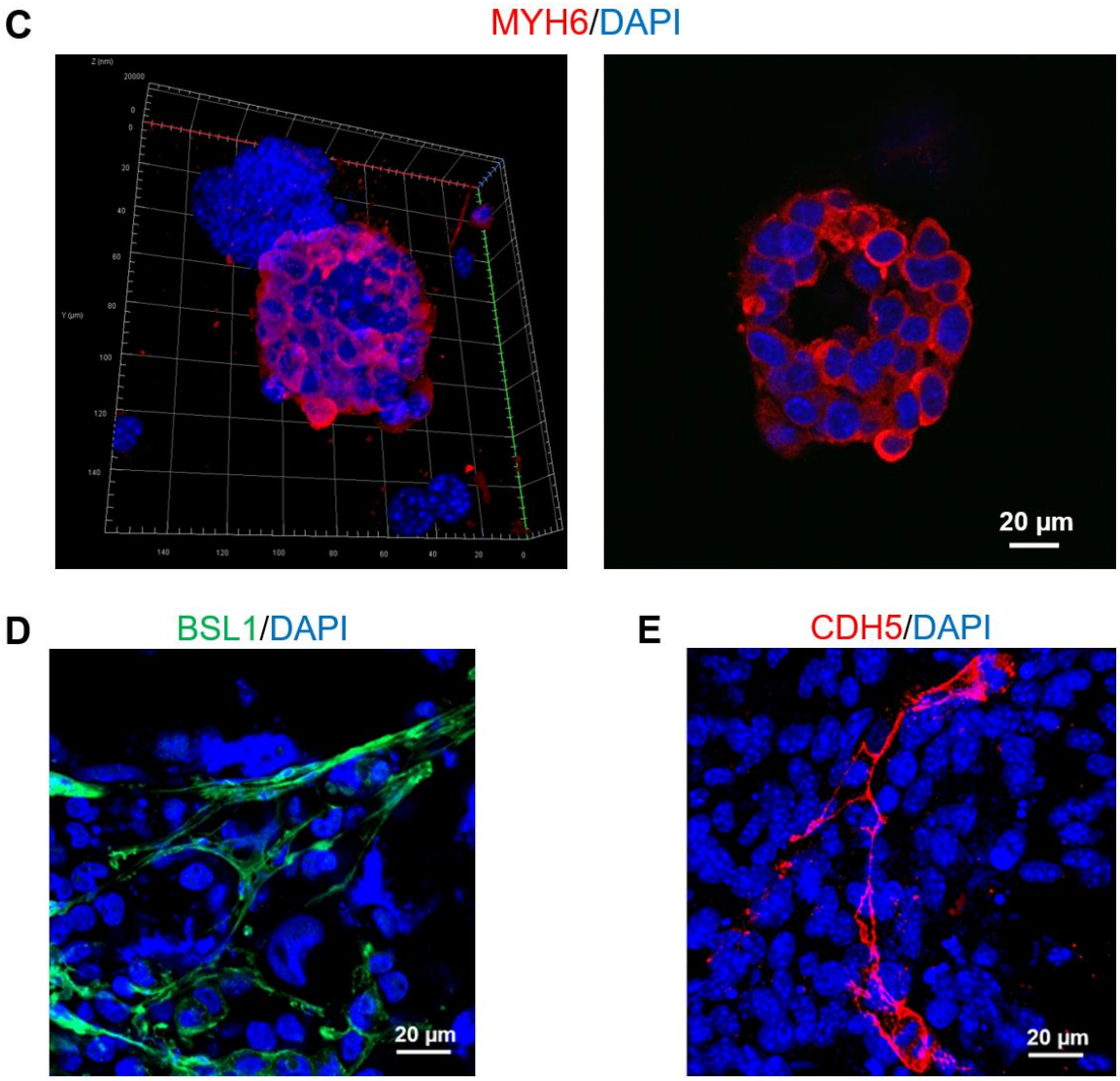
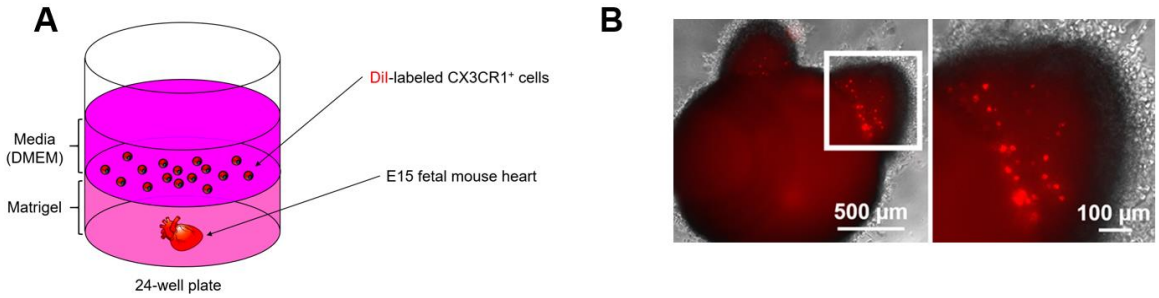


Figure 2. *In vitro* cardiovascular differentiation of mESC-derived CX3CR1^{+} cells. A. A schematic of the experiment for these Figures. mESCs were cocultured with OP9 cells and then were sorted using MACS on day 4. Cells were then subjected to cardiac conditions for further differentiation. All cells were harvested at day 15 and subjected to nuclear staining DAPI. Confocal images of mESC-derived CX3CR1^{+} cells stained with CM markers TNNT2 (B) and ACTN2 (C), and EC markers BSL1 (D) and CDH5 (E).

mESC-derived CX3CR1$^+$ cells contributed to the cardiovascular lineage populations present in the fetal mouse heart *ex vivo*

To further test the differentiation potential of mESC-derived CX3CR1$^+$ cells, they were cocultured with fetal mouse hearts *ex vivo* (Figure 3A). Using hematopoietic induction, CX3CR1$^+$ cells were sorted using Fluorescent activated cell sorting (FACS) to obtain a purer population of cells. These cells were the labeled with DiI for tracing. Within the first four days of the coculturing, DiI$^+$ cells were observed to be on the fetal mouse heart (Figure 3B). This suggested that the CX3CR1$^+$ cells demonstrated a cardiogenic affinity by migrating toward the fetal heart. After additional coculture, the fetal hearts were harvested and subjected to cryosection. Immunohistochemistry of the heart tissue demonstrated that our DiI$^+$ cells were also positive for CM markers ACTN2 and TNNT2 (Figure 3C). Additionally, immunostaining for ECs using PECAM1 was also positive in some of the DiI$^+$ cells (Figure 3D). These data suggest that the CX3CR1$^+$ cells were contributing to the existing CM and EC populations in the fetal mouse heart.



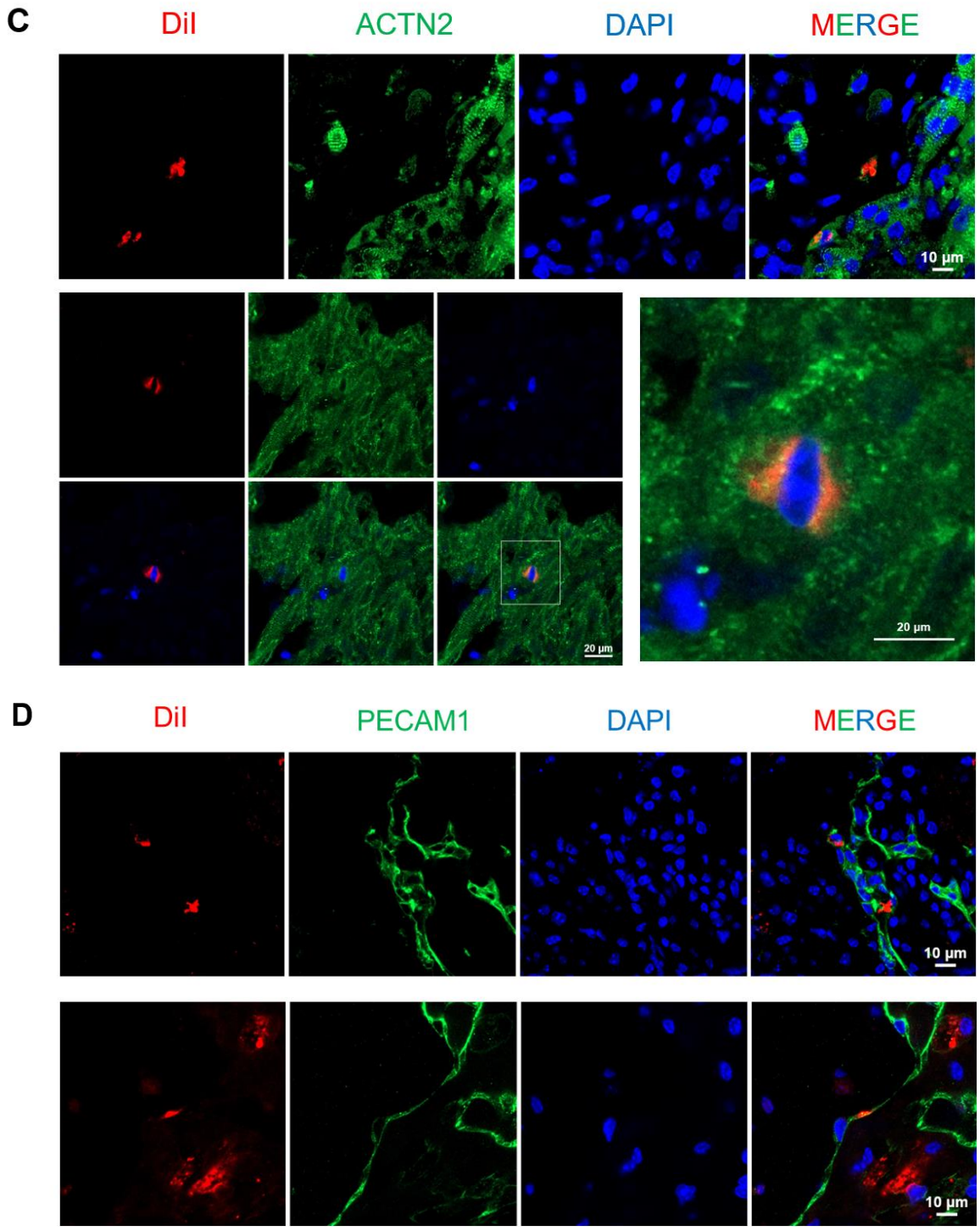


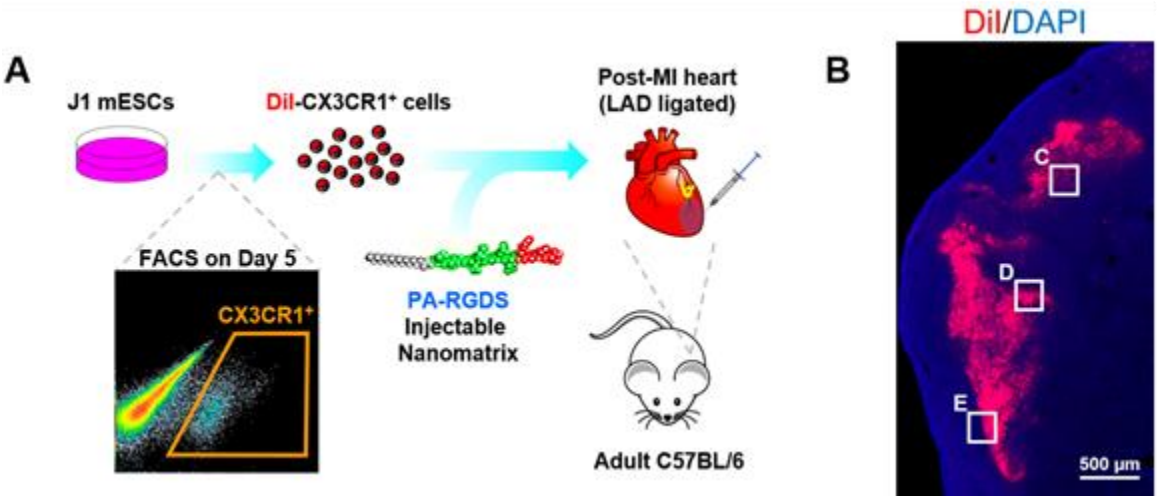
Figure 3. mESC-derived CX3CR1^{+} cells migration to the fetal heart and cardiovascular commitment *ex vivo*. A. A schematic processed by Kyuwon Cho for the experiments of this Figure. mESC-derived CX3CR1^{+} were sorted by FACS and were labeled with DiI.

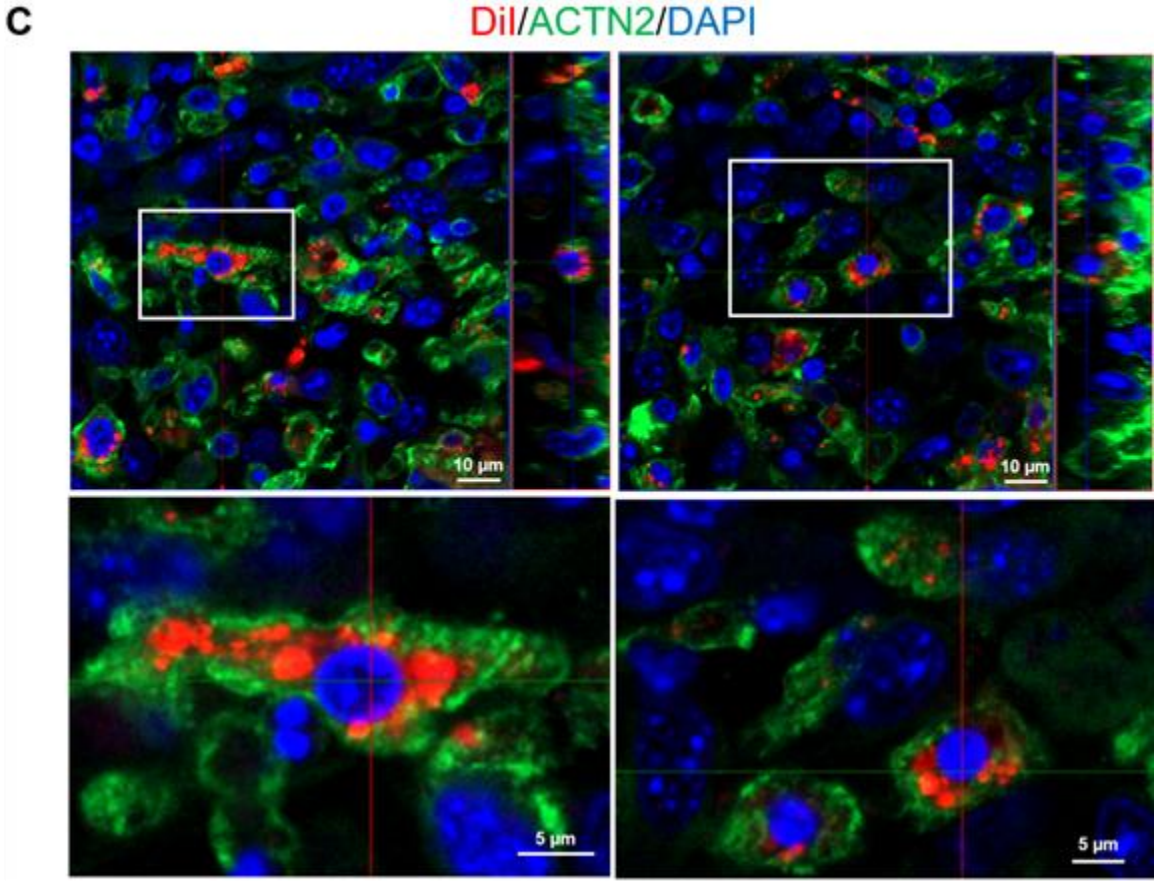
These cells were then transferred on top of a Matrigel layer containing a fetal mouse heart at E15.5. B. Epifluorescence microscopy demonstrate the incorporation of DiI$^+$ cells in the fetal mouse heart. C. Representative confocal microscopy images of the fetal mouse heart stained with CM marker ACTN2. Images were overlapped to visualize co-expression of DiI and ACTN2. D. Representative confocal microscopy images of the fetal mouse heart stained with EC marker PECAM1. Overlapping images visualize the co-expression of PECAM1 and DiI.

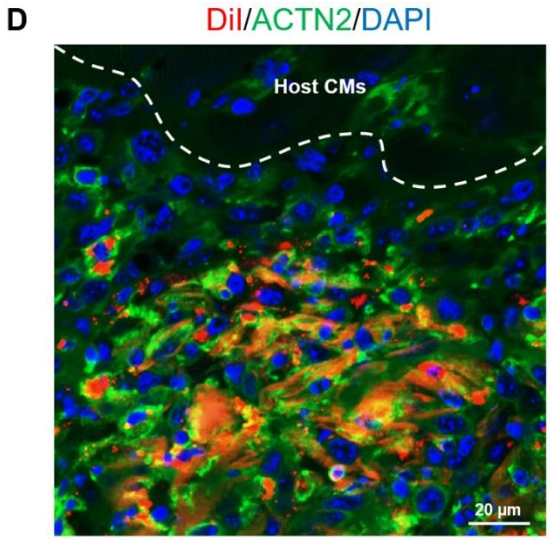
mESC-derived CX3CR1$^+$ cells contributed to the cardiovascular lineage populations in the post-MI adult mouse heart *in vivo*

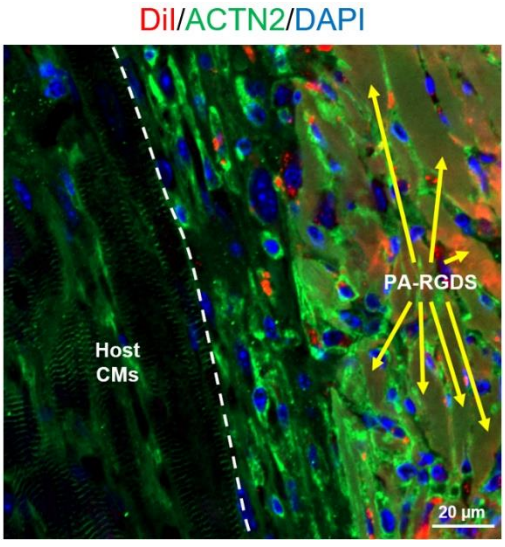
The CX3CR1$^+$ cell population was further tested for their differentiation and therapeutic potential by using them *in vivo.* J1 mESCs were differentiated and sorted using FACS on day 5. These cells were then stained with DiI for tracking. An adult mouse was subjected to MI and then to injections with a biodegradable nanomatrix, PA-RGDS, containing CX3CR1$^+$ cells directly into its heart. The biomaterial was used to enhance the retention capabilities of the injected CX3CR1$^+$ cells. After ten days, the mouse heart was harvested and subjected to cryosection. Confocal imaging of the heart tissue revealed that the DiI$^+$ cells were surviving in the heart even after ten days of administration (Figure 4B). Additionally, the cells demonstrated localization throughout the ventricles in different gradients indicating that they were migrating in the mouse heart *in vivo*. Immunohistochemistry of the tissue samples further revealed that the DiI$^+$ cells had committed to the CM population indicated by the double positive DiI and ACTN2 cells (Figure 4C). There was a clear distinction between the host tissue and the biomaterial

which allowed the imaging of the morphological difference between injected and non-injected areas of the mouse heart (Figure 4 D-E). Furthermore, some DiI^{+} cells were also positive for PECAM1 indicating that the CX3CR1^{+} cells were also contributing to the EC population (Figure 4F). Porous areas along the periphery of the ventricles were observed. Further magnification of these areas revealed tube-like structures that suggest the presence of vessels (Figure 4G). After even closer imaging of these areas, DiI^{+} cells demonstrated engraftment into some of these vessel-like structures which is indicated by the colocalized DiI and PECAM1 staining (Figure 4 H-I).



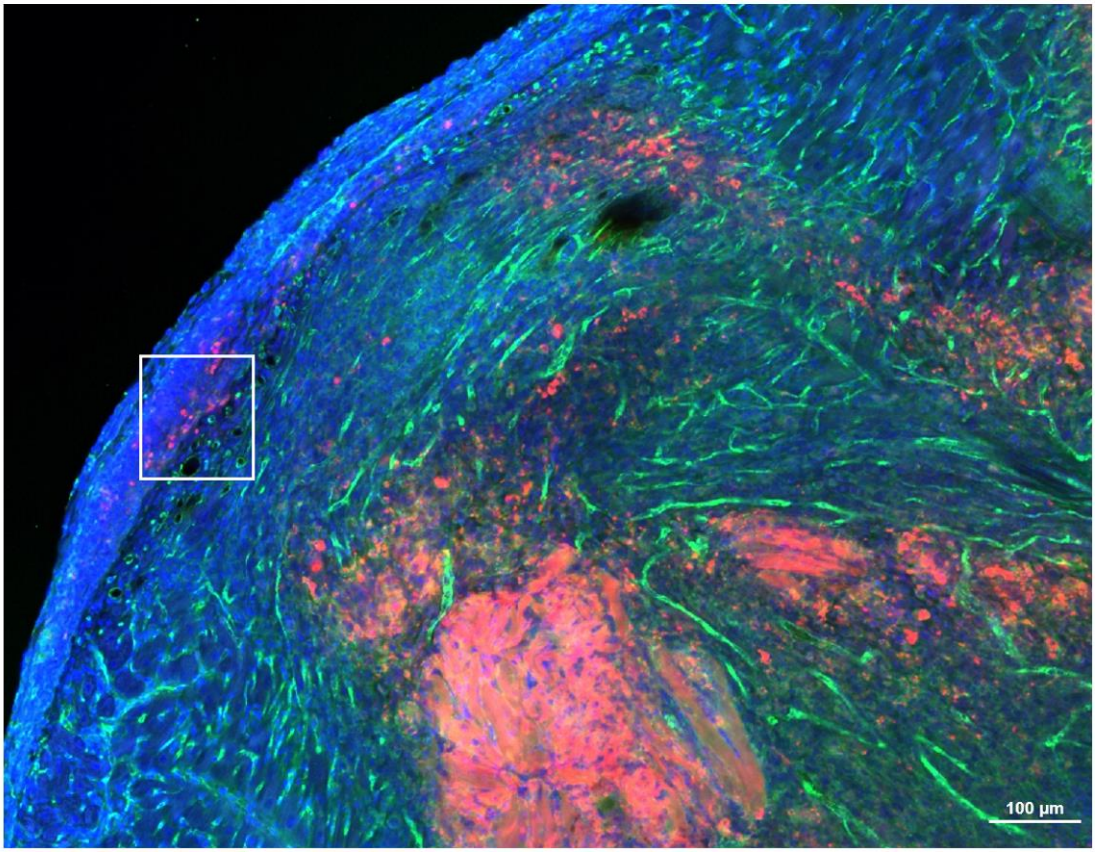

C
DiI/ACTN2/DAPI
10 μm
10 μm
5 μm
5 μm


D
DiI/ACTN2/DAPI
Host CMs
20 μm


E
DiI/ACTN2/DAPI
PA-RGDS
Host
CMs
20 μm

F

DiI/PECAM1/DAPI



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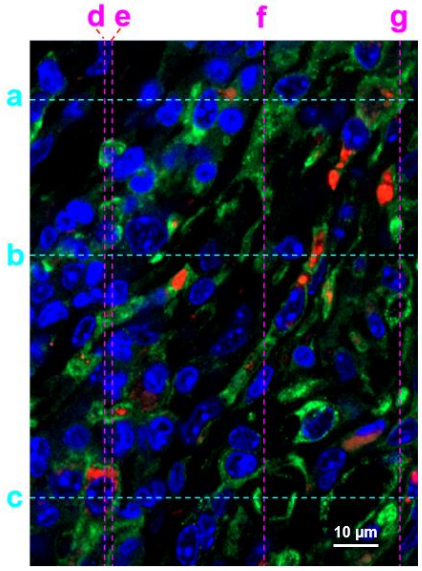
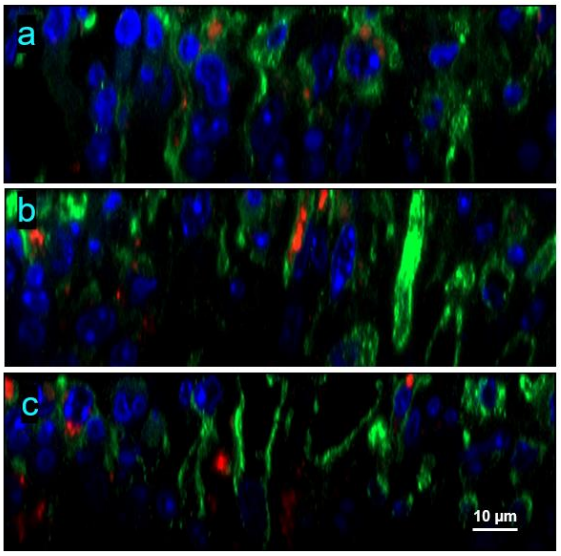


H

DiI/PECAM1/DAPI



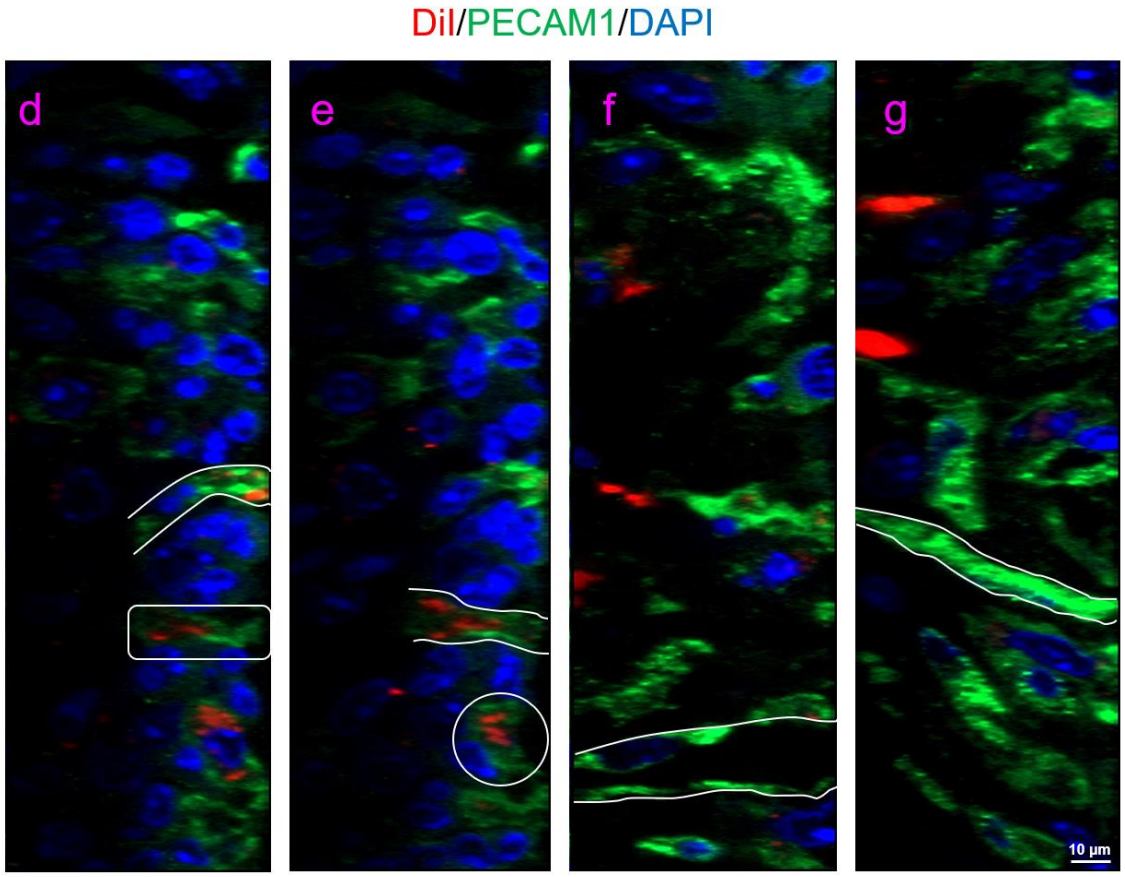


Figure 4. mESC-derived CX3CR1^{+} cells incorporated themselves into the adult mouse cardiovascular system *in vivo*. A. A schematic processed by Kyuwon Cho demonstrating the experimental approach for this Figure. J1 mESCs were cocultured with OP9 cells until day 5 for hematopoietic inductions. Cells were then sorted using FACS at day 5 using CX3CR1 as a selection marker. This cell population was labeled using DiI. DiI labeled cells were then injected with biomaterial PA-RGDS into the MI induced mouse heart. Heart was harvested on day 10 post injection and tissue was sectioned and imaged using confocal microscopy. DAPI was used as a nuclear stain. B. Representative image of whole mouse heart demonstrating the survival of DiI^{+} cells within the ventricles of the heart. C. Representative images of the mouse heart stained for CM marker ACTN2.

Colocalization of DiI and ACTN2 can be viewed with accompanying magnified images. C. Additional staining using an EC marker PECAM1 demonstrating circular morphology along the heart tissue. D. Images showing the morphology of the host myocardium in comparison to the myocardium that was injected with biomaterial and DiI^+ cells. F. Magnified image of the boxed area in (C) highlighting the PECAM1 staining in different sections. G-I. Magnified areas demonstrating the engraftment of DiI^+ into vessel-like structures.

III. Discussion

This study established a novel method by which $CX3CR1^+$ multipotent progenitors could be generated from mESCs. Additionally, we characterized the function of the mESC-derived $CX3CR1^+$ cells *in vitro* and *ex vivo* when placed in a cardiac microenvironment. An *in vivo* MI mouse model with microinjections of our cell population demonstrated that these cells could survive in the adult heart and recapitulate their potential to differentiate into cardiovascular lineages. Furthermore, our cells are unique in that they can be sorted solely using a surface protein. Thus, these data imply that PSC derived-$CX3CR1^+$ cells are a feasible multipotent cell population that can contribute to the cardiovascular lineage populations thus have a great prospect for cell therapy.

Based on the results in Figure 4, it can be seen that the number of $CX3CR1^+$ cells that are present in the mouse heart is great in numbers when compared to previous studies using mESC-derived CMs for cell therapy[12]. In 2014, Ban et al. published promising results demonstrating long-term engraftment of mESC-derived CMs using biomaterial in myocardium[12]. Hence, they could observe the beneficial effects of stem cell

derived therapies. Comparably, our study demonstrates an even stronger retention and engraftment effect. In the Ban et al study, 2 X 10^5 cells were used for injection and beneficial effects and retention was observed even after many weeks post injection of cells into the myocardium. Interestingly, confocal imaging of the myocardium reveals that at 20μm there are clusters of mESC derived CMs in the mammalian heart. In our study, 3 X 10^5 CX3CR1$^+$ cells were injected and observed to be in much larger bulks using a 100μm scaling bars (Figure 4). These comparable data suggest that CX3CR1$^+$ cells exhibit a greater survivability ability *in vivo* in the mouse MI model. However, it should be noted that although this can be visually assessed, the time points at which the hearts were harvested are greatly different. Therefore, as a future experiment, mouse MI hearts with CX3CR1$^+$ cells injected will be harvested at later time points for a more accurate comparison.

As the data suggest *ex vivo*, mESC-derived CX3CR1$^+$ cells demonstrated migratory patterns toward the fetal mouse heart. It is worthwhile noting, that *in vivo*, these cells recapitulated the phenomenon. This is to no surprise, in previous studies it has been reported that CPCs have displayed migration capacity *ex vivo* via PI3K and MAPK pathways[13]. In (Figure 4B) we can see that the Cx3CR1$^+$ cells have begun to sporadically accumulate around the ventricles of the heart seemingly away from injection sites. This is an indicator that our cells are exhibiting migration characteristics within the myocardium. However, the role, function, and reason are yet to be tested. One reasonable hypothesis would be that these cells could be migrating towards regions of the heart that are lacking in oxygen supply or have become necrotic. One could test this

using Trichrome staining to visualize whether these cells are localizing around fibrotic areas of the heart.

In addition to CM incorporation, areas around the heart that were densely packed with DiI^{+} cells demonstrated increased vasculature (Figure 4 F-I). This suggest that the CX3CR1^{+} cells have angiogenic potential by influencing its microenvironment to enhance EC to proliferation for further vascularization. Three mechanisms could explain these observations: 1) de novo ECs differentiated from CX3XR1^{+} cells, 2) Fusion or extension of preexisting vasculature with CX3CR1^{+} cells, and 3) paracrine signaling from CX3CR1^{+} cells inducing increased proliferation of pre-existing ECs. Therefore, coculturing ECs with labeled Cx3cr1 cells *in vitro* and measuring proliferation rates along with performing immunocytochemistry could further explore which mechanisms are in play. Furthermore, we can use a transgenic mouse model that has the cardiovascular system labeled with green fluorescent protein and inject them with DiI labeled CX3CR1^{+} cells. After a few weeks of treatment, mouse hearts could be subjected to confocal imaging and immunostaining and checked for colocalization of GFP and DiI. We can also digest the harvested hearts after treatment and sort the DiI cells using FACS and check for proangiogenic growth factors such as VEGFA, TGFβ, or PDGFA using qPCR. Thus, with the intrinsic ability for the CX3CR1^{+} cells to incorporate themselves into preexisting cardiovascular systems, it would be beneficial to further optimizing culturing conditions and determining the therapeutic effect on cardiac function of the adult mammalian heart in future research.

Despite the amount of promising results displayed by this study, limitations and questions remain unanswered. First, the MI mouse model died after 10 post treatment. In

order to draw more conclusive data, more MI mouse models would be needed with heart harvesting at different time points as seen in previous studies[12]. Additionally, it remains unclear what benefits these CX3CR1$^+$ cells have on the injured mammalian heart. Further, analysis on ventricular ejection fraction, echocardiogram, and trichrome staining would be essential to characterize the cells therapeutic potential. Conversely, it would be essential if any detrimental effects can be noted. For example, because the Cx3cr1 lineage demonstrates multipotency, it could be likely that the cells could contribute to the formation of fibrosis post heart injury. Thus, trichrome staining on different heat harvesting time points wit DiI labeled cells could provide insight into this possibility. If this is true, then RNA sequencing on this cell population would be essential for determining additional markers that can be used to actively sort *Cx3cr1* cells with desired properties such as angiogenic ones.

Another limitation to this study is the risk of the mESC-derived CX3CR1$^+$ cells exhibiting tumorgenicity after transplantation. As seen in Figure 4, the survival of these cells undeniable and their sporadic presence within the heart can seem daunting. Therefore, to limit or avoid the possibility of tumorgenicity additional sorting marker may be used to tightly and actively select a healthy population of CX3CR1$^+$ cells.

In summary, we demonstrated the cardiovascular differentiation capacity of mESC-derived CX3CR1$^+$ cells *in vitro*. Additionally, we devised a new method to generate these multipotent cells from mESCs using the surface protein CX3CR1. These CX3CR1$^+$ cells were able to integrate themselves into the existing cardiovascular lineage populations both *ex vivo* and *in vivo* thus highlighting their potential for cell therapy for heart regeneration.

IV. Methods

Mice

All protocols for animal experiments were approved by the Institutional Animal Care and Use Committees of Emory University. All the mice used in this study were purchased from the Jackson Laboratory.

Immunohistochemistry and Immunocytochemistry

Mouse tissues were fixed in 2% paraformaldehyde (PFA) at 4°C for a minimum of 12 hours and submerged in 30% sucrose solution at 4°C for a day. Tissues were then prepared with OCT compound, sectioned, and washed using phosphate-buffered saline (PBS). Permeabilization and blocking of tissue sections was performed using PBS with 0.5% Triton X-100 and 2.5% bovine serum albumin (BSA) for 1 hour at room temperature. Tissue samples were then incubated with anti-TNNT2, anti-ACTN2, and anit-PECAM1 at 4°C overnight. Tissue sections were then washed three times using PBS with 0.1% Tween 20 and then treated with appropriate secondary antibodies at room temperature for 2 hours. DAPI was used for nuclear staining, and visualization of samples was performed using a confocal laser scanning microscope.

For immunocytochemistry, cells were fixed in 4% PFA at room temperature for 10 minutes. Samples were permeabilized and blocked using PBS with 0.1% Triton X-100 and 2.5% BSA at room temperature for 1 hour. Cell slides were incubated with anti-TNNT2, anti-MYH6, fluorescein-labeled BSL1, and anti-CDH5 at 4°C overnight. Imaging was performed in correspondence to the method mentioned above.

Mouse ESC culture and differentiation

J1 mouse ESCs were maintained as described by Ban et al.[14]. OP9 stromal cells were maintained in α-MEM media with 20% fetal bovine serum (FBS) on a 0.1% gelatin-coated culturing dish. Upon confluency of OP9 cells, treatment with 10 µg/ml of Mitomycin C for 2 hours was carried out. mESCs were pre-plated for 1 hour onto a culturing dish for separation from STO feeder cells. Non-adherent cells were collected and were dissociated into single cell suspension using Accutase. These single cell mESCs were then placed onto the OP9 cells at 1 X 10^5 cells per well in 6 well plates. Co-culture was carried out using α-MEM media with 10% FBS and changed every other day for hematopoietic induction. During macrophage induction, the mESCs were dissociated once again using Accutase and cultured in DMEM media with 10% FBS and 20 ng/mL recombinant murine GM-CSF.

For cardiac induction, cells were dissociated using Accutase into single cell suspensions. They were subjected to MACS using APC-conjugated anti-CX3CR1 and anti-APC magnetic beads on day 4 according to manufacturer instructions (Miltenyi Biotec). After sorting, cells were cultured in α-MEM media with 20% FBS, 50 ng/ml of BMP4, 5 ng/mL of VEGFA, 10 µM of IWR-1, 4 µM of CHIR99021, and 2 µM of BIO. Additional differentiation was carried out by culturing the cells in α-MEM with 3% FBS, Insulin-Transferrin-Selenium and 50 µg/ml L-Ascorbic acid.

Flow cytometry

Using accutase, mESCs were dissociated into single cells. They were then resuspended in ice-cold PBS and incubated with fluorescent labeled antibodies diluted in a 1:100 ratio. The following antibodies were used: PE-conjugated isotype control, APC-conjugated

isotype, CX3CR1. Excess reagents were washed off with ice-cold PBS, cells were then analyzed by BD LSRII Flow Cytometer. Data was processed using FLOWJO software.

***Ex vivo* coculture of the fetal mouse heart with mESC-derived CX3CR1$^+$ cells**

E15.5 mouse hearts from CD-1 IGS pregnant mice (Charles River, 022) were cultured *ex vivo* as described [15]. On day 4-5 CX3CR1$^+$ cells were sorted using FACS. Differentiating mESCs co-cultured with OP9 cells for 4-5 days were dissociated into single cells using Accutase. Resuspensded of cells in ice-cold PBS were incubated with anti-CX3CR1 antibody conjugated with APC at a ratio of 1:100 for 30 minutes. Ice-cold PBS was used to wash excess antibody, and cells were sorted using SH800S Cell Sorter. Sorted cells were then labeled with Chloromethylbenzamido (CellTracker^{TM} CM-DiI Dye) according to Thermo Fisher instructions. Labeled CX3CR1$^+$ cells were resuspended in DMEM high glucose media with 10% FBS, 1% non-essential amino acids solution, 1% GlutaMAX^{TM}, and loaded on top of Matrigel that contained the fetal mouse heart. A density of 1 X 10^5 cells was used per well in a 24 well-plate. Labeled cells were visualized via epifluorescence microscope.

***In vivo* MI mouse heart injection with mESC-derived CX3CR1$^+$ cells and PA-RGDS**

The same PA-RGDS biomaterial, along with cell injections was performed in accordance with Ban et al[12]. MI of mouse was induced as described previously[16]. Additionally, the number of mESC derivied-CX3CR1$^+$ cells injected was 3 X 10^5. All cells injected were obtained form the same batched and labeled with DiI prior to injection. Harvesting of mouse heart occurred at 10 days post injections. Tissues were isolated, sectioned and subjugated to immunohistochemistry as mentioned previously.

V. References

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