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Selective Differentiation into Hematopoietic and Cardiac Cells from Pluripotent Stem Cells Based on the Expression of Cell Surface Markers

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Abstract

Flk1-expressing (+) mesodermal cells are useful source for the generation of hematopoietic cells and cardiomyocytes from pluripotent stem cells (PSCs). However, they have been reported as a heterogenous population that includes hematopoietic and cardiac progenitors. Therefore, to provide a method for a highly efficient production of hematopoietic cells and cardiomyocytes, cell surface markers are often used for separating these progenitors in Flk1$^+$ cells. Our recent study has shown that the expression of coxsackievirus and adenovirus receptor (CAR), a tight junction component molecule, could divide mouse and human PSC- and mouse embryo-derived Flk1$^+$ cells into Flk1$^+$CAR$^-$ and Flk1$^+$CAR$^+$ cells. Flk1$^+$CAR$^-$ and Flk1$^+$CAR$^+$ cells efficiently differentiated into hematopoietic cells and cardiomyocytes, respectively. These results indicate that CAR is a novel cell surface marker for separating PSC-derived Flk1$^+$ mesodermal cells into hematopoietic and cardiac progenitors. We herein describe a differentiation method from PSCs into hematopoietic cells and cardiomyocytes based on CAR expression.

Keywords: Mesoderm, Differentiation, CAR, Hematopoietic cells, Cardiomyocytes, Flk1

1 Introduction

During mouse embryogenesis, Flk1-expressing (+) cells are well known as mesodermal cells, which give rise to hematopoietic cells and cardiomyocytes (1, 2). Flk1$^+$ cells can be differentiated from pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), and produce hematopoietic cells and cardiomyocytes (2–6). Therefore, PSCs-derived Flk1$^+$ cells are a useful source for the generation of hematopoietic cells and cardiomyocytes. On the other hand, Flk1$^+$ cells have been reported to be a heterogenous population containing at least hematopoietic and cardiac progenitor cells (2), suggesting that separation of Flk1$^+$ cells into distinct mesodermal progenitors would allow us to establish a method for a highly efficient production of hematopoietic cells and cardiomyocytes.

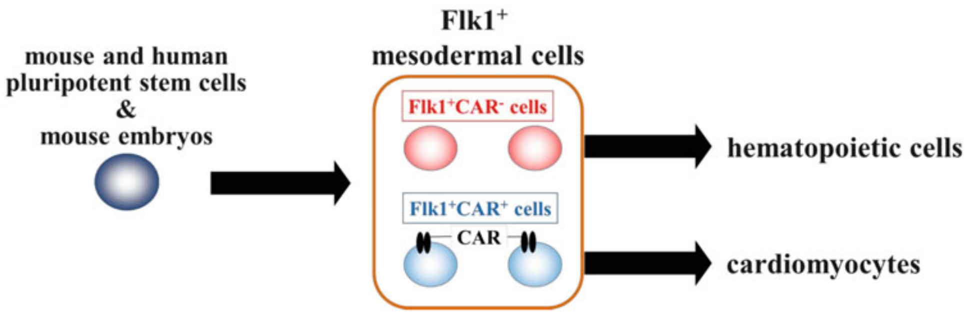

Fig. 1 Differentiation potentials of two Flk1$^+$ subsets. Mouse and human PSC- and mouse embryo-derived Flk1$^+$ mesodermal cells could be separated into two populations (Flk1$^+$CAR$^-$ cells and Flk1$^+$CAR$^+$ cells). Flk1$^+$CAR$^-$ cells and Flk1$^+$CAR$^+$ cells have the potential to differentiate into hematopoietic cells and cardiomyocytes, respectively

In our recent study, we have focused on coxsackievirus and adenovirus receptor (CAR), which is originally identified as a cell surface receptor for coxsackie B viruses and C-type adenoviruses and is also known as a tight junction component molecule (7, 8). It has been reported that the CAR expression is low or undetectable in hematopoietic cells, and is high in the heart (8–12), raising a possibility that Flk1$^+$ cells could be separated into hematopoietic and cardiac progenitor cells based on CAR expression. To test this possibility, we examined CAR expression in the mesodermal differentiation of PSCs, and found that mouse PSC- and embryo-derived Flk1$^+$ cells could be divided into two types of cells; CAR$^+$ and CAR$^-$ cells (13). Flk1$^+$CAR$^+$ cells have the cardiac differentiation potential, and Flk1$^+$CAR$^-$ cells efficiently differentiated into hematopoietic cells (13). In addition, CAR$^+$ and CAR$^-$ cells were also identified in human PSCs-derived KDR$^+$ (human counterpart to Flk1) mesodermal, and KDR$^+$CAR$^+$ and KDR$^+$CAR$^-$ cells have cardiac and hematopoietic differentiation potential, respectively (13). Therefore, the tight junction molecule CAR would be a useful marker for separating mouse and human PSC- and embryo-derived Flk1$^+$ mesodermal cells into hematopoietic and cardiac progenitors (Fig. 1). In this chapter, we provide a novel method for hematopoietic and cardiac differentiation with PSCs and mouse embryos.

2 Materials

2.1 Mouse ESCs/iPSCs

2.1.1 Cell Lines

1. Mouse ES cell line, BRC6 (Riken Bioresource Center).
2. Mouse iPS cell line, 38C2 (a gift from Dr. Shinya Yamanaka, Kyoto University) (14).

2.1.2 Cell Maintenance

1. ESC-Sure mESC Complete Medium (Applied StemCell, Inc.).
2. 1,000× Leukemia inhibitory factor (LIF; Wako).
3. 2-Mercaptoethanol (2-ME; Nacalai Tesque).
4. Culture medium (mESC/iPSC medium): ESC-Sure mESC Complete Medium supplemented with LIF (1×) and 2-ME (100 μM).
5. Phosphate-Buffered-Saline without Ca^{2+} and Mg^{2+} (PBS).
6. 0.25 % trypsin–EDTA solution (Life Technologies).
7. StemSure 0.1 w/v% gelatin solution (Gelatin; Wako).
8. 60-mm cell culture dishes (Nunc).
9. Mitomycin C-treated mouse embryonic fibroblasts (MEFs; Millipore).

2.1.3 Embryoid Body Formation

1. Dulbecco's modified Eagle's medium (DMEM; Wako).
2. Fetal Bovine Serum (FBS; Life Technologies).
3. 100× Non-Essential Amino Acids (NEAA; Life Technologies).
4. 100 mM L-glutamine (Life Technologies).
5. 100× Nucleoside (Millipore).
6. 2-ME.
7. Penicillin–Streptomycin (Pen-Strep; Life Technologies).
8. Differentiation medium (mEB medium): DMEM containing FBS at 15 % supplemented with NEAA (1×), L-glutamine (2 mM), nucleoside (1×), 2-ME (100 μM), and Pen-Strep.
9. Lipidure-coat 96-well plates (Thermo Fisher Scientific).

2.1.4 Cell Sorting

1. PBS.
2. FBS.
3. FACS buffer: PBS containing FBS at 2 %.
4. 0.25 % trypsin–EDTA solution.
5. 70 μm cell strainer (BD Bioscience).
6. Antibodies (*see* Tables 1 and 2).

2.1.5 OP9 Stromal Cell Maintenance

1. OP9 stromal cells (Riken Bioresource Center).
2. α-Minimum essential medium (αMEM; Sigma).
3. FBS.
4. L-glutamine.
5. NEAA.
6. Pen-Strep.

Table 1
List of primary antibodies used in this study

Species	Antigen	Clone	Host	Conjugated molecules	Application	Company
Mouse	CAR	4C9	Rat	–	FACS	–
	Flk1	Avas12a1	Rat	Biotin	FACS	e-Bioscience
	CD45	30-F11	Rat	FITC	FACS	e-Bioscience
	Ter119	TER-119	Rat	PE	FACS	e-Bioscience
Human	CAR	RmcB	Mouse	–	FACS	Millipore
	KDR	89106	Mouse	PE	FACS	R&D Systems
	CD45	HI30	Mouse	PECy7	FACS	BD Bioscience
	CD144	16B1	Mouse	PE	FACS	e-Bioscience
Mouse and human	cTNT	11–13	Mouse	–	Immuno staining	Thermo Scientific

Table 2
List of secondary antibodies used in this study

Secondary antibodies	Application	Company
Streptavidin-Brilliant Violet 421	FACS	BioLegend
Rat IgG-DyLight 649	FACS	BioLegend
Mouse IgG-Brilliant Violet 421	FACS	BioLegend
Mouse IgG-Alexa Fluor 594	Immunostaining	Life Technologies

7. Culture medium (OP9 medium): αMEM containing FBS at 20 % supplemented with L-glutamine (2 mM), NEAA (1×), and Pen-Strep.
8. PBS.
9. 0.25 % trypsin–EDTA solution.
10. 100-mm cell culture dishes (Nunc).

2.1.6 Hematopoietic Differentiation from Mouse ESC/iPSC-Derived Cells

1. OP9 medium.
2. Mouse stem cell factor (mSCF; Peprotech).
3. Human Flt3-ligand (hFlt3-L; Peprotech).
4. Mouse thrombopoietin (mTPO; Peprotech).
5. Mouse interleukin-3 (mIL-3; R&D Systems).
6. Human interleukin-6 (hIL-6; Peprotech).
7. 2-ME.

Table 3
List of primers used in this study

Species	Gene	Forward (5′–3′)	Reverse (5′–3′)
Mouse	*Gapdh*	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA
	Scl	AACAACAACCGGGTGAAGAG	GGGAAAGCACGTCCTGTAGA
	Runx1	CCAGGTAGCGAGATTCAACGA	CAACTTGTGGCGGATTTGTAAA
	Gata-1	TTGTGAGGCCAGAGAGTGTG	TTCCTCGTCTGGATTCCATC
	Gata-2	TAAGCAGAGAAGCAAGGCTCGC	ACAGGCATTGCACAGGTAGTGG
	Fli-1	CCAACGAACGGAGAGTCATT	ATTCCTTGCCATCCATGTTC
	Tbx5	CTACCCCGCGCCCACTCTCAT	TGCGGTCGGGGTCCAACACT
	Mesp1	GCTCGGTCCCCGTTTAAGC	ACGATGGGTCCCACGATTCT
	Gata-4	CCACGGGCCCTCCATCCAT	GGCCCCCACGTCCCAAGTC
	Myl4	AAGAAACCCGAGCCTAAGAAGG	TGGGTCAAAGGCAGAGTCCT
	cTNT	GCGGAAGAGTGGGAAGAGACAGAC	GCACGGGGCAAGGACACAAG
	αMHC	GCTGGGCTCCCTGGACATTGAC	CCTGGGCCTGGATTCTGGTGAT
Human	*Gapdh*	GGTGGTCTCCTCTGACTTCAACA	GTGGTCGTTGAGGGCAATG
	Runx1	TGGCTGGCAATGATGAAAAC	CACTTCGACCGACAAACCTGA
	Scl	AGCCGGATGCCTTCCCTAT	GGGACCATCAGTAATCTCCATCT
	Gata-2	GCAACCCCTACTATGCCAACC	CAGTGGCGTCTTGGAGAAG
	Isl1	TTGTACGGGATCAAATGCGCCAAG	AGGCCACACAGCGGAAACA
	Tbx5	AAATGAAACCCAGCATAGGAGCTGGC	ACACTCAGCCTCACATCTTACCCT
	cTNT	TTCACCAAAGATCTGCTCCTCGCT	TTATTACTGGTGTGGAGTGGGTGTGG

8. Differentiation medium: OP9 medium supplemented with mSCF (50 ng/mL), hFlt3-L (50 ng/mL, Peprotech), mTPO (10 ng/mL), mIL-3 (5 ng/mL), hIL-6 (5 ng/mL), and 2-ME (50 μM).
9. 24-well culture plates (Nunc).

2.1.7 Cardiac Differentiation from Mouse ESC/iPSC-Derived Cells

1. OP9 medium.
2. 2-ME.
3. Differentiation medium: OP9 medium supplemented with 2-ME (50 μM).
4. 24-well, 48-well culture plates (Nunc).

2.2 Mouse Embryos

2.2.1 Cell Sorting

1. Cell dissociation buffer, enzyme-free, PBS (Life Technologies).

Other materials are described in Section 2.1.4.

2.2.2 OP9 Stromal Cell Maintenance

See Section 2.1.5.

2.2.3 Hematopoietic Differentiation from Mouse Embryo-Derived Cells

See Section 2.1.6.

2.2.4 Cardiac Differentiation from Mouse Embryo-Derived Cells

See Section 2.1.7.

2.3 Human ESCs/iPSCs

2.3.1 Cell Lines

1. Human ES cell line, KhES-3 (provided by Dr. Norio Nakatsuji, Kyoto University) (15).
2. Human iPS cell line, 201B7 (provided by Dr. Shinya Yamanaka, Kyoto University) (16).

2.3.2 Cell Maintenance

1. Repro Stem medium (ReproCELL, Tokyo, Japan).
2. Human fibroblast growth factor-2 (hFGF2, Katayama Kagaku Kogyo).
3. Culture medium (hESC/iPSC medium): Repro Stem medium supplemented with hFGF2 (5 ng/mL).
4. StemSure 0.1 w/v% gelatin solution (Gelatin; Wako).
5. 100-mm cell culture dishes.
6. Mitomycin C-treated mouse MEFs.

2.3.3 Embryoid Body Formation

1. StemPro-34 SFM (Life Technologies).
2. StemPro-34 nutrient supplement (Life Technologies).
3. Ascorbic acid (AA, Sigma).
4. Monothioglycerol (MTG, Sigma).
5. Rock inhibitor (Y-27632; Wako).
6. Human bone morphogenetic protein 4 (hBMP4; R&D Systems).
7. Human Activin-A (hActivin-A; R&D Systems).
8. hFGF2.
9. Human vascular endothelial growth factor (hVEGF, Peprotech, Rocky Hill, NJ).
10. Human Dickkopf1 (hDKK1, R&D Systems).
11. L-Glutamine.
12. Pen-Strep.
13. Basal medium: StemPro-34 SFM containing StemPro-34 nutrient supplement, L-glutamine (2 mM), and Pen-Strep.
14. Differentiation medium 1 (hEB-1 medium): basal medium supplemented with Y-27632 (10 μM).

15. Differentiation medium 2 (hEB-2 medium): basal medium supplemented with hBMP4 (2 ng/mL), Y27632 (10 μM), AA (50 μg/mL), and MTG (450 μM).
16. Differentiation medium 3 (hEB-3 medium): basal medium supplemented with hBMP4 (10 ng/mL), hActivin-A (6 ng/mL), hFGF2 (5 ng/mL), AA (50 μg/mL), and MTG (450 μM).
17. Differentiation medium 4 (hEB-4 medium): basal medium supplemented with hVEGF (10 ng/mL), hDkk1 (10 ng/mL), AA (50 μg/mL), and MTG (450 μM).
18. Dispase (Roche).
19. Minimum Essential Medium Eagle (MEM; Sigma).
20. Dispase solution: MEM containing dispase (0.1 mg/mL).
21. Gelatin.
22. 100-mm cell culture dishes.
23. 100-mm petri dishes (AS ONE).

2.3.4 Cell Sorting

See Section 2.1.4.

2.3.5 OP9 Stromal Cell Maintenance

See Section 2.1.5.

2.3.6 Hematopoietic Differentiation from Human ESC/iPSC-Derived Cells

1. OP9 medium.
2. mSCF.
3. hFlt3-L.
4. mTPO.
5. Human interleukin-3 (hIL-3; Peprotech).
6. hIL-6.
7. AA.
8. MTG.
9. 2-ME.
10. Differentation medium: OP9 medium supplemented with mSCF (100 ng/mL), hFlt3-L (100 ng/mL), mTPO (10 ng/mL), hIL-3 (10 ng/mL), hIL-6 (10 ng/mL), AA (50 μg/mL), MTG (450 μM), and 2-ME (50 μM).
11. 24-well culture plates.

2.3.7 Cardiac Differentiation from Human ESC/iPSC-Derived Cells

1. Basal medium (*see* Section 2.3.3, **Step 13**).
2. AA.
3. MTG.
4. hVEGF.

5. hFGF2.
6. Y-27632.
7. Differentiation medium-1: basal medium supplemented with hVEGF (10 ng/mL), hFGF2 (10 ng/mL), AA (50 μg/mL), and MTG (450 μM).
8. Differentiation medium-2: Differentiation medium-1 supplemented with Y-27632 (10 μM).
9. Gelatin.
10. 96-well culture plates (Nunc).

3 Methods

3.1 Differentiation of Mouse ESCs/iPSCs to Hematopoietic and Cardiac Cells (see Fig. 2)

3.1.1 Mouse ESC/iPSC Culture

1. Prepare MEF feeder layer on gelatin-coated 60 mm culture dishes.
2. Culture mouse ESCs (BRC6) and iPSCs (38C2) in 3–4 mL of mESC/iPSC medium on MEF feeder layer.
3. Change entire medium daily.
4. Passage the cells to fresh MEF feeder layer every 2–3 days as described below.
5. Aspirate the culture medium, wash with PBS twice, and then add 0.5 mL of 0.25 % trypsin–EDTA solution.
6. Incubate the culture at 37 °C for 3–5 min.
7. Gently dissociate the cell aggregates into single cells by pipetting.
8. Add 1.0 mL of mESC/iPSC medium and transfer the dissociated cells into 15 mL tube.
9. Centrifuge at 367 × *g* for 5 min at room temperature.
10. Resuspend the cells in 0.5 mL of mESC/iPSC medium.
11. Seed a part of the cell suspension (1:10–20) on fresh MEFs feeder layers.

3.1.2 Embryoid Body Formation

1. Harvest mouse ESCs/iPSCs and spin down as described above (*see* Section 3.1.1, **Steps 5–9**).
2. Aspirate the supernatant, suspend in 5 mL of mEB medium, and then plate on 60 mm cell culture dish.
3. Incubate the cells at 37 °C and 5 % CO_2 for 30 min to remove MEFs.
4. Transfer the suspension into 15 mL tube.
5. Centrifuge at 367 × *g* for 5 min at room temperature.
6. Resuspend the cells in 2–5 mL of mEB medium.

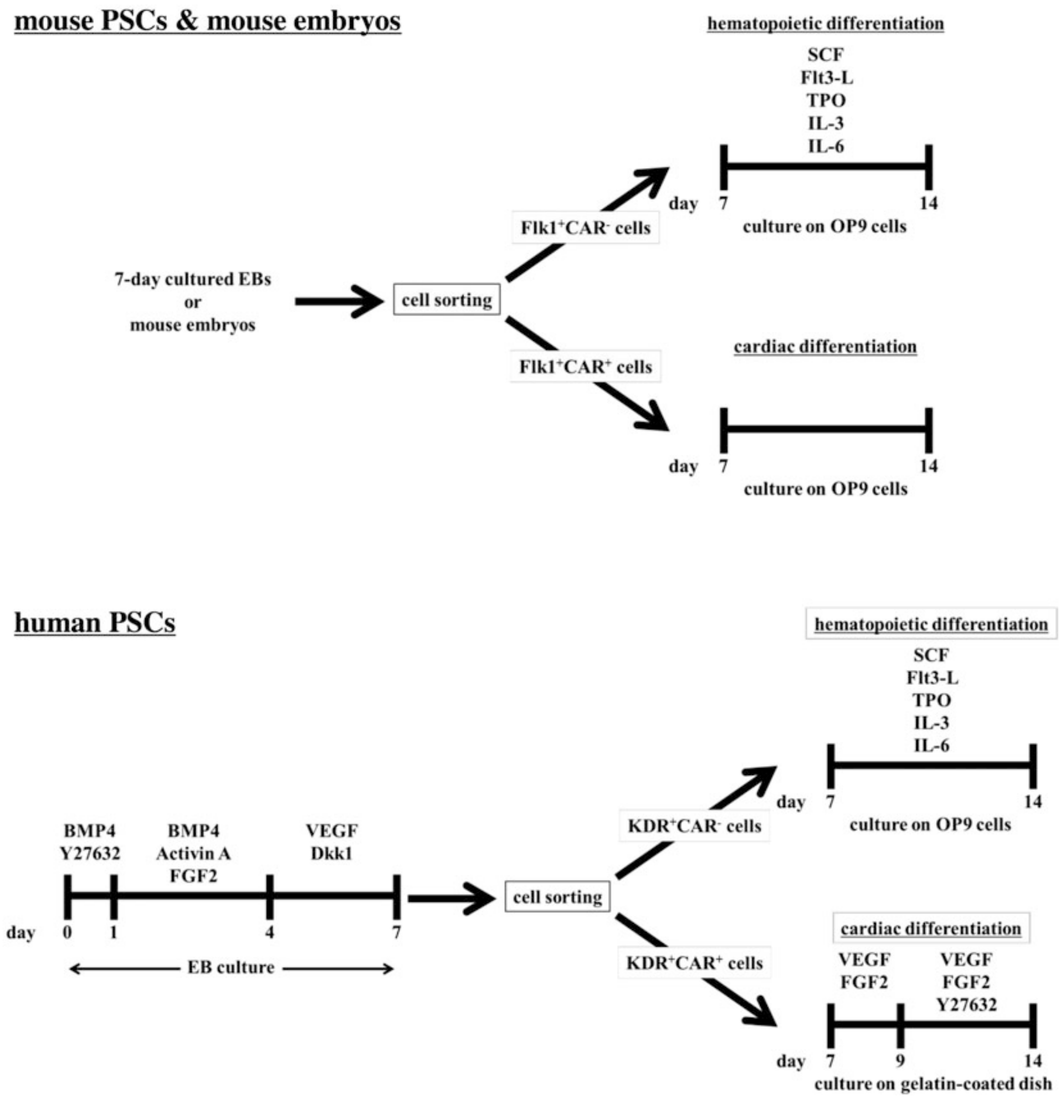

Fig. 2 Selective differentiation into hematopoietic and cardiac cells from pluripotent stem cells. Experimental protocols for hematopoietic and cardiac cells differentiation from mouse and human PSCs- and mouse embryo-derived cells are shown

7. Plate the dissociated cells on Lipidure-coated 96-well plates (BRC6 cells: 3.0×10^3 cells/well, 38C2 cells: 1.0×10^3 cells/well) with mEB medium (100 μL/well). We usually prepare five plates for cell sorting.
8. On day 2, add 100 μL of fresh mEB medium per well.
9. On day 5, change half of the medium (100 μL/well).
10. On day 7, harvest EBs and prepare them for cell sorting (*see* **Note 1**).

3.1.3 Cell Sorting

1. Transfer 7 day-cultured EBs into 50 mL tube.
2. Aspirate the culture medium, wash with PBS, and then add 1.0 mL of 0.25 % trypsin–EDTA solution.
3. Incubate the culture at 37 °C for 3–5 min.
4. Gently dissociate the EBs into single cells by pipetting.
5. Add 9.0 mL of FACS buffer (*see* Section 2.1.4, 3), gently mix the cell suspension by pipetting, and then pass through 70 μm cell strainer.
6. Transfer the suspension into 15 mL tube.
7. Centrifuge at 367 × *g* for 5 min at room temperature.
8. Resuspend the cells in 0.5 mL of FACS buffer.
9. Add an appropriate concentration of rat anti-mouse CAR antibody and incubate for 30 min at 4 °C.
10. Wash the cells with FACS buffer twice.
11. Resuspend the cells in 0.5 mL of FACS buffer.
12. Add an appropriate concentration of DyLight 649-conjugated anti-rat IgG antibody and incubate for 20 min at 4 °C.
13. Wash the cells with FACS buffer three times.
14. Resuspend the cells in 0.5 mL of FACS buffer.
15. Add an appropriate concentration of biotin-conjugated rat anti-mouse Flk1 antibody and incubate for 30 min at 4 °C.
16. Wash the cells with FACS buffer twice.
17. Resuspend the cells in 0.5 mL of FACS buffer.
18. Add an appropriate concentration of Brilliant Violet-conjugated streptavidin and incubate for 20 min at 4 °C.
19. Wash the cells with FACS buffer twice.
20. Resuspend the cells in 5–10 mL of FACS buffer and then pass through 70 μm cell strainer just before cell sorting.
21. Sorting two $Flk1^+$ subsets ($Flk1^+CAR^-$ cells and $Flk1^+CAR^+$ cells) with FACS Aria or FACS AriaII (BD Bioscience).

3.1.4 Hematopoietic Differentiation from Mouse ESC/iPSC-Derived Cells

1. A day before sorting, prepare 24-well plates with 80–90 % confluent monolayer of OP9 cells cultured in OP9 medium (*see* Section 2.1.5, 7).
2. Seed the FACS-sorted Flk1+CAR− cells on feeder layers of OP9 cells with differentiation medium (*see* Section 2.1.6, 8) at a density of 1.0–5.0 × 10^4 cells/well.
3. After 4–7 days in culture, confirm the hematopoietic differentiation of the FACS sorted $Flk1^+CAR^-$ cells by FACS and RT-PCR analyses (*see* **Note 2**).

3.1.5 Cardiac Differentiation from Mouse ESC/iPSC-Derived Cells

1. A day before sorting, prepare 24-well or 48-well plates with 80–90 % confluent monolayer of OP9 cells cultured in OP9 medium.
2. Seed FACS-sorted Flk1+CAR+ cells on feeder layers of OP9 cells with differentiation medium (*see* Section 2.1.7, 3, 500 μL/well) at a density of 1–5 × 10^4 cells/well (24-well plate) or 1–10 × 10^3 cells/well (48-well plate).
3. After 3–4 days in culture, add 500 μL of fresh differentiation medium.
4. On day 7, confirm the cardiac differentiation of FACS-sorted Flk1+CAR+ cells by immunocytochemical and RT-PCR analyses (*see* **Note 3**).

3.2 Differentiation of Mouse Embryo-Derived Cells to Hematopoietic and Cardiac Cells

3.2.1 Dissociation of Mouse Embryos

1. Prepare E8.5 mouse embryos.
2. Transfer into 1.5 mL tube, wash with PBS, and then add 0.3–0.5 mL of cell dissociation buffer.
3. Incubate the culture at 37 °C for 15 min.
4. Gently dissociate into single cells by pipetting.
5. Add 0.5–1.0 mL of FACS buffer and then mix the cell suspension gently by pipetting.
6. Centrifuge at 2,400 × *g* for 5 min at room temperature.
7. Resuspend the cells in 0.5 mL of FACS buffer.

3.2.2 Cell Sorting

See Section 3.1.3, **Steps 9–22**.

3.2.3 Hematopoietic Differentiation from Mouse Embryo-Derived Cells

See Section 3.1.4.

3.2.4 Cardiac Differentiation from Mouse Embryo-Derived Cells

See Section 3.1.5.

3.3 Differentiation of Human ESCs/iPSCs to Hematopoietic and Cardiac Cells

3.3.1 Human ESC/iPSC Culture

1. Prepare MEF feeder layers on gelatin-coated 100 mm culture dishes.
2. Culture human ESCs (KhES-3) and iPSCs (201B7) in 8–10 mL of the culture medium (*see* Section 2.3.2, **Step 3**) on MEF feeder layer.
3. Change entire medium daily.
4. Passage the cells to fresh MEF feeder layers every 5–7 days either with dispase or by colony picking.

3.3.2 Embryoid Body Formation

1. Prepare two or three dishes of 5–7 day-cultured human ESCs/iPSCs.
2. Replace the culture medium to 10 mL of basal medium (*see* Section 2.3.3, **Step 13**).
3. Incubate at 37 °C and 5 % CO_2 for 30 min.
4. Aspirate the basal medium and then add 1–2 mL of dispase solution (*see* Section 2.3.3, **Step 13**).
5. Incubate at 37 °C and 5 % CO2 for 5–10 min.
6. Aspirate the dispase solution, and then add 10 mL of basal medium.
7. Harvest the small clumps of hESC/iPSC colonies into 15 mL tube.
8. Centrifuge at 15 × *g* for 2 min at room temperature.
11. Carefully resuspend the clumps in 10 mL of hEB-1 medium (*see* Section 2.3.2, **Step 14**), and then plate on gelatin-coated 100-mm cell culture dish.
12. Incubate at 37 °C and 5 % CO_2 for 2 h to remove MEFs.
13. Harvest the clumps into 15 mL tube.
14. Centrifuge at 15 × *g* for 2 min at room temperature.
15. Resuspend the clumps in 10 mL of hEB-2 medium (*see* Section 2.3.2, **Step 15**) and then plate on a 100 mm petri dish.
16. On day 1, change the culture medium; harvest the clump-derived EBs into 15 mL tube, centrifuge at 15 × *g* for 2 min at room temperature, carefully and gently resuspend the EBs in 10 mL of hEB-3 medium (*see* Section 2.3.2, **Step 15**), and then plate on fresh 100 mm petri dish.
17. On day 4, change the culture medium to 10 mL of hEB-4 medium (*see* Section 2.3.2, **Step 16**) as described above.
18. On day 7, harvest EBs and prepare them for cell sorting (*see* **Note 4**).

3.3.3 Cell Sorting

1. Transfer 7 day-cultured EBs into 15 mL tube.
2. Aspirate the culture medium, wash with PBS, and then add 1.0 mL of 0.25 % trypsin–EDTA solution.
3. Incubate the culture at 37 °C for 10 min.
4. Gently dissociate the EBs into single cells by pipetting.
5. Add 9.0 mL of FACS buffer and then gently mix the cell suspension by pipetting.
6. Centrifuge at 367 × *g* for 5 min at room temperature.
7. Resuspend the cells in 0.5 mL of FACS buffer.
8. Add an appropriate concentration of mouse anti-human CAR antibody and incubate for 30 min at 4 °C.

9. Wash the cell solution with FACS buffer twice.
10. Resuspend the cells in 0.5 mL of FACS buffer.
11. Add an appropriate concentration of BV421-conjugated anti-mouse IgG antibody and incubate for 20 min at 4 °C.
12. Wash the cell solution with FACS buffer three times.
13. Resuspend the cells in 0.5 mL of FACS buffer.
14. Add an appropriate concentration of PE-conjugated human KDR antibody and incubate for 45 min at 4 °C.
15. Wash the cell solution with FACS buffer twice.
16. Resuspend the cells in 5–10 mL of FACS buffer, and then pass through 70 μm cell strainer just before cell sorting.
17. Sort two KDR^+ subsets (KDR^+CAR^- and KDR^+CAR^+ cells) with FACS Aria or FACS AriaII (BD Bioscience).

3.3.4 Hematopoietic Differentiation from Human ESC/iPSC-Derived Cells

1. A day before sorting, prepare 24-well plates with 80–90 % confluent monolayer of OP9 cells cultured in OP9 medium.
2. Seed FACS-sorted KDR+CAR– cells on OP9 feeder layer with differentiation medium (*see* Section 2.3.6, **Step 10**) at a density of 2.0–5.0 × 10^4 cells/well.
3. After 4–7 days in culture, confirm the hematopoietic differentiation of FACS sorted KDR^+CAR^- cells by FACS and RT-PCR analyses (*see* **Note 5**).

3.3.5 Cardiac Differentiation from Human ESC/iPSC-Derived Cells

1. Prepare gelatin-coated 96-well plates, wash with PBS three times, and then dry the plates.
2. Seed FACS-sorted KDR+CAR+ cells on the gelatin-coated 96-well plates with differentiation medium-1 (*see* Section 2.3.7, **Step** 7) at a density of 0.5–1.0 × 10^5 cells/well.
3. After 2 days in culture, change the culture medium to differentiation medium-2 (*see* Section 2.3.7, **Step 8**). Do the same step every 2 days.
4. On day 7, confirm cardiac differentiation of FACS-sorted KDR^+CAR^+ cells by immunocytochemical and RT-PCR analyses (*see* **Note 6**).

4 Notes

1. Because the percentage of $Flk1^+$ cells in EBs reached peak at day 7, and decreased thereafter, 7 day-cultured EBs were used for cell sorting.
2. To confirm hematopoietic differentiation of mouse ESCs/iPSCs, we evaluate the percentage of Ter119- or

CD45-positive cells by FACS analysis, and examine the expression of marker genes, such as *Scl*, *Runx-1*, *Gata-1*, *Gata-2*, and *Fli-1* with quantitative RT-PCR analysis (*see* Tables 1–3).

3. The cardiac differentiation of mouse ESCs/iPSCs are confirmed by both the expression of a cardiac marker cTNT with immunocytochemical analysis and the expression of marker genes, such as *Tbx-5*, *Mesp-1*, *Gata-4*, *Myl-4*, *cTNT*, *αMHC* with quantitative RT-PCR analysis (*see* Tables 1–3).
4. At day 7 in culture, quantitative RT-PCR confirmed increased expression of marker genes for hematopoietic and cardiac cells. We used *Runx-1* and *Scl* as hematopoietic cell markers, and used *Isl-1* and *Tbx-5* as cardiac cell markers (*see* Tables 3).
5. We detect the hematopoietic differentiation of human ESCs/iPSCs by FACS analysis using CD45 and by quantitative RT-PCR analysis of marker genes, such as *Scl*, *Runx1*, and *Gata-2* (*see* Tables 1–3).
6. Human ESC/iPSC-derived cardiomyocytes are examined by either immunocytochemistry for cTNT and quantitative RT-PCR of marker genes *Tbx-5, Isl-1*, and *cTNT* (*see* Tables 1–3).

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