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Corresponding Author:	Chulan Kwon Johns Hopkins School of Medicine Baltimore, MD UNITED STATES
Corresponding Author's Institution:	Johns Hopkins School of Medicine
Corresponding Author E-Mail:	ckwon13@jhmi.edu
Order of Authors:	Chulan Kwon
	Emmanouil Tampakakis
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TITLE:

In Vitro Generation of Mouse Heart Field-Specific Cardiac Progenitor Cells

AUTHORS & AFFILIATIONS:

Emmanouil Tampakakis[1], Chulan Kwon[1]

[1]*Division of Cardiology, Department Medicine, Johns Hopkins School of Medicine, Baltimore, MD, USA*

Corresponding Author:

Chulan Kwon

Email Address: ckwon13@jhmi.edu

KEYWORDS:

stem cells, heart fields, cardiac progenitors, cardiac spheroids, Tbx1, Hcn4, Cxcr4

SHORT ABSTRACT:

The purpose of this method is to generate heart field-specific cardiac progenitor cells in vitro in order to study the progenitor cell specification and functional properties, and to generate chamber specific cardiac cells for heart disease modelling.

LONG ABSTRACT:

Pluripotent stem cells offer great potential for understanding heart development and disease and for regenerative medicine. While recent advances in developmental cardiology have led to generating cardiac cells from pluripotent stem cells, it is unclear if the two cardiac fields—the first and second heart fields (FHF and SHF)—are induced in pluripotent stem cells systems. To address this, we generated a protocol for in vitro specification and isolation of heart field-specific cardiac progenitor cells. We used embryonic stem cells lines carrying Hcn4-GFP and Tbx1-Cre; Rosa-RFP reporters of the FHF and the SHF, respectively, and live cell immunostaining of the cell membrane protein Cxcr4, a SHF marker. With this approach, we generated progenitor cells which recapitulate the functional properties and transcriptome of their in vivo counterparts. Our protocol can be utilized to study early specification and segregation of the two heart fields and to generate chamber-specific cardiac cells for heart disease modelling. Since this is an in vitro organoid system, it may not provide precise anatomical information. However, this system overcomes the poor accessibility of gastrulation-stage embryos and can be upscaled for high-throughput screens.

INTRODUCTION:

The use of pluripotent stem cells (PSCs) has revolutionized the field of cardiac regeneration and personalized medicine with patient-specific myocytes for disease modeling and drug therapies[1-4]. More recently, in vitro protocols for the generation of atrial vs ventricular as well as pacemaker-like PSC-derived cardiomyocytes have been developed[5,6]. However, whether cardiogenesis can be recreated in vitro to study cardiac development and subsequently generate ventricular chamber-specific cardiac cells is still unclear.

During early embryonic development, mesodermal cells under the influence of secreted morphogens such as BMP4, Wnts and Activin A form the primitive streak[7]. Cardiac mesodermal cells marked by the expression of Mesp1, migrate anteriorly and latterly to form the cardiac crescent and then the primitive heart tube[7,8]. This migratory group of cells includes two very distinct populations of cardiac progenitor cells (CPCs), namely the first and the second heart field (FHF and SHF)[9,10]. Cells from the SHF are highly proliferative and migratory and are primarily responsible for the elongation and looping of the heart tube. Additionally, SHF cells differentiate to cardiomyocytes, fibroblasts, smooth muscle and endothelial cells as they enter the heart tube to form the right ventricle, right ventricular outflow tract and large part of both atria[7,10]. In contrast, FHF cells are less proliferative and migratory and differentiate mainly to cardiomyocytes as they give rise to the left ventricle and a smaller part of the atria[11]. Moreover, SHF progenitors are marked by the expression of Tbx1, FGF8, FGF10 and Six2 while FHF cells express Hcn4 and Tbx5[11-15].

PSCs can differentiate to all three germ layers and subsequently to any cell type in the body[4,16]. Therefore, they offer tremendous potential for understanding heart development and for modelling specific developmental defects resulting in congenital heart disease, the most frequent cause of birth defects[17]. A large subgroup of congenital heart disease includes chamber-specific cardiac abnormalities[18,19]. However, it still unclear whether these originate from anomalous heart field development. In addition, given the inability of cardiomyocytes to proliferate after birth, there have been extensive efforts to create cardiac tissue for heart regeneration[1,7,20]. Considering the physiological and morphological differences between cardiac chambers, generation of chamber-specific cardiac tissue using PSCs is of significant importance. While recent advances in developmental cardiology have led to robust generation of cardiac cells from PSCs, it is still unclear if the two heart fields can be induced in PSC systems.

To recapitulate cardiogenesis in vitro and study the specification and properties of CPCs, we previously used a system based on differentiating PSC-derived cardiac spheroids[21-24]. Recently, we generated mouse embryonic stem cells (mESCs) with GFP and RFP reporters under the control of the FHF gene Hcn4 and the SHF gene Tbx1, respectively (mESCs$^{Tbx1\text{-}Cre;\ Rosa\text{-}RFP;\ HCN4\text{-}GFP)}$ [25]. In vitro differentiated mESCs formed cardiac spheroids in which GFP+ and RFP+ cells appeared from two distinct areas of mesodermal cells and patterned in a complementary manner. The resulting GFP+ and RFP+ cells exhibited FHF and SHF characteristics, respectively, determined by RNA-sequencing and clonal analyses. Importantly, using mESCs carrying the Isl1-RFP reporter (mESC$^{Isl1\text{-}RFP}$), we discovered that SHF cells were faithfully marked by the cell-surface protein CXCR4, and this can enable isolation of heart field-specific cells without transgenes. The present protocol will describe the generation and isolation of heart field-specific CPCs from mESCs, which may serve as a valuable tool for studying chamber-specific heart disease.

PROTOCOL:

NOTE: In vitro generation of heart field-specific mouse cardiac progenitor cells (**Figure 1**).

1. Maintenance of mouse ESCs

1.1 Grow mESCs (mESCs$^{Tbx1\text{-}Cre;\ Rosa\text{-}RFP;\ HCN4\text{-}GFP}$, mESC$^{Isl1\text{-}RFP}$)[25] on 0.1% (w/v) gelatin coated T25 flasks in 2i medium (870 mL of glascow minimum essential medium (GMEM), 100 mL of fetal bovine serum (FBS), 10 mL of GlutaMAX, 10 mL of non-essential amino acids, 10 mL of sodium pyruvate, 3 µL of beta-mercaptoethanol, 20 µL of Lif (200 U/mL), 0.3 µM CHIR99021 and 0.1 µM PD0325901).

1.2 When the cells reach 70-80% confluence, rinse the cells once with phosphate buffer solution (PBS) and then dissociate into single cells by adding 1 mL of Trypsin and incubating at 37 °C for 3 min.

1.3 Neutralize Trypsin by adding 4 mL of 10% FBS in Dulbecco's Modified Eagle Medium (DMEM). Count the cells using an automated cell counter.

1.4 Centrifuge ~3 x 10^5 cells for 3 min at 270 x *g* and room temperature.

1.5 Aspirate the supernatant, resuspend the cells in 5 mL of 2i medium and replate on 0.1% (w/v) gelatin coated T25 flasks for maintenance.

2. Generation of cardiac progenitor cells using cardiac spheroids

2.1. Centrifuge 2.5 x 10^6 cells from step 1.3 for 3 min at 270 x *g* and room temperature.

2.2. Aspirate the supernatant and resuspend the cells in 25 mL of SFD medium (10^5 cells/mL). Depending on the scale of the experiment, mESC number can be adjusted accordingly.

NOTE: SFD medium contains 715 mL of Iscove's Modified Dulbecco's Medium (IMDM), 250 mL of Ham's F12, 5 mL of N2-supplement, 10 mL of B27 minus Vitamin A, 5 mL of 10% (w/v) BSA (in PBS), 7.5 mL of GlutaMAX and 7.5 mL of Penicillin-Streptomycin. Add ascorbic acid (50 µg/mL) and 3.9 x 10^{-3}% (v/v) of monothioglycerol prior to using.

2.3. Plate the cell suspension into one 150 mm x 25 mm sterile plate and incubate at 37 °C in the 5% CO_2 incubator for 48 h. Cardiac spheroids should be formed within 24 h.

2.4. Collect all the formed cardiac spheroids and centrifuge for 3 min at 145 x *g* and room temperature to selectively isolate spheroids and avoid single cells.

2.5. Aspirate the supernatant and resuspend the spheroids in 25 mL of SFD medium with 1 ng/mL of Activin A and 1.5 ng/mL of BMP4 for differentiation induction. Plate the spheroids in the same 150 mm x 25mm sterile plate and incubate them at 37 °C in the 5% CO_2 incubator for 24 h.

NOTE: Different concentrations of Activin A (0-3 ng/mL) and BMP4 (0.5-2 ng/mL) can be used for differentiation optimization depending on the mESC line.

2.6. Collect all the cardiac spheroids and centrifuge for 3 min at 145 x *g* and room temperature.

2.7. Aspirate the supernatant and resuspend the spheroids in 25 mL of SFD medium. Transfer the resuspended EBs in an ultra-low attachment 75 cm^2 flask and incubate them at 37 °C in the 5% CO_2 incubator for 48 h.

3. Isolation of heart field specific cardiac progenitor cells using fluorescent reporters

3.1. Centrifuge cardiac spheroids at 145 x *g* and room temperature for 3min and aspirate the supernatant. Add 1 mL of Trypsin and incubate at 37 °C for 3 min. Mix well by pipetting to dissociate the cells.

3.2. Add 4 mL of 10% FBS in DMEM to inactivate Trypsin and mix well by pipetting. To remove the non-dissociated EBs, filter the mix using a 70 μm strainer and centrifuge the filtrated cells for 3 min at 270 x *g* and room temperature.

3.3. To sort CPCs carrying fluorescent reporters (CPCs derived from mESCs$^{Tbx1\text{-}Cre;\ Rosa\text{-}RFP;\ HCN4\text{-}GFP}$), aspirate the supernatant and add 500 μL of FACS sorting solution (1% (v/v) FBS, 200 mM HEPES and 10 mM of EDTA in PBS) to resuspend.

3.4. To remove all cell clusters prior to sorting, filter the cells again using a 5 mL polystyrene round-bottom tube with a 40 μm cell strainer. Keep the cells on ice until sorting.

3.5. Sort the cells to isolate Tbx1-Cre; Rosa-RFP and HCN4-GFP positive CPCs using a fluorescent activated cell sorter (FACS). Collect the sorted cells in 1 mL of FBS. Keep the cell sample and sorted cells at 4 °C.

4. Isolation of heart field specific cardiac progenitor cells using Cxcr4 as a cell surface protein marker

4.1. To isolate first vs second heart field CPCs based on the expression of the surface protein receptor Cxcr4, use the mESC$^{Isl1\text{-}RFP}$ line. Aspirate the supernatant from step 3.3 and resuspend the single CPCs in 300 μL of 10% FBS in PBS containing 1:200 (vol/vol) PerCP-efluor 710 conjugated anti-Cxcr4 antibody.

4.2. Incubate at room temperature for 5min and wash by adding 1-2 mL of cold PBS. Centrifuge the single CPCs for 3 min at 270 x *g* and room temperature and wash two more times followed by centrifugation.

4.3. Aspirate the supernatant and add 500 μL of FACS sorting solution to resuspend the single CPCs and filter as in step 3.4.

4.4. Isolate Cxcr4+ and Cxcr4- cells using FACS. Collect the sorted cells in 1 mL of FBS. Keep the cell sample and sorted cells at 4 °C.

5. Analysis of isolated heart field specific cardiac progenitor cells

5.1. Centrifuge sorted CPCs for 3 min at 270 x *g* and room temperature. Sorted cells can be used for gene and protein expression analyses or they can be recultured for analyses at later time points.

5.2. To re-culture isolated CPCs, aspirate the supernatant, resuspend the cells in SFD medium and replate ~3 x 10^4 cells per well of a 384-well plate coated with 0.1% (w/v) gelatin. If increased cell death is noted after sorting, add 10 µM of Y-27632 (ROCK inhibitor) to the sample. Two days after reculture, spontaneous beating should be noted.

5.3. To analyze the ability of plated CPCs to differentiate to cardiomyocytes, collect the cells at day 12 of differentiation. Use Trypsin as described in steps 1.2-1.5 to isolate single CMs. Resuspend the cells in 4% (w/v) paraformaldehyde (PFA) and incubate for 30 min at room temperature to fix the cells.

5.4. Centrifuge the cells for 3 min at 895 x *g*, and room temperature. Aspirate the supernatant and resuspend the cells in PBS to wash the PFA. Repeat this step once more.

5.5. Aspirate the supernatant and resuspend the cells in 10% FBS in PBS. Incubate half of the cell sample with mouse anti-Troponin T antibody (1:500) and use the rest of the sample as a negative control. Incubate for 30 min at room temperature.

5.6. Wash the cells twice as described in step 5.4 using PBS. Aspirate the supernatant and resuspend both cell samples in 10% FBS in PBS with 1:500 donkey anti-mouse IgG (H+L) secondary antibody, Alexa Fluor 647 conjugate. Incubate for 30 min at room temperature.

5.7. Wash twice with PBS as in step 5.6. Aspirate the supernatant and resuspend the cells in 200 µL of PBS. Use a flow cytometer to analyze the cells.

REPRESENTATIVE RESULTS:

After approximately 132 h of differentiation, Tbx1-RFP and Hcn4-GFP CPCs can be detected using a fluorescent microscope (**Figure 2**). Generally, GFP and RFP cells appear approximately around the same time. The two populations of CPCs continue to expand in close proximity and commonly in a complementary pattern. Adjusting the concentrations of Activin A and BMP4 will alter the percentages of FHF vs SHF CPCs (**Figure 3**). CPC specification in vitro was primarily determined by the concentration of BMP4. Therefore, our cardiac spheroid system can be used to study CPC specification.

Similarly using the Isl1-RFP reporter mESC line, after 132 h of differentiation, Isl1-RFP+ CPCs appear. After immunostaining of CPCs for CXCR4, Isl1-RFP+, Cxcr4+ vs Isl1-RFP+, Cxcr4- cells can

be isolated (**Figure 4**).

To analyze the ability of mESC-derived CPCs to differentiate to cardiomyocytes, immunostaining for cardiac Troponin T can be performed at day 12 of differentiation. In agreement with the model that FHF cells differentiate mainly to myocytes, cells derived from Hcn4-GFP+ CPCs are mainly myogenic (**Figure 5A, B**). Similarly, cells derived from Isl1+, CXCR4- CPCs also give rise to cardiomyocytes at much higher percentages in comparison to Isl1+, CXCR4- CPCs (**Figure 5C**).

Occasionally, mESCs fail to differentiate efficiently and form very low numbers heart field-specific CPCs (**Figure 6**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic representation of in vitro specification of heart field-specific cardiac progenitor cells. mESCs form spheroids within 48 h. Then exposure to Activin A and BMP4 for 40 h will lead to mesodermal induction. Cardiac progenitor cells develop approximately 36 h later. Progenitors of the second or first heart field can be sorted using fluorescent activated cell sorting. Second heart field cells are marked by Tbx1-RFP expression vs first heart field that are marked by Hcn4-GFP. Alternatively, Isl1-RFP marks CPCs and using live immunostaining against Cxcr4 one can sort Isl1+, Cxcr4+ vs Isl1+, Cxcr4- CPCs that represent second vs first heart field cells respectively.

Figure 2: Representative image of cardiac spheroids after CPC specification. RFP marks Tbx1+ and GFP marks Hcn4+ CPCs. The two cell populations are formed in close proximity in a complimentary pattern. Scale bars indicate 50 μm.

Figure 3: Flow cytometric analysis of cardiac spheroids after exposure to different concentrations of Activin A and BMP4. Adjusting the concentrations of the two morphogens leads to different percentages of Tbx1+ and Hcn4+ CPCs. The two populations were mainly affected by adjusting BMP4 concentration.

Figure 4: Flow cytometric analysis of cardiac progenitor cells expressing Isl1 and are immunostained for Cxcr4. Cardiac progenitors were first gated based on their Isl1 expression and then Isl1+, Cxcr4+ vs Isl1+, Cxcr4- cells were sorted.

Figure 5: Flow cytometric analysis of cells derived from heart field-specific CPCs stained for cardiac Troponin T. (A). Consistent with the higher myogenic potential of FHF cells, a high percentage of Hcn4-GFP+ cells differentiate to myocytes. (**B).** Analysis of all mESC-derived cardiomyocytes, where the vast majority are Hcn4-GFP+. (**C).** Cxcr4- CPCs differentiate to a higher percentage of cardiomyocytes.

Figure 6: Representative cytometric analyses of failed/low efficiency *in vitro* differentiations. (**A).** Flow cytometry analysis after 132 h of differentiation showing no formation of Hcn4-GFP cells and a very low percentage of Tbx1-RFP+ cells. (**B).** Low differentiation efficiency of mESCs

expressing very low levels of Isl1.

DISCUSSION:

In our protocol, we describe a methodology to generate cardiac spheroids and isolated heart field-specific CPCs. Those can be used to study mechanisms of CPC specification and their properties, as well as for cardiac chamber-specific disease modelling. One previously published work used a mESC line with two fluorescent reporters (Mef2c/Nkx2.5) to study cardiogenesis in vitro, however, both those markers are expressed at embryonic day 9.5-10 when cardiomyocytes are already formed[26]. To our knowledge, there are currently no methods for the isolation of heart field-specific CPCs in vitro. More importantly, our protocol can also be applied to human stem cells, where CXCR4 can be used to isolate SHF CPCs that express high levels of Isl1[25]. In addition, our double, fluorescent reporter mESC line can be used to screen libraries of compounds and transcription factors that can affect heart field specification or cell polarity in CPCs.

One of the critical steps in the protocol is the starting number of mESCs. Using low or high numbers will significantly affect the size of cardiac spheroids and differentiation efficiency. We recommend testing different cell numbers (7.5-10 x 10^4 cells /mL) for different mESC lines. Alternatively, if the size of the cardiac spheroids remains significantly variable, plates with wells containing microwells of specified size can also be used to increase reproducibility. Investigators should also be mindful of the specific timing and duration of mesodermal induction as well as the timing of cell sorting. Moreover, for different mESC lines, optimization of the morphogen concentrations will need to be performed prior to testing their ability to generate CPCs in cardiac spheroids. The use of older/expired cytokines or cell culture medium, or inconsistent concentrations of morphogens will affect the differentiation efficiency. Finally, mESC lines that have been passaged for more than ~15-20 times, do appear to lose their ability to differentiate efficiently.

Our differentiation system allows specific modifications. Cxcr4 can be used as a sole marker of SHF CPCs in mESC lines without a fluorescent reporter. However, investigators should still optimize the differentiation protocol to increase the percentage of Isl1+ CPCs prior to sorting Cxcr4+ vs Cxcr4- CPCs[25]. In addition, Activin A can be substituted with canonical Wnt agonists/activators such as Wnt3a or CHIR99021 (GSK3b inhibitor) to increase further the specification of SHF CPCs[25].

This protocol enables the study of CPC specification using well-defined conditions, time-lapse monitoring, and unrestricted numbers of cells. Thus, it is more facile, efficient and less costly in comparison to analyzing embryos. Nevertheless, it is still an in vitro system where the absolute gene expression values of heart-field specific CPCs may not tightly correlate with in vivo gene expression levels. Thus, in our system, solely BMP4 could specify CPCs from both heart fields and can significantly alter their respective ratios. Additionally, variability may exist regarding the differentiation efficiencies.

In conclusion, using mESC fluorescent reporter lines or immunostaining of cell membrane proteins, we recapitulated cardiogenesis in vitro and isolated heart field-specific CPCs. This

allows the study of early signals that mediate CPC specification and functional properties as well as modelling heart field/chamber-specific congenital cardiac diseases.

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DISCLOSURES:

The authors have nothing for disclosures.

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Figure 1

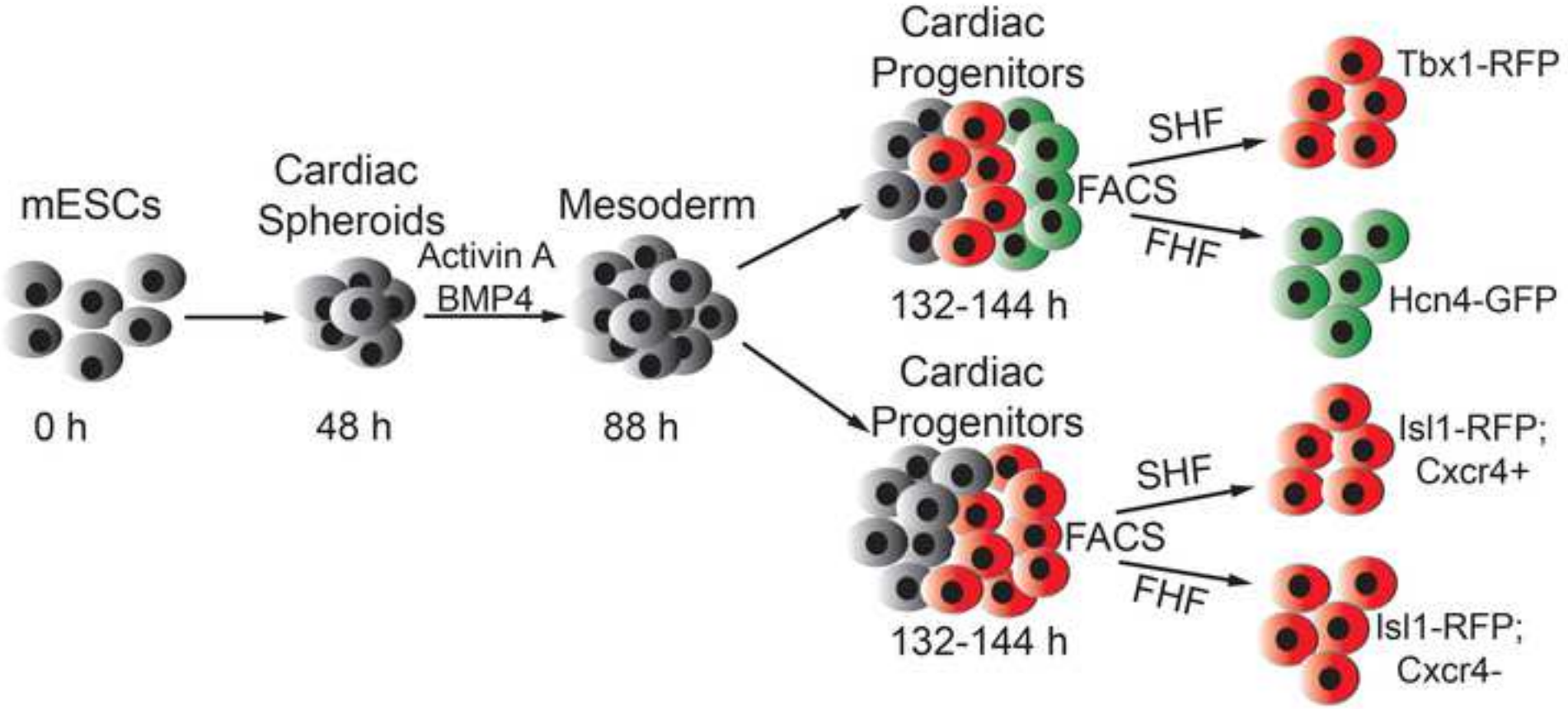
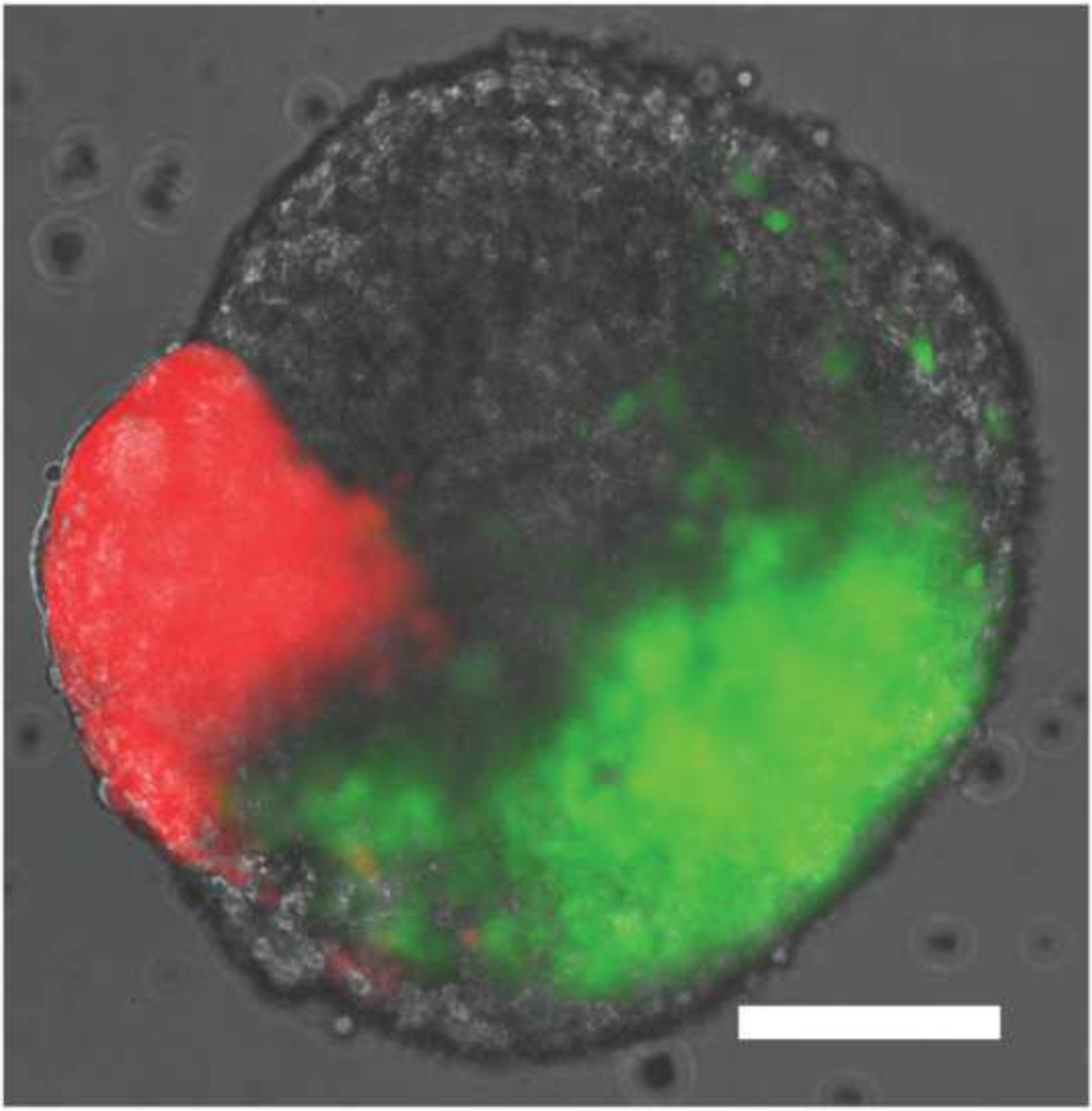


Figure 2



124-140 h

Figure 3

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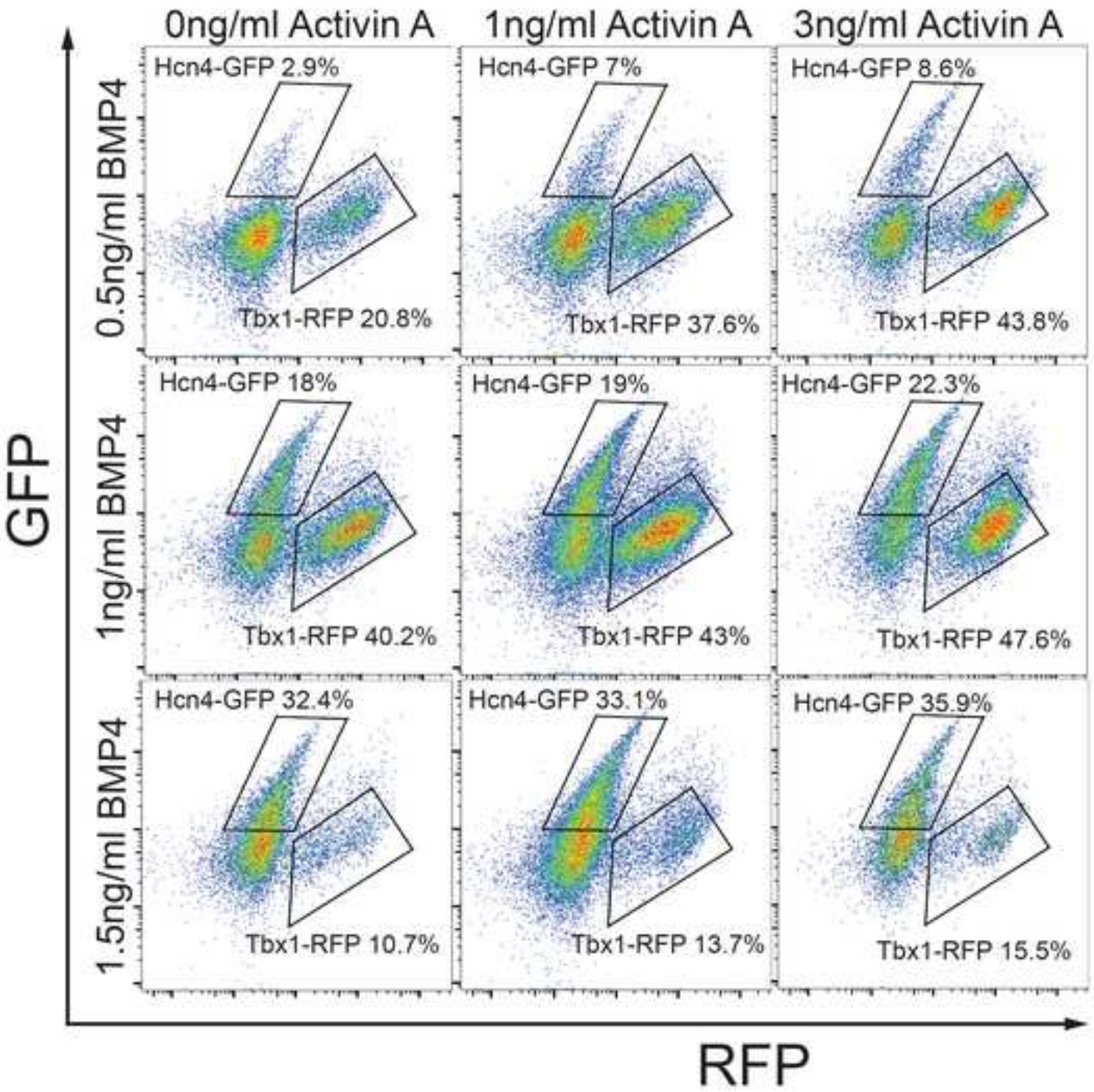

Figure 4

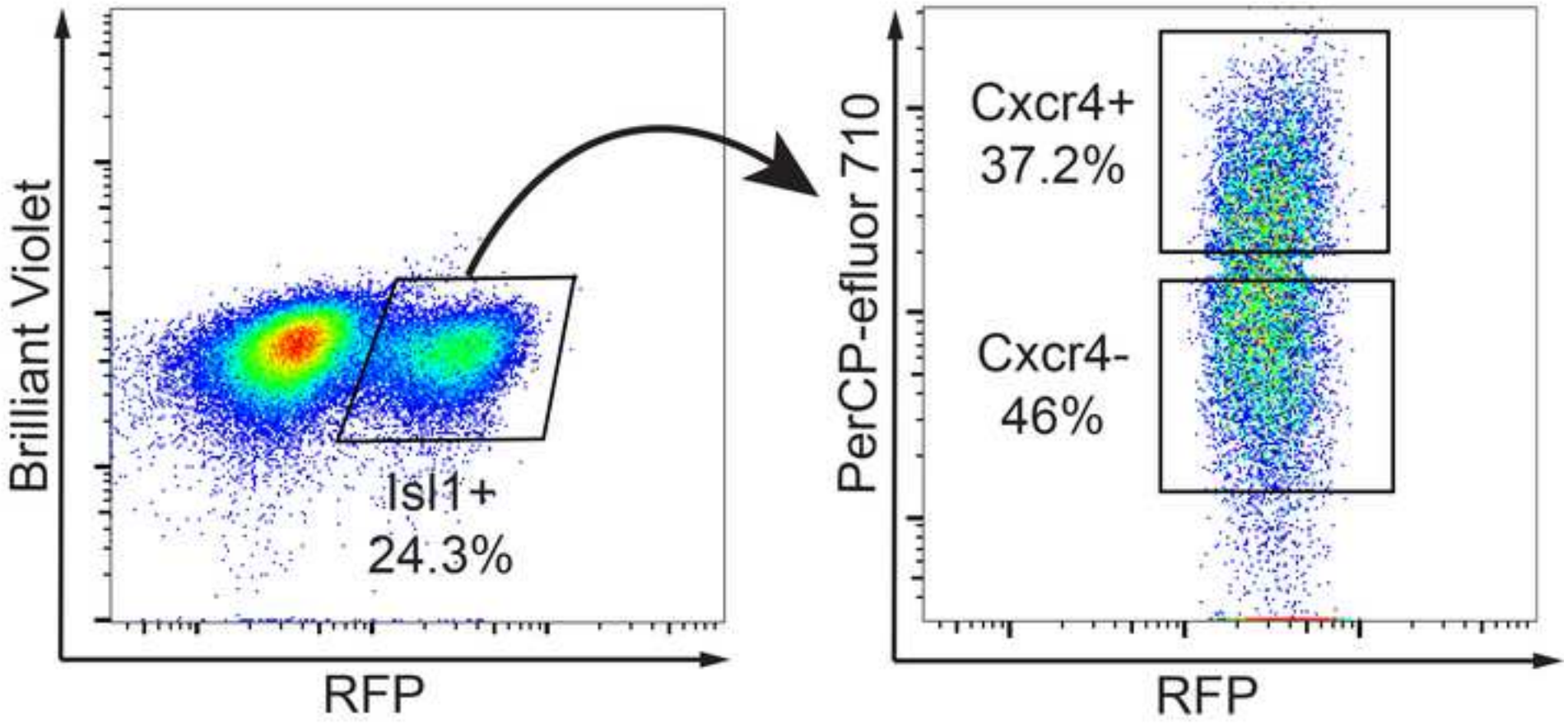

Figure 5

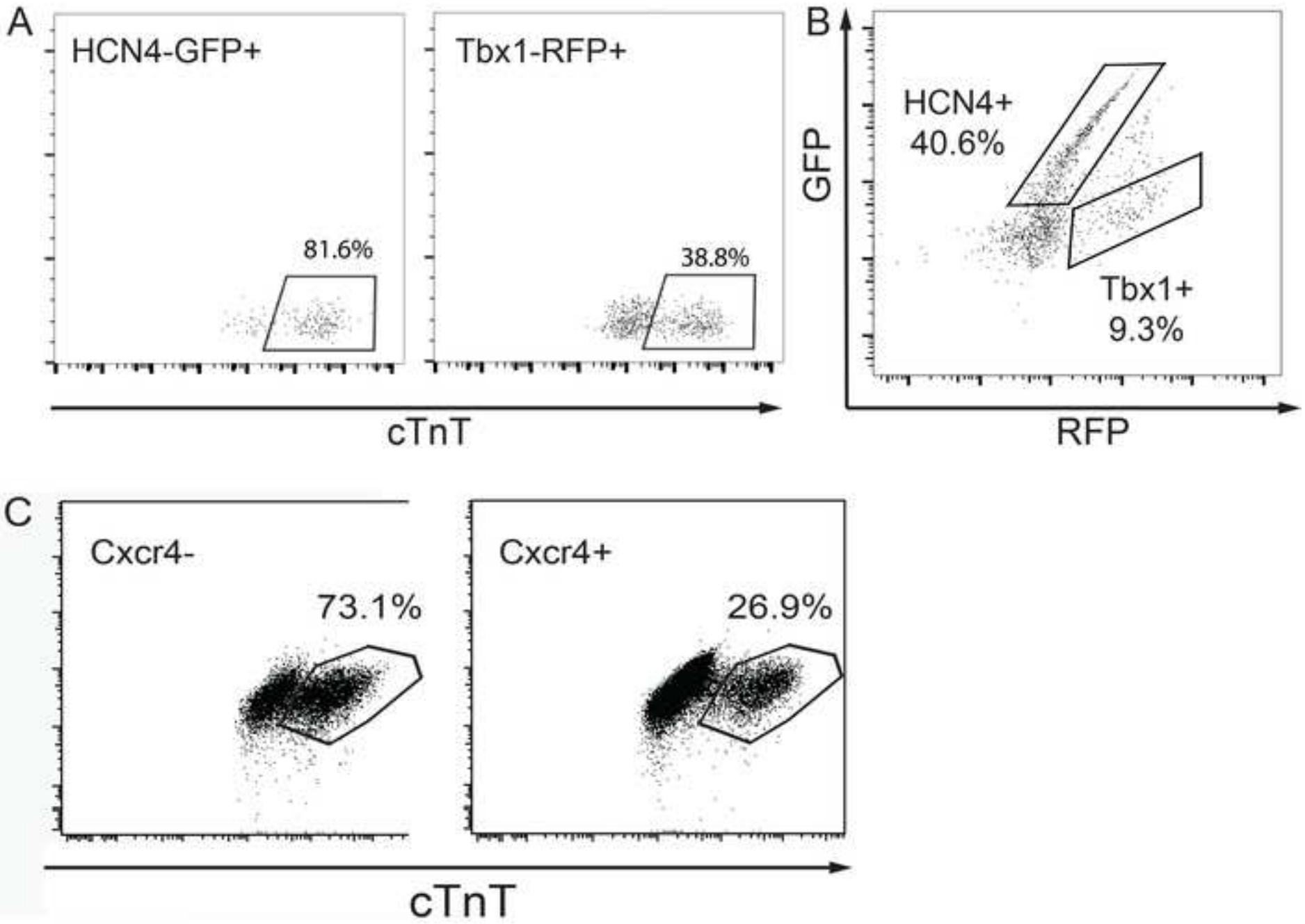

Figure 6

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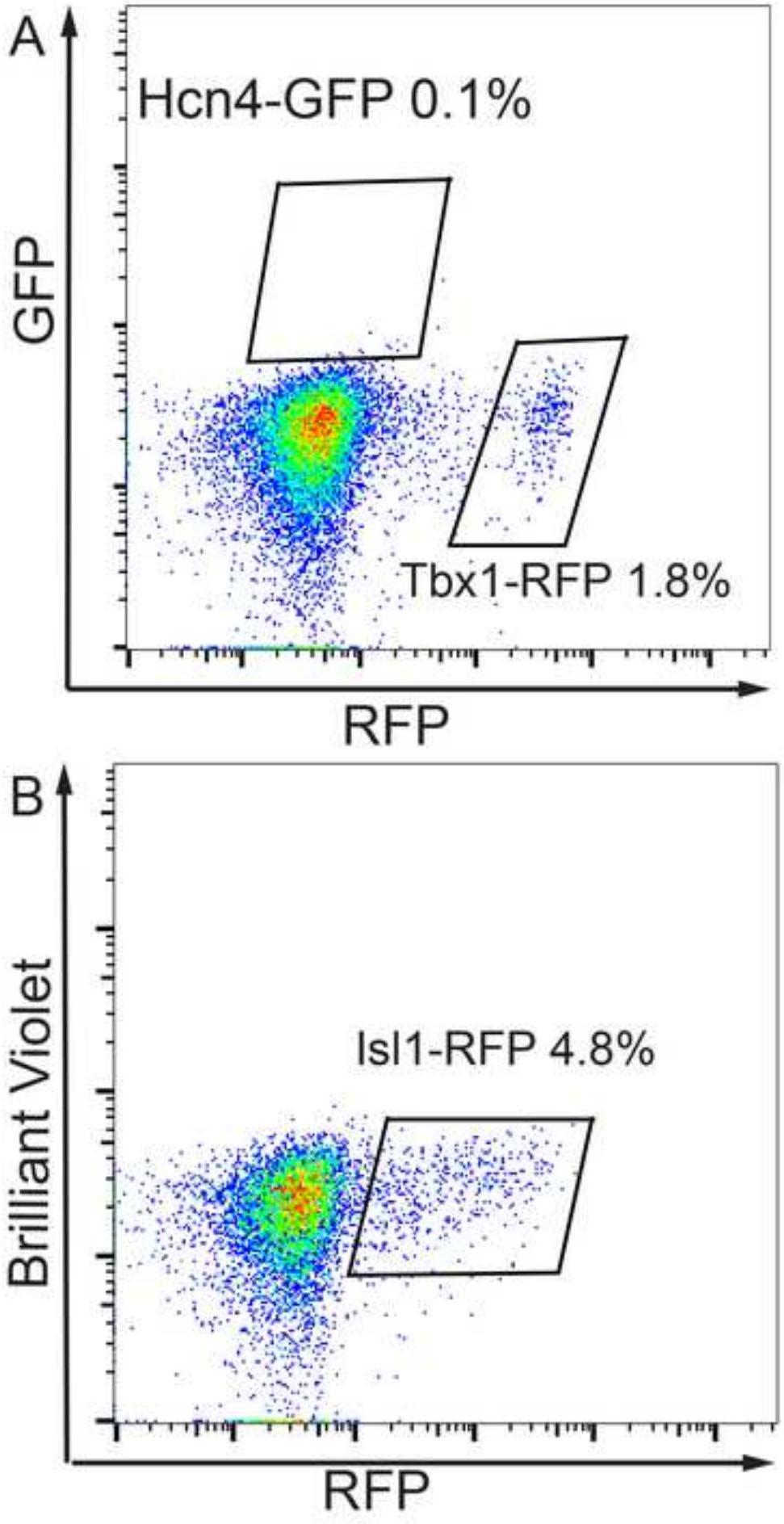

Medium

2i medium

SFD medium

FACS sorting solution 10X

Recipe

870 ml GMEM, 100 ml FBS, 10 ml GlutaMAX, 10 ml NEM NEAA, 10 ml Sodium Pyruvate, 3 µl beta-mercaptoethanol, 20 µl of Lif (200 U/ml), 0.3 µM CHIR99021 and 0.1 µM PD0325901. Filter to sterilize

715 ml IMDM, 250 ml Ham's F12, 5 ml N2-Supplement (0.5% v/v), 10 ml B27 minus Vitamin A, 5 ml of 10% (w/v) BSA (in PBS), 7.5 ml GlutaMAX and 7.5 ml Pen-Strep. Filter to sterilize and store at 4 ^{o}C

1% (v/v) FBS, 200 mM HEPES and 10 mM of EDTA in PBS. Filter to sterilize and store at 4 ^{o}C

Name of Material/ Equipment

β-mercaptoethanol

0.1% (w/v) Gelatin

100mM Sodium Pyruvate

100X Pen/Strep

1X PBS w/o Calcium and Magnesium

20% Paraformaldehyde

5 ml Polystyrene round-bottom tube with a 40µm cell strainer

Activin A

Ascorbic Acid

B27 minus vitamin A (50x)

BMP4

Bovine Serum Albumin

Cell sorter

Cell strainer 70µm

Centrifuge Sorvall Legend XT

CHIR99021

CO2 Incubator

Corning Ultra Low Attachment T75 flask

Countless II FL automated cell counter

Donkey anti-mouse IgG secondary antibody, Alexa Fluor 647 conjuga

Dulbecco's Modified Eagle's Medium high glucose (DMEM)
EDTA

ESGRO (LIF)
EVOS FL microscope
Fetal Bovine Serum
Glasgow's MEM (GMEM)
GlutaMAX (100 x)
Ham's F12
HEPES
IMDM
Monothioglycero (MTG)
Mouse anti-Troponin T antibody
N2-SUPPLEMENT
Non-essential amino acid solution (NEAA
PD0325901
PerCP-efluor 710 conjugated anti-Cxcr4 antibody
Suspension culture dish 150 mm x 25mm
T25 flasks
TrypLE (Trypsin)
Y-27632 (ROCK inhibitor)

Company	Catalog Number
Sigma	M6250
EMD Millipore	ES-006-B
Gibco	11360
Gibco	15070-063
Thermo Fisher Scientific	21-040-CV
Thermo Fisher Scientific	50-980-493
BD Falcon	35223
R & D Systems	338-AC-010
Sigma	A-4544
Thermo Fisher Scientific	12587010
R & D Systems	314-BP
Sigma	A2153
Sony	SH800
Thermo Fisher Scientific	08-771-2
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Thermo Fisher Scientific	
Thermo Fisher Scientific	A-31571, Lot #1757130
Gibco	11965-092
Sigma	E6758

Millipore	ESG1106	
Thermo Fisher Scientific	AMF4300	
Invitrogen	SH30071.03	
Gibco		11710035
Gibco	35050-061	
Gibco	10-080-CV	
Sigma	H3375	
Gibco		12440053
Sigma	M-6145	
Thermo Fisher Scientific	MS-295-P1	
Gibco	17502-048	
Invitrogen	11140-050	
Selleckchem	S1036	
Thermo Fisher Scientific	46-9991-82	
Corning		430597
Corning		353109
Gibco		12604
Stem cell technologies		72304

Comments/Description

Sony or any other fluorescence-activated cell sorter

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Title of Article:	In vitro generation of mouse heart field-specific cardiac progenitor cells
Author(s):	Emmanouil Tampakakis, Chulan Kwon

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CORRESPONDING AUTHOR

Name:	Chulan Kwon
Department:	Medicine
Institution:	Johns Hopkins University
Title:	Associate Professor of Medicine
Signature:	Chulan Kwon
Date:	2/11/2019

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6. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials. We made all necessary changes.
7. Please define all abbreviations before use, e.g., PBS, etc. We defined all abbreviations
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Reviewers' comments:

Reviewer #1:

Manuscript Summary:
In this ms, the authors described a robust method to induce and isolate PHF- and SHF-derived cardiomyocytes from in vitro mES cells.

Major Concerns:
No major concern

Minor Concerns:
1. It will be really helpful if the authors can list the companies and cat# of the reagents that are used in this protocol. Respectfully, these are included in the table.
2. It will also be very helpful if the authors put the receipts of all the special medium used in this study (such SFD medium, 2i medium, etc) as a supplement table. We added one more table with the recipes.
3. Step 2.5, will the authors give some suggestions regarding the range of the concentrations of Activin A and BMP4? We added a range of concentrations.

4. The author will need to provide the information regarding the cTnt and Cxcr4 antidodies (company name and cat #). Respectfully, these are included in the table.

Reviewer #2:

Manuscript Summary:
The recent advances in developmental cardiology have led to generating cardiac cells from pluripotent stem cells, but it is unclear if the progenitor cells of first and second heart fields (FHF and SHF) are induced in this pluripotent stem cells systems. To address this, the authors generated a protocol for in vitro specification and isolation of heart field-specific cardiac progenitor cells. To distinguish the cells of FHF and SHF, the authors used embryonic stem cells lines carrying Hcn4-GFP and Tbx1-Cre; Rosa-RFP reporters of the FHF and the SHF, respectively, and live cell immunostaining of the cell membrane protein Cxcr4, a SHF marker. With this approach, they can establish a protocol to generate progenitor cells, which recapitulate the functional properties and transcriptome of their in vivo counterparts.

Major Concerns:
No major concern.

Minor Concerns:

The authors did not introduce "mESCsTbx1-Cre; Rosa-RFP; HCN4-GFP, mESCIsl1-RFP" in the abstracts or introduction, but use it in the protocol. It will be better for the authors to introduce this line before they use it in the protocol. We made the changes and introduced the two lines.

Line 276: The description of "when cardiomyocytes are already formed" is not accurate. Respectfully, the first cardiomyocytes during heart development do appear around embryonic day 9.5-10.

In Figure 3, the differentiation of the cells treated with different concentration of BMP4 is dramatically affected by the concentration of BMP4. It will be better for the authors to explain or discuss. We want to thank the reviewer for his comment and note that we now discuss this in the text.