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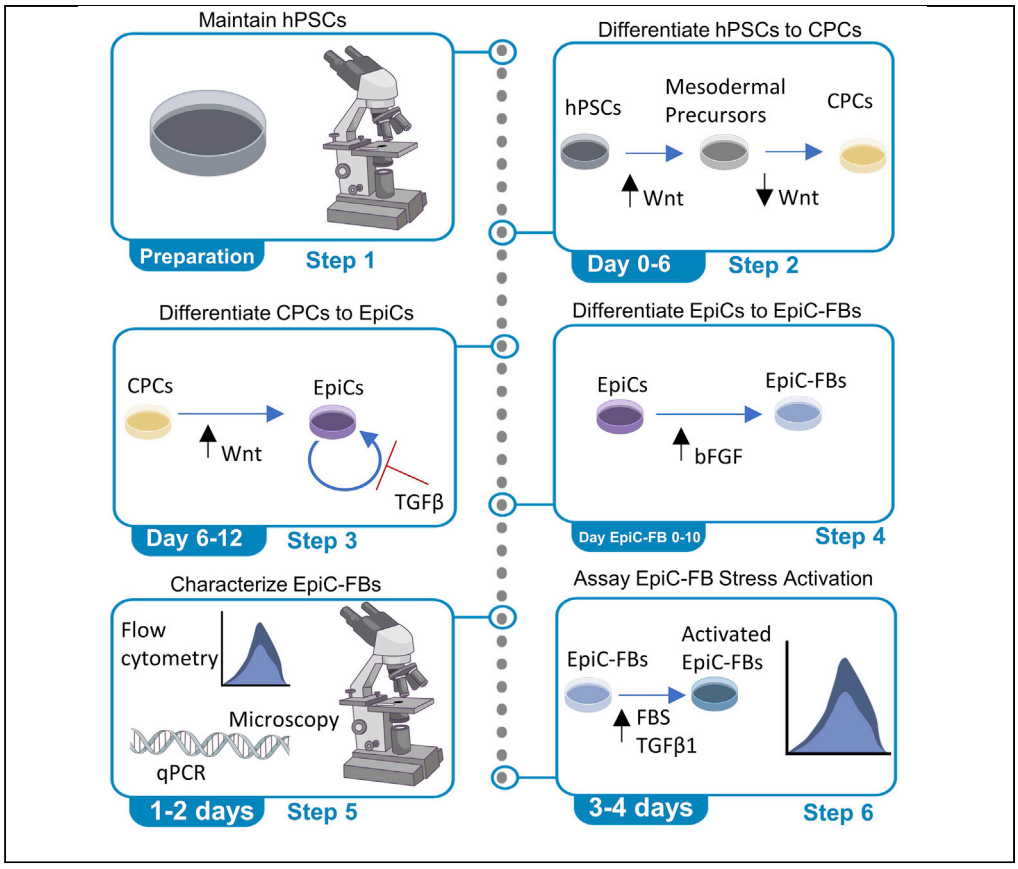
Protocol

Directed differentiation of human pluripotent stem cells to epicardial-derived fibroblasts



Cardiac fibroblasts (CFBs) are a key therapeutic target due to their supportive roles during heart development and response to injury and disease. Here, we describe a robust protocol to differentiate human pluripotent stem cells (hPSCs) into CFBs through an epicardial intermediate. We discuss in detail the characterization of the resulting epicardial-derived fibroblasts (EpiC-FBs) using immunofluorescence microscopy, flow cytometry, and qPCR. We anticipate that these EpiC-FBs can be applied to drug testing, disease modeling, and tissue engineering.

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Highlights

A human pluripotent stem cell (hPSC)-derived cardiac fibroblast model

Detailed protocol to differentiate hPSCs to epicardial-derived fibroblasts (EpiC-FBs)

In-depth description of quality control assays for EpiC-FB differentiations

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Directed differentiation of human pluripotent stem cells to epicardial-derived fibroblasts

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SUMMARY

Cardiac fibroblasts (CFBs) are a key therapeutic target due to their supportive roles during heart development and response to injury and disease. Here, we describe a robust protocol to differentiate human pluripotent stem cells (hPSCs) into CFBs through an epicardial intermediate. We discuss in detail the characterization of the resulting epicardial-derived fibroblasts (EpiC-FBs) using immunofluorescence microscopy, flow cytometry, and qPCR. We anticipate that these EpiC-FBs can be applied to drug testing, disease modeling, and tissue engineering.

For complete details on the use and execution of this protocol, please refer to Bao et al. (2016), Floy et al. (2021), and Lian et al. (2015).

BEFORE YOU BEGIN

Institutional permissions

The human embryonic stem cell lines used in this study are approved for research use by the National Institutes of Health. The study was approved by the University of Wisconsin – Madison Stem Cell Research Oversight Committee. Others who wish to replicate this protocol will need approval from their respective funding agencies and/or institutions.

Preparations

Timing: 2 days to a week

A detailed schematic of human pluripotent stem cell (hPSC) differentiation through cardiac progenitor cells (CPCs) and epicardial cells (EpiCs) to epicardial-derived cardiac fibroblasts (EpiC-FBs) is shown in Figure 1. For additional details on hPSC differentiation to CPCs and EpiCs, please refer to (Bao et al., 2016; Lian et al., 2015). For details on hPSC-derived EpiC-FB phenotype compared to primary and hPSC-derived second heart field progenitor-derived cardiac fibroblasts, please refer to (Floy et al., 2021). We have successfully performed the EpiC-FB differentiation in the H9, H9-cTnT-eGFP, and H9-7TGP human embryonic stem cell (hESC) lines, and the 19-9-11, WTC-CAAX-RFP, and WTC-LMNB1-eGFP human induced pluripotent stem cell (hiPSC) lines. We expect similar differentiation results in other hPSC lines. To perform the differentiation follow these steps:

1. Obtain all key reagents mentioned in the key resources table.
2. Prepare all stock solutions listed below and working aliquots.
3. Order primers and antibodies listed in the key resources table to assess differentiation efficiency.
4. Prepare media following the steps below when needed. Media can be stored at 4°C for up to 2 weeks.

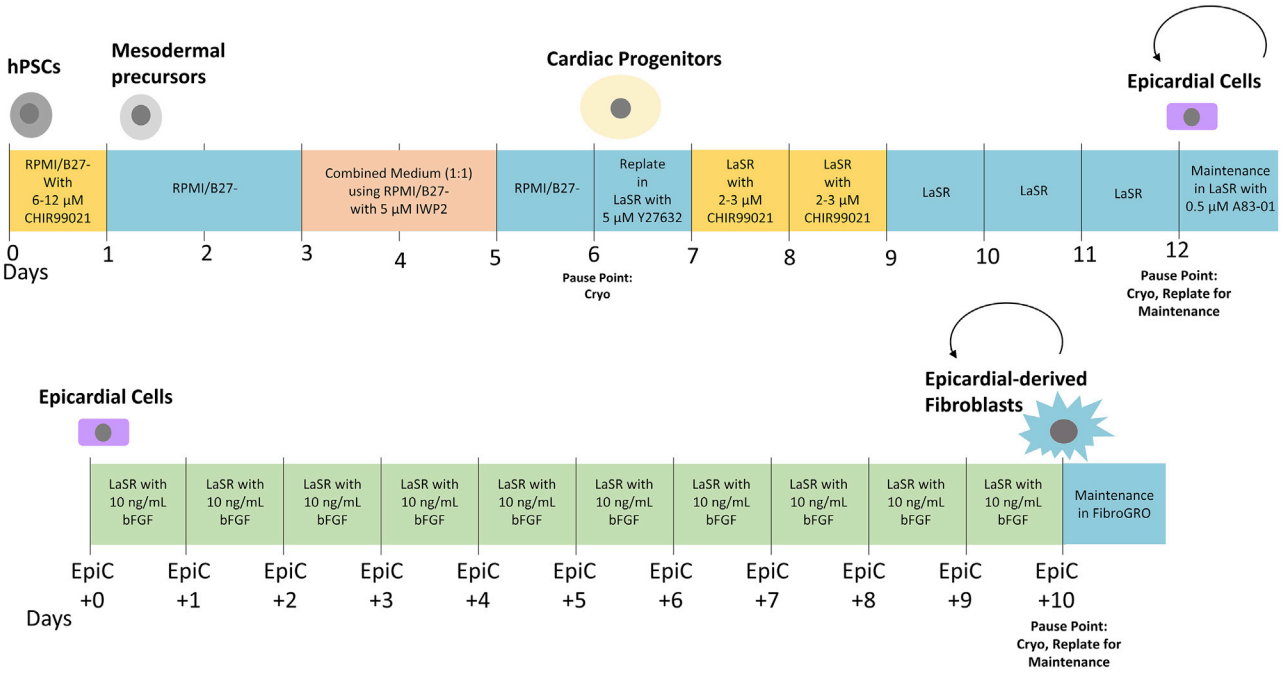

Figure 1. Schematic of EpiC-FB differentiation protocol

5. Obtain/thaw hPSCs and maintain as undifferentiated stem cell cultures in mTeSR1 medium following the protocol below. hPSCs need to be passaged at least twice (>1 week in culture) before starting the differentiation.
6. Obtain/thaw primary human cardiac fibroblasts as a control, if desired.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
WT1, Host: Rabbit IgG, Dilution 1:200, Milk Buffer for Immunofluorescence Microscopy	Abcam	Cat#Ab89901, RRID:AB_2043201
CD90, Host: Mouse IgG1, Dilution: 1:100, BSA Buffer for Immunofluorescence Microscopy	BioLegend	Cat#328102, RRID:AB_940393
VIM, Pre-conjugated 488, Dilution 1:100, BSA Buffer for Immunofluorescence Microscopy	R&D Systems	Cat#IC2105G, RRID:AB_2889353
TE7, Host: Mouse IgG1, Dilution 1:100, BSA Buffer for Immunofluorescence Microscopy	Millipore	Cat#CBL271, RRID:AB_93449
FSP1, Host: Rabbit IgG, Dilution 1:500, BSA Buffer for Immunofluorescence Microscopy	Millipore	Cat#ABF32, RRID:AB_11203822
Fibronectin, Host: Mouse IgG1, Dilution 1:200, BSA Buffer for Immunofluorescence Microscopy	Santa Cruz	Cat#Sc-8422, RRID:AB_627598
SMA, Host: Mouse IgG2a, Dilution 1:100	Invitrogen	Cat#MA5-11544 RRID: AB_10981631
Alexa Flour 488 goat anti-mouse IgG1, Dilution 1:1000	Invitrogen	Cat#A11001, RRID:AB_2534069
Alexa Flour 647 goat anti-mouse IgG1, Dilution 1:1000	Invitrogen	Cat#A21240, RRID:AB_141658
Alexa Flour 488 chicken anti-rabbit IgG, Dilution 1:1000	Invitrogen	Cat#A21441, RRID:AB_2535859
Alexa Flour 647 donkey anti-rabbit IgG, Dilution 1:1000	Invitrogen	Cat#A31573, RRID:AB_2536183
Hoechst 33342 Solution, Dilution: 5 µg/mL	Life Technologies	Cat#H3570

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
A83-01	R&D Systems	Cat#2939
Accutase	Innovative Cell Technologies	Cat#AT104
Advanced D-MEM/F-12	Life Technologies	Cat#12634
B27 supplement minus Insulin	Life Technologies	Cat#A1895601
B27 supplement with Insulin	Life Technologies	Cat#17504044
Bovine Serum Albumin	Sigma-Aldrich	Cat#A9418
CHIR99021	Selleckchem	Cat#S2924-25mg
Dimethylsulfoxide (DMSO), sterile	Sigma	Cat#D8418
Dulbecco's modified Eagle's medium/nutrient mixture F-12 (D-MEM-F12)	Gibco	Cat#11330032
Dulbecco's (DPBS) PBS (without calcium, magnesium)	Sigma-Aldrich	Cat#D8537
ELIMINase decontaminant	Fisher Scientific	Cat#04-355-32
Fetal bovine serum	Life Technologies	Cat#16000-044
FibroGRO Complete Media Kit for Culturing Human Fibroblasts	EMD Millipore	Cat#SCMF001
2% Gelatin solution	Sigma	Cat#G1393
GlutaMAX supplement	Life Technologies	Cat#35050-061
Hydrochloric Acid (HCl)	Sigma	Cat#320331
Human fibroblast growth factor 2 (bFGF)	R&D Systems	Cat#233-FB
Insulin (from bovine pancreas)	Sigma-Aldrich	Cat#I0516-5ML
IWP2	Tocris	Cat#3533-10mg
Knockout Serum Replacement	Life Technologies	Cat#10828-028
L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate	Sigma-Aldrich	Cat#A8960-5G
Matrigel, growth factor reduced	BD Biosciences	Cat#354277
Methanol	Fisher Chemical	Cat#A412-4
mTeSR1 complete kit (basal medium plus 5× supplement)	STEMCELL Technologies	Cat#05857
Non-fat dry milk	Bio-Rad	Cat#170-6404XTU
Omniscript RT Kit	Qiagen	Cat#205111
$OligoDT_{20}$ Primers	Life Technologies	Cat#18418-012
16% Paraformaldehyde	Electron Microscopy Sciences	Cat#15710-S
PowerUP SYBR green master mix	Applied Biosystems	Cat#A25742
QIAshredder columns	Qiagen	Cat#79654
RNase-free DNase set	Qiagen	Cat#79254
RNase-out	Life Technologies	Cat#10777-019
RNeasy mini kit	Qiagen	Cat#74104
ROCK inhibitor Y-27632	Tocris	Cat#1254
RPMI medium 1640	Life Technologies	Cat#11875-119
TGFβ1 Human Recombinant Protein	PeproTech	Cat#100-21-10UG
Triton X-100	Sigma	Cat#T8532
Versene	Life Technologies	Cat#15040-066
Water, sterile, cell culture	Thermo Fisher	Cat#15230147
Experimental models: Cell lines		
Human: H9 (WA09) embryonic stem cells, female	WiCell	RRID:CVCL_9773
Human: H9-cTnT-eGFP (H9-hTnnT2-pGZ-TD2) embryonic stem cells, female	WiCell	N/A
Human: H9-7TGP human embryonic stem cells, female	University of Wisconsin-Madison	Contact Sean Palecek
Human: 19-9-11 (iPS-DF19-9-11T.H) induced pluripotent stem cells, male	WiCell	RRID: CVCL_K054
Human: WTC-LMNB1-eGFP (WTC-mEGFP-LMNB1-cl210) induced pluripotent stem cells, male	Coriell Institute (part of Allen Institute Cell Collection)	RRID:CVCL_IR32
Human: WTC-CAAX-RFP (WTC-mTagRFPT-CAAX-Safe harbor locus AAVS1-cl91) induced pluripotent stem cells, male	Coriell Institute (part of Allen Institute Cell Collection)	RRID:CVCL_VK84
Human: NHCF-V – Adult Ventricular Cardiac Fibroblasts, male	Lonza	Cat#CC-2904
Oligonucleotides (5′ to 3′)		
GATA4-FW: AAACGGAAGCCCAAGAACCT	This paper	N/A

(*Continued on next page*)

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
GATA4-RV: GAGAACGTCTGGGACACGG	This paper	N/A
GAPDH-FW: GAAGGTGAAGGTCGGAGTCAACG	This paper	N/A
GAPDH-RV: TCCTGGAAGATGGTGATGGGAT	This paper	N/A
TBX18-FW: CCCAGGACTCCCTCCTATGT	This paper	N/A
TBX18-RV: TAGGAACCCTGATGGGTCTG	This paper	N/A
TBX20-FW: GAGGGAAAGTGTGGAGAGCC	This paper	N/A
TBX20-RV: AAGGCTGACCCTCGATTTGG	This paper	N/A
Other		
5 mL Round-bottom tube with cell-strainer cap	Falcon	Cat#352235
15 mL Centrifuge tube	BD Biosciences	Cat#352095
50 mL Centrifuge tube	BD Biosciences	Cat#352073
8 well chamber, removable slide	ibidi	Cat#80841
Aria Mx Real-time PCR System	Agilent	Cat#G8830A
Corning tissue culture plates (6 well)	Corning	Cat#3516
Corning tissue culture plates (12 well)	Corning	Cat#3513
Corning tissue culture plates (24 well)	Corning	Cat#3526
Corning tissue culture plates (96 well)	Corning	Cat#3596
Nalgene 1.2 mL CryoTube	Thermo Fisher	Cat#5000-0012
Mr Frosty freezing container	Thermo Scientific	Cat#5100-001
Sterile biosafety cabinets	N/A	N/A
Liquid waste disposal system	N/A	N/A
BD C6 Plus Cytometer	BD Sciences	N/A
Sterilized pasteur pipettes	Fisher Scientific	Cat#13-678-20D
Humidified tissue culture incubator (37°C, 5% CO_2)	N/A	N/A
Hemocytometer	Hausser Scientific	Cat#02-671-52
Inverted phase contrast microscope	N/A	N/A
Microcentrifuge tube (1.5 mL)	Fisher Scientific	Cat#05-408-129
VWR Scientific 1205 Dual Heated Water Bath Incubator	VWR	Cat#14405
Serological pipettes 5 mL	Fisher Scientific	Cat#13-678-11D
Serological pipettes 10 mL	Fisher Scientific	Cat#13-678-11E
Serological pipettes 25 mL	Fisher Scientific	Cat#13-678-11
Stericup filtration system	Millipore	Cat#SCGPU05RE
Barrier Filter Pipette Tips	Thermo Fisher	Cat#2139-05-HR

MATERIALS AND EQUIPMENT

We recommend that cell culture is performed in a sterile environment without the use of antibiotics. Aseptic technique should be sufficient to maintain sterile conditions, and STEMCELL Technologies, producers of mTeSR1 hPSC maintenance medium, recommend antibiotic-free undifferentiated hPSC maintenance. Antibiotics have been shown to induce changes in gene expression in cultured cells, and these changes may affect stem cell growth or differentiation (Cohen et al., 2006; Ryu et al., 2017). We suggest purchasing sterile cell culture materials such as sterile centrifuge tubes, micro-centrifuge tubes, DNAse/RNAse free barrier pipet tips, and serological pipettes. If unsterile, these reagents can be sterilized by autoclaving prior to use. We also suggest that all new items being introduced to the biosafety cabinet are sprayed with 70% (vol/vol) ethanol in deionized water. Routine spraying of gloved hands with 70% (vol/vol) ethanol will also reduce chances of contamination. Additionally, we recommend never passing items or hands over open containers of media, pipettes, or cells.

Media should be prepared under sterile and endotoxin-free conditions or can be sterilized using a 0.22 μm filter prior to use. We recommend that media is warmed on the day of use for at least 30 min at 15°C–25°C. We do not suggest keeping media stocks that have been warmed at 37°C. This will

Table 1. Volumes of medium required for hPSC maintenance, CPC differentiation, EpiC differentiation, EpiC maintenance, EpiC-FB differentiation, and EpiC-FB maintenance

	Volume of media for CPC differentiation	Volume of media for hPSC maintenance, EpiC differentiation, and EpiC-FB maintenance	Volume of media for EpiC maintenance and EpiC-FB differentiation	Number cells required to initiate CPC differentiation	Number cells required to initiate EpiC differentiation	Number cells required to initiate EpiC-FB differentiation
6 well plate	(Not recommended)	2 mL/well	1 mL/well	(Not recommended)	190-480k CPCs/well	50%–100% Confluent EpiCs
12 well plate	2 mL/well	1 mL/well	0.5 mL/well	0.5–2 M hPSCs/well	76-152k CPCs/well	50%–100% Confluent EpiCs
24 well plate	1 mL/well	0.5 mL/well	0.25 mL/well	0.25–1 M hPSCs/well	38-76k CPCs/well	50%–100% Confluent EpiCs
48 well plate	0.5 mL/well	0.25 mL/well	0.125 mL/well	0.125–0.5 M hPSCs/well	19-38k CPCs/well	50%–100% Confluent EpiCs

reduce activity of protein components in the media which are crucial for cell maintenance and viability. If quick use is desired, transfer the desired amount to a conical tube and warm at 37°C until medium is at approximately 15°C–25°C. Recommended media volumes for various plate sizes are shown in Table 1. For lifting cells, Versene solution should also be warmed at 15°C–25°C for 30 min, but Accutase should not be warmed prior to use.

1% Paraformaldehyde

Reagent	Final concentration	Amount
Paraformaldehyde (16% (wt/vol))	1% (wt/vol)	62.5 µL
DPBS	n/a	1 mL
Total	**n/a**	**1.0625 mL**

We do not recommend storing this solution.

⚠ CRITICAL: Causes skin irritation, causes serious eye irritation, may cause an allergic skin reaction, suspected of causing cancer, harmful if inhaled or ingested. Wear protective gloves, wear protective eyewear, and do not mix with bleach.

4% Paraformaldehyde

Reagent	Final concentration	Amount
Paraformaldehyde (16% (wt/vol))	4% (wt/vol)	1 mL
DPBS	n/a	3 mL
Total	**n/a**	**4 mL**

We do not recommend storing this solution.

⚠ CRITICAL: Avoid inhalation, contact with eyes, skin, and clothing. Suspected of causing cancer. Wear protective gloves, wear protective eyewear, and do not mix with bleach.

90% Methanol

Reagent	Final concentration	Amount
Methanol	90% (vol/vol)	45 mL
MilliQ Water	n/a	5 mL
Total	**n/a**	**50 mL**

Store at −20°C for up to 6 months.

⚠ CRITICAL: Flammable. Avoid inhalation, contact with eyes, skin, and clothing. Keep away from heat/sparks/open flames/hot surfaces, wear protective gloves, wear protective eyewear, and keep container sealed tightly.

A83-01 (10 mM)		
Reagent	Final concentration	Amount
A83-01	10 mM	10 mg
DMSO	n/a	2.37 mL
Total	**n/a**	**2.37 mL**

Aliquot and store at −20°C for up to 3 years. Keep a working aliquot at 4°C. Working aliquot can be thawed at 37°C immediately prior to use if necessary.

⚠ CRITICAL: Avoid inhalation, contact with eyes, skin, and clothing. Keep away from heat/sparks/open flames/hot surfaces, wear protective gloves, wear protective eyewear, and keep container sealed tightly.

Alternatives: 10 mM SB431542.

bFGF (0.1 mg/mL)		
Reagent	Final concentration	Amount
bFGF	0.1 mg/mL	25 μg
DPBS	n/a	250 μL
Total	**n/a**	**250 μL**

Aliquot and store at −20°C for up to 3 months. Keep a single working aliquot at 4°C for up to 1 month. Thaw the working aliquot at 15°C–25°C. Avoid freeze-thaw cycles.

⚠ CRITICAL: Avoid inhalation, contact with eyes, skin, and clothing. Wear protective gloves, wear protective eyewear, and keep container sealed tightly.

CHIR99021 (36 mM)		
Reagent	Final concentration	Amount
CHIR99021	36 mM	25 mg
DMSO	n/a	1.49 mL
Total	**n/a**	**1.49 mL**

Aliquot and store at −20°C for up to 3 months. Keep a single working aliquot at 4°C for up to 2 weeks. Working aliquot can be thawed at 37°C immediately prior to use. Avoid freeze-thaw cycles.

⚠ CRITICAL: Avoid inhalation, contact with eyes, skin, and clothing. Fatal if ingested. Wear protective gloves, wear protective eyewear, and keep container sealed tightly.

D-MEM/F-12 containing 10% (vol/vol) FBS		
Reagent	Final concentration	Amount
FBS	10% (vol/vol)	50 mL
D-MEM/F-12	n/a	450 mL
Total	**n/a**	**500 mL**

Aliquot and store at −20°C for up to 3 months. Keep a single working aliquot at 4°C for up to 2 weeks. Working aliquot can be thawed at 37°C immediately prior to use. Avoid freeze-thaw cycles.

⚠ CRITICAL: Avoid inhalation, contact with eyes, skin, and clothing. Fatal if ingested. Wear protective gloves, wear protective eyewear, and keep container sealed tightly.

FibroGRO Medium (commercially available kit)		
Reagent	Final concentration	Amount
FibroGRO Basal Medium		480 mL
rhFGF-b provided in kit	5 ng/mL	1.0 mL
Ascorbic Acid provided in kit	50 µg/mL	0.5 mL
Hydrocortisone Hemisuccinate provided in kit	1.0 µg/mL	0.5 mL
rh Insulin provided in kit	5 µg/mL	0.5 mL
Fetal bovine serum	2% (vol/vol)	10 mL
GlutaMAX	3.66% (vol/vol)	18.75 mL
Total	**n/a**	**511.25 mL**

Store at 4°C for up to 6 months. Avoid warming at 37°C for extended periods of time, because bFGF is unstable at this temperature.

Flow Buffer 1		
Reagent	Final concentration	Amount
Bovine serum albumin	0.5% (wt/vol)	2.5 g
DPBS	n/a	500 mL
Total	**n/a**	**500 mL**

Store at 4°C for up to 6 months. We recommend preparing aliquots of Flow Buffer 1, because contamination will occur if not kept sterile.

Flow Buffer 2		
Reagent	Final concentration	Amount
Bovine serum albumin	0.5% (wt/vol)	2.5 g
1% Triton X-100	0.09% (vol/vol)	50 mL
DPBS	n/a	500 mL
Total	**n/a**	**550 mL**

Store at 4°C for up to 6 months. We recommend preparing aliquots of Flow Buffer 2, because contamination will occur if not kept sterile.

Freezing Medium		
Reagent	Final concentration	Amount
D-MEM/F-12	60% (vol/vol)	30 mL
Fetal Bovine Serum	30% (vol/vol)	15 mL
DMSO	10%	5 mL
Total	**n/a**	**50 mL**

Store at −20°C for up to 2 years or at 4°C for up to 2 months.

⚠ CRITICAL: Avoid inhalation, contact with eyes, skin, and clothing. Keep away from heat/sparks/open flames/hot surfaces, wear protective gloves and wear protective eyewear.

4 mM HCl containing 0.1% (wt/vol) BSA		
Reagent	Final concentration	Amount
HCl (1 M)	4 mM	8 µL
BSA	0.1% (wt/vol)	4 mg
DI Water	n/a	1.992 mL
Total	**n/a**	**2 mL**

Aliquot and store at −20°C.

⚠ CRITICAL: Avoid inhalation, contact with eyes, skin, and clothing. Wear protective gloves, wear protective eyewear, and keep container sealed tightly.

Hoechst staining solution		
Reagent	Final concentration	Amount
Hoechst 33342 stock solution (10 mg/mL)	5 μg/mL	2.5 μL
DPBS	n/a	5 mL
Total	**n/a**	**5 mL**
We do not recommend storing this solution.		

⚠ CRITICAL: Avoid inhalation, contact with eyes, skin, and clothing. Suspected of causing genetic defects if inhaled and harmful if swallowed. Wear protective gloves, wear protective eyewear, and keep container sealed tightly.

Alternatives: 0.1–1 μg/mL DAPI (4′,6-diamindino-2-phenylindole).

IWP2 (5 mM)		
Reagent	Final concentration	Amount
IWP2	5 mM	10 mg
DMSO	n/a	4.28 mL
Total	**n/a**	**4.28 mL**
Aliquot and store at −20°C for up to 1 year. Keep a single working aliquot at 4°C for up to 2 weeks. Working aliquot can be thawed at 37°C immediately prior to use. Avoid freeze-thaw cycles.		

⚠ CRITICAL: Avoid inhalation, contact with eyes, skin, and clothing. Wear protective gloves, wear protective eyewear, and keep container sealed tightly.

Alternatives: 5 mM IWP4.

LaSR Basal Medium		
Reagent	Final concentration	Amount
Advanced D-MEM/F-12	n/a	500 mL
GlutaMAX	1.23 (vol/vol)%	6.25 mL
L-ascorbic acid 2-phosphate	0.006 (wt/vol)%	0.03 mg
Total	**n/a**	**506.25 mL**
Store at 4°C for up to 2 weeks.		

Matrigel Stock Solution Aliquot		
Reagent	Final concentration	Amount
Matrigel, growth factor reduced	2.5 mg	Depends on lot
Total	**n/a**	**Depends on lot, approximately 250 μL**
Aliquot according to manufacturer's datasheet concentration, store at −80°C for up to 6 months. When aliquoting Matrigel, warm Matrigel at 4°C for 8–24 h. Chill microcentrifuge tubes and pipette tips in the freezer at −20°C for 8–24 h. We recommend working quickly when aliquoting as Matrigel is sensitive to temperature.		

Milk Buffer		
Reagent	Final concentration	Amount
Non-fat dry milk	5% (wt/vol)	0.5 g
1% Triton X-100	0.4% (vol/vol)	4 mL
DPBS	n/a	6 mL
Total	**n/a**	**10 mL**
We do not recommend storing this solution.		

mTeSR1 medium (commercially available mTSR1 complete kit)		
Reagent	Final concentration	Amount
Basal medium	n/a	400 mL
5× supplement provided from mTeSR1 complete kit	1×	100 mL
Total	**n/a**	**500 mL**
Matrigel 5× supplement should be thawed at 4°C. Store at 4°C for up to 2 weeks or −20°C for up to 6 months. Avoid warming at 37°C for extended periods of time, because bFGF is unstable at this temperature.		

RPMI/B27 minus insulin		
Reagent	Final concentration	Amount
RPMI 1640	n/a	500 mL
B27 supplement minus insulin	1.96% (vol/vol)	10 mL
Total	**n/a**	**510 mL**
B27 supplement minus insulin should be thawed overnight at 4°C. Store at 4°C for up to 2 weeks. Preparation of RPMI/B27 plus insulin is equivalent.		

TGFβ1 (15 μg/mL)		
Reagent	Final concentration	Amount
TGFβ1	15 μg/mL	10 μg
4 mM HCl containing 0.1% (wt/vol) BSA	n/a	666 μL
Total	**n/a**	**666 μL**
Aliquot and store at −20°C for up to 6 months. Keep a single working aliquot at 4°C for up to 1 month. Thaw the working aliquot at 15°C–25°C. Avoid freeze-thaw cycles.		

⚠ CRITICAL: Avoid inhalation, contact with eyes, skin, and clothing. Wear protective gloves, wear protective eyewear, and keep container sealed tightly.

1% Triton X-100		
Reagent	Final concentration	Amount
Triton X-100	1% (vol/vol)	5 mL
DPBS	n/a	495 mL
Total	**n/a**	**500 mL**
Store at 4°C for up to 6 months.		

⚠ CRITICAL: Avoid inhalation, contact with eyes, skin, and clothing. Wear protective gloves, wear protective eyewear, and keep container sealed tightly.

Y-27632 ROCK inhibitor (5 mM)		
Reagent	Final concentration	Amount
Y-27632	5 mM	10 mg
DPBS	n/a	6.24 mL
Total	**n/a**	**6.24 mL**

Aliquot and store at −20°C for up to 1 year. Keep a single working aliquot at 4°C for up to 1 month. Avoid freeze-thaw cycles.

⚠ CRITICAL: Avoid inhalation, contact with eyes, skin, and clothing. Wear protective gloves, wear protective eyewear, and keep container sealed tightly.

STEP-BY-STEP METHOD DETAILS

Preparing plates

Timing: 1.25 h (Hands-on time: 15 min) for step 1

Timing: 1 day (Hands-on time: 15 min) for step 2

These steps describe preparation of tissue culture plates for cell culture.

1. Preparing Matrigel-coated plates.

Note: This step describes preparation of Matrigel-coated plates that can be used for hPSC maintenance, hPSC differentiation, CPC differentiation, EpiC maintenance, and EpiC differentiation.

a. Pipette 32 mL of D-MEM/F-12 medium into a 50 mL conical tube.
b. Obtain tissue culture plates to be coated. One aliquot of Matrigel solution (2.5 mg, approximately 250 μL) is enough to coat 5 plates.
c. Remove Matrigel stock solution from the −80°C freezer immediately prior to preparing plates.
d. Using a P1000, take up 1 mL of D-MEM/F-12 medium from the conical tube prepared in step 1a and pipette to mix with the single Matrigel solution aliquot until thawed (~10–30 s). Return all contents to the conical tube.
e. Using a 10 mL pipette, mix the Matrigel solution.
f. Add 1 mL of Matrigel solution to each well of the 6 well plate. Add 0.5 mL per well to 12 well plates, etc.
g. If storing the plates for more than 3 days, add extra D-MEM/F-12 so plates do not dry out.

Note: We suggest adding 1–2 mL of D-MEM/F-12 every 3–7 days to prevent the Matrigel wells from drying out.

h. Place the plate in the incubator (37°C, 5% CO_2, humidified). Move the plate in 3 quick, short, back-and-forth and side-to-side motions to ensure the Matrigel solution fully coats the surface of the wells. Matrigel plates should be incubated for at least 1 h prior to use.

⚠ CRITICAL: Matrigel-coated plates can be stored in the incubator (37°C, 5% CO_2, humidified) for up to 2 weeks. If dry spots appear on the plate, those well(s) should not be used for further cell culture.

2. Preparing gelatin-coated plates.

Note: This step describes preparation of gelatin-coated plates that can be used for CPC differentiation, EpiC maintenance, and EpiC differentiation.

a. Obtain 5 tissue culture plates to be coated.
b. Transfer 32 mL of sterile water to a conical tube.
c. Add 160 μL gelatin solution to the conical tube.
d. Using a 10 mL pipette, mix the gelatin solution. Add 1 mL of gelatin solution to each well of a 6 well plate. Add 0.5 mL per well for 12 well plates, etc.

Note: We suggest adding 1–2 mL of sterile water to prevent the gelatin wells from drying out if the plates are to be stored for more than a day.

e. Transfer the plate to the incubator (37°C, 5% CO_2, humidified). In the incubator, move the plate in 3quick, short, back-and-forth and side-to-side motions to disperse the solution across the surface of the wells.
f. On the day of using gelatin plates, aspirate the gelatin solution. Leave lid off the plate for 2–3 h in the sterile biosafety cabinet. Do not use this biosafety cabinet while the plate is drying and do not dry the plate in the incubator. We have observed reduced cell attachment if the gelatin plates are not correctly prepared.

⚠ CRITICAL: Gelatin-coated plates can be kept in the incubator for up to 3 days if they are not allowed to dry out. If dry spots appear on the plate, those well(s) should not be used for further cell culture.

Thawing and freezing cells

Timing: 1 h (Hands-on time: 30 min) for step 3

Timing: 1 h (Hands-on time: 30 min) for step 4

Timing: 1 h (Hands-on time: 30 min) for step 5

Timing: 1 day (Hands-on time: 30 min) for step 6

These steps describe thawing of hPSC lines for producing healthy colonies suitable for EpiC-FB differentiation, CPCs and EpiCs for further differentiation to CFBs, and EpiC-FBs. Additionally, these steps describe freezing of hPSCs, CPCs, EpiCs, and EpiC-FBs for storage in liquid nitrogen.

3. Thawing of human pluripotent stem cells.
   a. Warm mTeSR1 medium at 15°C–25°C for approximately 30 min.
   b. Remove a Matrigel-coated 6 well plate from the incubator.
   c. Transfer 5 mL of mTeSR1 to a 15 mL conical tube.
   d. Transfer 10 mL of mTeSR1 to another 15 mL conical tube. Add 10 μL of 5 mM Y27632 (ROCK inhibitor, final concentration of 5 μM) to the mTeSR1.

⚠ CRITICAL: Including ROCK inhibitor is important for high hPSC recovery after freezing and thawing. If ROCK inhibitor is omitted, hPSCs will not adhere.

   e. Remove a frozen cell cryotube of hPSCs from liquid nitrogen and immerse the cryotube in a 37°C water bath without submerging the cap. Swirl the cryotube gently for 2–3 min until the contents are completely thawed.
   f. Spray the cryotube with 70% (vol/vol) ethanol and move it into a tissue culture hood. Use a sterile 1 mL pipette to gently transfer the cells from the cryotube to the sterile 15 mL conical tube containing 5 mL of 15°C–25°C mTeSR1 medium prepared in step 3c.

Table 2. Recommended number of cells per cryotube and number of wells to thaw a cryotube into for hPSCs, CPCs, EpiCs, and EpiC-FBs

Cell type	Suggested number of wells per cryotube	Approximate cell count per cryotube	Number of wells to thaw cryotube of cells into	Medium to thaw cells into	Maintenance medium
hPSCs	1 confluent well from a 6 well plate	1–2 M	3–6 wells of a 6 well plate, will be confluent in 2–4 days depending on hPSC line, hPSC passage number, etc.	mTeSR1 + 5 mM Y-27632	mTeSR1
CPCs	4–6 wells from 12 well plate	6–12 M	4–6 6 well plates (for EpiC differentiation)	LaSR + 5 mM Y-27632	Directly differentiate into EpiCs, cannot be maintained as CPCs
EpiCs	Entire confluent 6 well plate	1 M	1 6 well plate *generally notice a lot of cell death, and observed better survival if replated at a higher density, will be confluent in 3–6 days	LaSR + 5 mM Y-27632 + 0.5 μM A83-01	LaSR + 0.5 μM A83-01
EpiC-FBs	1 confluent well from a 6 well plate	1 M	1–2 6 well plates, Will be confluent in 4–7 days	FibroGRO	FibroGRO

Note: Submerging the cryotube cap in the water bath will increase the likelihood of contamination.

Note: Keeping the cryotube in the water bath too long will reduce cell viability.

g. Centrifuge the cells at 200 × *g* for 5 min at 15°C–25°C.
h. Label the Matrigel-coated plate with hPSC line, hPSC passage number, date frozen, date thawed, and initials.
i. Aspirate the supernatant from the 15 mL conical tube and aspirate the liquid from the wells of the Matrigel-coated plate.
j. Resuspend the cell pellet in 10 mL of mTeSR1 medium containing 5 μM Y27632 prepared in step 3d. Slowly add 1.5 mL of the cell suspension into each well of the Matrigel-coated 6 well plate.
k. Return the plate to the 37°C, 5% CO_2 incubator. Move the plate in 3 quick, short, back-and-forth and side-to-side motions to evenly disperse the cells across the surface of the wells. Please see Table 2 for further information regarding cryopreservation. Troubleshooting 1.

4. Thawing of cardiac progenitor cells and epicardial cells.
   a. Warm LaSR medium at 15°C–25°C for 30 min.
   b. Remove a Matrigel-coated or gelatin-coated 12 well plate from the incubator.
   c. Transfer 5 mL of LaSR medium to a 15 mL conical tube.
   d. Transfer 12 mL of LaSR to another 15 mL conical tube. For CPCs, add 12 μL of 5 mM Y27632 (ROCK inhibitor, final concentration of 5 μM). For thawing EpiCs, add add 12 μL of 5 mM Y27632 (ROCK inhibitor, final concentration of 5 μM) and 0.6 μL of 10 mM A83-01 (TGFβ inhibitor, final concentration of 0.5 μM).

⚠ CRITICAL: Including ROCK inhibitor is important for efficient EpiC and CPC attachment after thawing.

Optional: For thawing EpiCs, add 120 μL FBS of tothe medium (final concentration of 1% (vol/vol) FBS). This helps to increase viability and attachment of EpiCs.

   e. Remove a frozen cryotube of CPCs or EpiCs from liquid nitrogen storage and immerse the cryotube in a 37°C water bath without submerging the cap. Swirl the cryotube gently for 2–3 min until the contents are completely thawed.

Note: Submerging the cryotube cap in the water bath will increase the likelihood of contamination.

Note: Keeping the cryotube in the water bath too long will reduce cell viability.

f. Spray the cryotube with 70% (vol/vol) ethanol and transfer it into a tissue culture hood. Use a sterile 1 mL pipette to gently transfer the cells into a sterile 15 mL conical tube containing 5 mL of warmed LaSR medium prepared in step 4c.
g. Centrifuge the cells at 200 × *g* for 5 min at 15°C–25°C.
h. Label plate with hPSC line, hPSC passage number, date thawed, date frozen, and initials. When thawing EpiCs, also record the EpiC passage number.
i. Aspirate the supernatant from the conical tube, and aspirate the liquid from the wells of the fresh Matrigel or gelatin-coated plate.
j. Resuspend the cell pellet in the medium prepared in step 4d. Slowly add 1 mL of the cell suspension into each well of the 12 well Matrigel or gelatin-coated plates.
k. Return the plate to the 37°C, 5% CO_2 incubator. Move the plate in 3 quick, short, back-and-forth and side-to-side motions to disperse the cells across the surface of the wells. Please see Table 2 for further information regarding cryopreservation. Troubleshooting 3, Troubleshooting 4.

5. Thawing of epicardial-derived fibroblasts.
   a. Warm FibroGRO medium at 15°C–25°C for 30 min.
   b. Obtain a 6 well tissue culture plate (uncoated).
   c. Transfer 5 mL of FibroGRO to a 15 mL conical tube.
   d. Remove a frozen cryotube of EpiC-FBs from liquid nitrogen and immerse the cryotube in a 37°C water bath without submerging the cap. Swirl the cryotube gently for 2–3 min until the contents are completely thawed.

   Note: Submerging the cryotube cap in the water bath will increase the likelihood of contamination.

   Note: Keeping the cryotube in the water bath too long will reduce cell viability.

   e. Spray the cryotube with 70% (vol/vol) ethanol and transfer it into a tissue culture hood. Use a sterile 1 mL pipette to gently transfer the cells into a sterile 15 mL conical tube containing 5 mL of warmed FibroGRO medium prepared in step 5c.
   f. Centrifuge the cells at 200 × *g* for 5 min at 15°C–25°C.
   g. Label plate with hPSC line, hPSC passage number, EpiC passage number, EpiC-FB passage number, date thawed, date frozen, and initials.
   h. Aspirate and discard the supernatant from the conical tube.
   i. Resuspend the cell pellet in 12 mL of FibroGRO. Slowly add 2 mL of the cell suspension into each well of the 6 well tissue culture plate (uncoated).
   j. Transfer the plate to the 37°C, 5% CO_2 incubator. Move the plate in 3 quick, short, back-and-forth and side-to-side motions to disperse the cells across the surface of the wells. Troubleshooting 10.

6. Freezing of cells.

Note: This step describes freezing of cells for long term storage in liquid nitrogen.

⚠ CRITICAL: Do not refreeze cells that were recently thawed as this will decrease overall viability. We suggest expanding cells for at least two passages after thaw before refreezing.

a. Prepare freezing medium: 10% DMSO, 30% FBS, 60% medium (for hPSCs use mTeSR1, for others use D-MEM/F-12) supplemented with 5 μM Y27632. Prepare 1 mL of freezing medium/cryotube of frozen cells. Freezing medium can be stored for several months at −20°C.
b. Obtain a Mr Frosty freezing container.

⚠ CRITICAL: The Mr Frosty freezing container must be at 15°C–25°C. If not, this will alter the freezing rate of the cells and reduce overall viability.

c. Label cryotubes with hPSC line, cell type, hPSC passage number, freezing date, initials, and number of cells/cryotube. For EpiCs and EpiC-FBs, add information on respective passage numbers.
d. Treat wells to be frozen with Accutase until cells are singularized and lifted. Add 1 mL of Accutase per well to each well of a 6 well plate or 0.5 mL of Accutase per well to each well of a 12 well plate. Incubate plate at 37°C, 5% CO_2 for 5–7 min for hPSCs, 10–30 min for CPCs, 5–10 min for EpiCs, and 5–10 min for EpiC-FBs.

Note: If there is a large amount of cell debris in the wells, we recommend washing with 1 mL/well sterile, DBPS (at 15°C–25°C) prior to addition of Accutase.

e. Prepare the quench solution by filling a conical with 1:1 (vol/vol) of medium (for hPSCs, use mTeSR1, for others use D-MEM/F-12) to Accutase solution.
f. After cells have begun to lift off the plate, use a 1 mL pipette to pipette up and down singularizing the cells, and transfer Accutase-lifted cell solution to the conical containing the quench medium.
g. Count the total number of cells in the conical using a hemocytometer.
h. Centrifuge the cells at 200 × *g* for 5 min at 15°C–25°C.
i. Aspirate supernatant from the conical tube. Resuspend the cell pellet at desired concentration in freezing medium such that there is 1 mL of freezing medium/cryotube. Quickly aliquot into cryotubes.
j. Quickly cap all freezing cryotubes and transfer them into the Mr Frosty freezing container. Do not tighten Mr Frosty freezing container cap fully.

Note: Mr Frosty freezing container cap may become difficult to unscrew if tightened too much at 15°C–25°C.

k. Chill the Mr Frosty freezing container in −80°C freezer for 8–24 h.
l. The next day, move all cryotubes to liquid nitrogen storage. We do not suggest leaving cryotubes in the −80°C for long term storage.

Note: Table 2 shows the suggested number of cells to freeze per cryotube and the number of wells to thaw a single cryotube into for hPSCs, CPCs, EpiCs, and EpiC-FBs.

Maintenance of human pluripotent stem cells

Timing: 45 min (Hands-on time: 15 min) for step 7

Timing: 45 min (Hands-on time: 15 min) for step 8

These steps describe daily maintenance of hPSCs. Additionally, these steps describe passaging of hPSCs colonies using Versene. hPSCs split at a 1:6 ratio should be passaged at 70%–90% confluency approximately every 3–4 days. If split at a 1:12 ratio, hPSCs should be passaged approximately every 4–5 days.

7. Daily maintenance of human pluripotent stem cells.
   a. Daily, warm mTeSR1 at 15°C–25°C for approximately 30 min.
   b. Aspirate the medium from each well of the 6 well plate and replace it with 2 mL of fresh warmed mTeSR1 medium. Repeat this medium replacement daily until cells are ready for passage.
8. Passaging human pluripotent stem cells using Versene.
   a. Warm Versene and mTeSR1 at 15°C–25°C for approximately 30 min. Obtain a Matrigel-coated 6 well plate.
   b. Aspirate the medium from a single well of a 6 well plate of hPSCs and add 1 mL of warmed Versene. At this point, 1–2 million cells should be present in each well.

   Note: We suggest using a single well from a confluent 6 well plate of hPSCs for Versene passaging to expand the hPSC line and using the remaining 5 wells to start a differentiation.

   c. Incubate the plate at 37°C, 5% CO_2 and wait for 5 min.

   Note: Versene incubation time may need to be adjusted. If cells do not detach easily by mechanical dissociation after a 5 min Versene incubation, incubate longer during the next passage. If cells are floating in the Versene in less than 5 min, incubate for less time during the next passage.

   d. Transfer 10 mL of mTeSR1 to a 15 mL conical tube.
   e. Aspirate the Versene.
   f. Using a P1000, remove 1 mL of mTeSR1 from the conical containing mTeSR1 medium prepared in step 8d. Dispense the mTeSR1 medium over the surface of the plate well with the pipette tip perpendicular to the surface until all the colonies are detached (~2–3 washes). After the cells are detached from the surface of the well, transfer the contents of the single well into the sterile conical tube containing 10 mL of mTeSR1.

   ⚠ CRITICAL: hPSCs must be gently passaged. If there are only a few cells per colony after Versene passaging, we suggest using a 5 mL or 10 mL glass pipette instead of a P1000 at this step.

   g. Aspirate the liquid from a fresh 6 well Matrigel-coated plate.
   h. Gently mix the conical contents using a 10 mL pipette.
   i. Seed 1.5 mL of the cell suspension into each well of a Matrigel-coated plate.

   ⚠ CRITICAL: The split ratio is variable, although generally between 1:6 and 1:18 is appropriate when using Versene for passaging. A general rule is to observe the last split ratio and adjust the ratio according to the appearance of the hPSC colonies. If the cells have an undifferentiated morphology (colony diameter <500 μm) and colonies have enough space (more than 10 μm) between them to expand, split them using the same ratio. If they are overly dense and crowded (colony diameter >500 μm, spacing between colonies <10 μm), increase the ratio; decrease the ratio if the cells are sparse (colony diameter <50 μm, spacing between colonies >500 μm). Ideally, hPSCs should be split every 3–5 days.

   j. Return the plate to the incubator after plating the cells. Move the plate in 3 quick, short, back-and-forth and side-to-side motions to evenly disperse the cells across the surface of the wells.

   Note: hPSC culture can be maintained by repeating these steps until cardiac differentiation. We have been able to accomplish successful differentiations across a range of passages from 30 to 70.

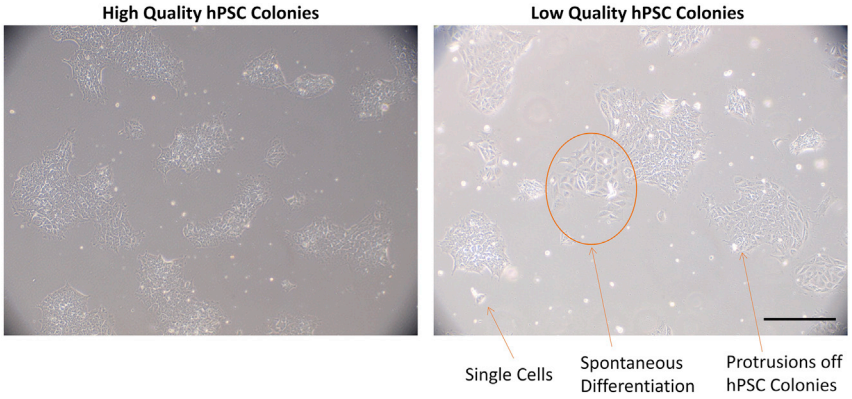

Figure 2. Brightfield images of 19-9-11 hiPSCs

Left image shows high quality 19-9-11 colonies two days after passaging at a 1:6 split ratio with Versene. Right image shows low quality hPSC colonies with arrows pointing out single cells, spontaneous differentiation, and protrusions off the edges of the colonies. Scale bar is 100 μm.

⚠ CRITICAL: hPSC colonies should be constantly monitored for spontaneous differentiation, changes in colony morphology, and/or large changes in growth rate. Example images are shown in Figure 2. If these events occur, we suggest thawing a new cryotube of hPSCs. If this problem is consistent throughout multiple banked cryotubes, we suggest performing colony picking or obtaining a new cryotube of hPSCs from commercial sources. If this problem is consistent across multiple hPSC lines, we suggest obtaining new Matrigel and mTeSR1 stocks.

Differentiation of human pluripotent stem cells to cardiac progenitor cells

Timing: 8 days

Timing: 1 h (Hands-on time: 30 min) for step 9

Timing: 45 min (Hands-on time: 15 min) for step 10

Timing: 45 min (Hands-on time: 15 min) for step 11

Timing: 45 min (Hands-on time: 15 min) for step 12

Timing: 45 min (Hands-on time: 15 min) for step 13

Timing: 45 min (Hands-on time: 15 min) for step 14

These steps describe the differentiation of hPSCs to cardiac progenitor cells (CPCs). CPCs can be further differentiated into cardiomyocytes or EpiCs. For background on the development of this protocol and differentiation to cardiomyocytes, please see (Lian et al., 2015). In this part of the protocol, we will describe differentiation of hPSCs into CPCs in terms of a single 12 well plate which requires approximately 12 million hPSCs or 5 wells of a confluent 6 well plate. This differentiation can be scaled to a 24 well plate or a 48 well plate as shown in Table 1. Additionally, we will use a common convention in hPSCs differentiations where we denote Day 0 as the day where we induce differentiation using CHIR99021 and use negative numbers to denote expansion of hPSCs.

9. Day -2.
   a. Warm mTeSR1 at 15°C–25°C for approximately 30 min.

b. Remove hPSCs cultured on Matrigel-coated 6 well plates in mTeSR1 medium to confluency from the incubator.
c. Aspirate the medium from each well and add 1 mL of Accutase to 5 wells of a 6 well plate.
d. Incubate the plate at 37°C, 5% CO_2 until cells are suspended, approximately 5 min.

Note: Gentle agitation of the plate should be sufficient to resuspend the hPSCs in the Accutase solution.

e. Prepare the quench medium by adding 5 mL of mTeSR1 to a conical tube.
f. Transfer all of the cell suspensions to the conical tube prepared in step 9e using a P1000.
g. Count the total cell number in the conical tube using a hemocytometer.
h. Centrifuge the cells at 200 × *g* for 5 min at 15°C–25°C.
i. Prepare the plating medium by adding 27 mL of mTeSR1 with 27 μL of 5 mM Y27632 (ROCK inhibitor, final concentration of 5 μM) in a conical tube.
j. Obtain a 12 well Matrigel-coated plate and label with hPSC line, "Cardiac", "Day -2", seeding density, hPSC passage number, date, and initials.
k. Aspirate liquid from a fresh Matrigel-coated plate and add 2 mL of mTeSR1 with 5 μM Y27632 to each well using the plating medium prepared in step 9i.
l. After centrifuging the cell suspension, aspirate the supernatant from the conical tube and resuspend the cell pellet in mTeSR1 with 5 μM Y27632 at a cell density of 1 million cells per 100 μL using the plating medium prepared in step 9i. Add the corresponding volume of cell suspension to each well. For 1 million cells, add 100 μL. Troubleshooting 1.

⚠ CRITICAL: The starting seeding cell density is crucial for efficient cardiac differentiation. The initial plating density and/or the time of expansion before initiation of differentiation may require optimization for different hPSC lines or expansion conditions. We suggest optimizing the differentiation by testing various seeding densities and CHIR99021 concentrations at Day 0. We recommend plating at a cell density of 0.5 million cells per well of 12 well plate and increasing this stepwise to 2 million cells per well for a specific hPSC lines in the first experiment, and then expanding the cells for 2 days before initiation of differentiation. After determining the optimal seeding density for a given hPSC lines, this seeding density can be used for subsequent differentiation experiments. Another alternative is to try expanding the cells for 3 days before initiating the differentiation on day 0 rather than 2 days.

m. Return the plate to the 37°C, 5% CO_2 incubator after plating the cells. Move the plate in 3 quick, short, back-and-forth and side-to-side motions to disperse the cells evenly across the surface of the wells.

10. Day -1.
    a. Warm mTeSR1 at 15°C–25°C for approximately 30 min.
    b. On Day −1 of differentiation, aspirate the medium and add 2 mL of mTeSR1 to each well of the 12 well plate.
    c. Return the plate to the 37°C, 5% CO_2 incubator.

11. Day 0.
    a. Warm RPMI/B27 minus insulin medium at 15°C–25°C for approximately 30 min.
    b. On Day 0 of differentiation, prepare a conical tube with 25 mL of RPMI/B27 minus insulin supplemented with 5.55 μL of 36 mM CHIR99021 (final concentration of 8 μM).
    c. Aspirate the previous medium and then add 2 mL of RPMI/B27 minus insulin with CHIR99021 to each well of the 12 well plate. Record the time.

⚠ CRITICAL: Recording the time when RPMI/B27 minus insulin with CHIR99021 is added is important, as replacing medium should to be done exactly 24 h after CHIR99021 addition.

Although we identified 8 μM CHIR99021 as the optimal concentration for the H9 hESC line, other hPSC lines will likely respond to CHIR99021 treatment differently. Thus, optimization of CHIR99021 concentration may be required. We recommend screening in a 12-well or 24-well plate a range of seeding densities as discussed in step 9l and treating that range with 6–14 μM CHIR99021 in 2 μM steps in the first experiment. We suggest follow-up experiments in the range of desired seeding densities and CHIR99021 concentrations to identify the optimal conditions and verify these across multiple differentiations. Additionally, we have identified that optimal length of CHIR99021 treatment varies between hPSC lines and may range from 23-48 h. We have also observed variability among CHIR99021 lots, so we recommend optimizing differentiation for each lot.

d. Return the plate to the 37°C, 5% CO_2 incubator. Troubleshooting 2.

12. Day 1.
    a. Warm RPMI/B27 minus insulin at 15°C–25°C for approximately 30 min.
    b. After 24 h (Day 1 of differentiation), aspirate the medium from each well of the 12 well plate and replace it with 2 mL of warmedRPMI/B27 minus insulin. Timing here is critical. We recommend that medium is changed within 5 min of the 24 h mark.
    c. Return the plate to the 37°C, 5% CO_2 incubator.

13. Day 3.
    a. Warm RPMI/B27 minus insulin at 15°C–25°C for approximately 30 min.
    b. On Day 3 of differentiation (72 h after addition of CHIR99021, although timing here is not as critical, ±2 h is acceptable), prepare a conical tube with 12.5 mL of fresh RPMI/B27 minus insulin medium. Add 25 μL of 5 mM IWP2 (final concentration in conditioned medium of 5 μM) to the medium.
    c. Do not shake the plate after taking from the incubator. Collect 12.5 mL of conditioned medium from the induced cells, add the conditioned medium to the conical tube prepared in step 13b, and mix.
    d. Before aspirating the remaining medium in the well of the 12 well plate, gently rock the plate back and forth to suspend cell debris, ensuring that the debris will be removed via aspiration. Aspirate the remaining medium from the 12 well plate.

Note: If there is a large amount of cell debris still remaining in the wells, we recommend washing each well with 1 mL DPBS (at 15°C–25°C) prior to adding the prepared medium.

    e. Add 2 mL of the combined medium containing IWP2 to each well and return the cells to the 37°C, 5% CO_2 incubator.

14. Day 5.
    a. Warm RPMI/B27 minus insulin at 15°C–25°C for approximately 30 min.
    b. On Day 5 of differentiation, aspirate the medium from each well of the 12 well plate and add warmed RPMI/B27 minus insulin at a volume of 2 mL per well. Timing here is less critical, we recommend ±3 h from Day 3.

Note: If there is a large amount of cell debris in the wells, we recommend washing each well with 1 mL DPBS (at 15°C–25°C) prior to adding fresh medium.

    c. Return the plate to the 37°C, 5% CO_2 incubator.

15. Day 6 – CPCs.

Pause point: Cryopreserved CPCs can be stored for more than 3 years in liquid nitrogen.

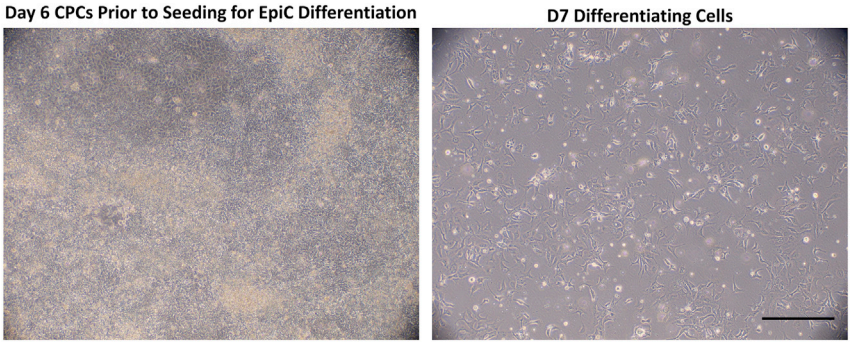

Figure 3. Brightfield images of 19-9-11 hiPSC-derived D6 CPCs (left) and D7 cells (right) after replating for EpiC differentiation

Scale bar is 100 μm.

a. On Day 6, CPCs will be present. These cells can be frozen or differentiated into EpiCs. For freezing CPCs, follow the previous instructions for cryopreservation (step 6). For differentiating CPCs into EpiCs, following the upcoming instructions (steps 16–18).

Note: An example brightfield image of D6 CPCs prior to replating for differentiation to EpiCs and one day later (D7 differentiating cells) is shown in Figure 3.

Note: CPCs can be further differentiated into cardiomyocytes with a medium change to LaSR medium or RPMI supplemented with 2% B27 with insulin medium every 2–3 days until spontaneous beating is observed around Day 12. The cardiomyocytes can be maintained for more than 6 months. (Bao et al., 2016; Lian et al., 2015).

Differentiation of cardiac progenitor cells to epicardial cells

Timing: 6 days

Timing: 1 h (Hands-on time: 30 min) for step 16

Timing: 45 min (Hands-on time: 15 min) for step 17

Timing: 45 min (Hands-on time: 15 min) for step 18

Timing: 1 h (Hands-on time: 30 min) or cryopreservation for step 19

These steps describe differentiation of hPSC-derived CPCs into EpiCs. For additional details on development of this protocol, see (Bao et al., 2016). In this part of the protocol, we will describe the EpiC differentiation in a single 6 well plate which requires approximately 2 M CPCs or 1 well of a 12 well plate. This protocol can be scaled to other plate sizes as shown in Table 1.

16. Day 6 – CPCs.
    a. Warm LaSR medium at 15°C–25°C for approximately 30 min.
    b. Remove a gelatin-coated or Matrigel-coated 6 well plate from the 37°C, 5% CO_2 incubator. If using gelatin-coated plates, make sure that the plates have dried completely before replating.
    c. Prepare plating medium by adding 13 mL of LaSR basal medium and 13 μL of 5 mM Y27632 (ROCK inhibitor, final concentration of 5 μM).
    d. Remove CPCs from the incubator, aspirate medium from a single well of a 12 well plate, and add 0.5 mL of Accutase.

e. Incubate the plate at 37°C for 10–30 min. We recommend gently agitating the plate every 5 min. If the cells are detached and in solution, proceed to the next step.

Note: Trypsin is also a suitable reagent for singularizing CPCs but requires a much shorter incubation time and needs to be quenched with serum-containing medium.

Note: D5 CPCs are also suitable for differentiation to EpiCs. However, we have found that for most hPSC lines D6 CPCs generate a higher percentage of $WT1^+$ EpiCs.

f. Prepare a conical with 0.5 mL of LaSR medium for quenching.
g. Transfer the cell suspension from the well to the conical tube using a P1000. Pipette the cell suspension on the plate to singularize the cells and detach any remaining cells from the plate.
h. Count the total cell number in the conical using a hemocytometer.

Note: If necessary, splitting 1 well of CPCs to a 6 well plate is acceptable. This is approximately the correct density; however, this depends on the seeding density for the cardiac differentiation. Some hPSC lines are more sensitive to than others to the replating, so the seeding density may need to be adjusted. A range of seeding densities, approximately 20,000–40,000 cells/cm^2, is acceptable for differentiation to EpiCs.

i. Centrifuge the cells at 200 × *g* for 5 min at 15°C–25°C.
j. Add 2 mL of plating medium (prepared in step 16c) to each well of the 6 well plate.
k. Label the 6 well plate with hPSC line, hPSC passage number, date, "Day 6 CPCs to EpiC", and initials.
l. After centrifuging the cell suspension, aspirate the supernatant from the conical tube and resuspend the cell pellet in plating medium (prepared in step 16c) at a cell density of 1 million cells per 100 μL. Add 300k cells to each well of a 6 well plate.

Note: If CPCs are thawed from cryopreservation, we recommend replating 350k cells in each well to account for a reduction in cell viability due to freezing.

m. Return the plate to the 37°C, 5% CO_2 incubator after plating the cells. Move the plate in 3 quick, short, back-and-forth and side-to-side motions to disperse the cells evenly across the surface of the wells. Troubleshooting 3.

17. Day 7 and 8.
    a. Warm LaSR medium at 15°C–25°C for approximately 30 min.
    b. Prepare differentiation medium by adding 12.5 mL of LaSR and 1.04 μL of 36 mM CHIR99021 (final concentration of 3 μM) to a conical tube.
    c. Aspirate medium and add 2 mL of LaSR supplemented with CHIR99021 to each well.
    d. Return the plate to the 37°C, 5% CO_2 incubator.

18. Day 9, 10, and 11.
    a. Warm LaSR medium at 15°C–25°C for approximately 30 min.
    b. Aspirate the medium and add 2 mL of LaSR to each well.
    c. Return the plate to the 37°C, 5% CO_2 incubator.

19. Day 12 – EpiCs.

Pause point: EpiCs at Day 12 or any passage afterward can be stored in liquid nitrogen for long periods of time.

a. Warm LaSR at 15°C–25°C for approximately 30 min.

b. Remove gelatin-coated or Matrigel-coated plates from the incubator. If using gelatin-coated plates, make sure that the plates have dried completely before replating.
c. Prepare plating medium by adding 12.5 mL of LaSR, 12.5 μL of Y27632 (final concentration 5 μM), and 0.625 μL of A83-01 (TGFβ inhibitor, final concentration 0.5 μM) to a 15 mL conical tube.

Optional: Add 125 μL of FBS to the plating medium (final concentration of 1% (vol/vol) FBS). This helps to increase viability and attachment of EpiCs.

d. Remove Day 12 EpiCs from the incubator, aspirate medium from 2 wells, and add 1 mL Accutase to those wells. Incubate the plate for 8 min at 37°C, 5% CO_2.

Note: Trypsin can also be used to singularize EpiCs. We recommend using a shorter incubation time and quenching in serum containing medium.

e. Prepare quenching medium by adding 2 mL of LaSR medium to a 15 mL conical tube.
f. Collect the cell suspension from 2 wells and pool in the conical tube using a P1000.

Note: Vigorous pipetting is required to disperse the cells since the cells typically detach as sheets. It is acceptable if the cells are not completely singularized at this stage.

g. Centrifuge the cells at 200 × *g* for 5 min at 15°C–25°C.
h. Label 6 well Matrigel-coated or gelatin-coated plate with hPSC line, hPSC passage number, date, "EpiC P1", and initials.
i. Aspirate supernatant from the conical tube. Using a 10 mL pipette, add 12.5 mL of prepared plating medium from step 19c to the cell pellet and resuspend the cells. Transfer 2 mL of the cell suspension to each well of the 6 well plate.

Note: We recommend splitting at a ratio of 1:3, however up to 1:12 is acceptable.

j. Return the plate to the 37°C, 5% CO_2 incubator after plating the cells. Move the plate in 3 quick, short, back-and-forth and side-to-side motions to disperse the cells evenly across the surface of the wells. Troubleshooting 5.

Maintenance of epicardial cells

Timing: 1 h (Hands-on time: 30 min) or cryopreservation for step 20

Timing: 45 min (Hands-on time: 15 min) for step 21

20. Epicardial Cell Maintenance.
    a. Daily, warm LaSR medium at 15°C–25°C for approximately 30 min.
    b. Prepare EpiC maintenance medium by mixing 50 mL of LaSR medium and 2.5 μL of 10 mM A83-01 (TGFβ inhibitor, final concentration of 0.5 μM).

Note: 50 mL aliquots of LaSR with 0.5 μM A83-01 can be stored for 3–4 days at 4°C.

    c. Daily, aspirate medium from each well and add 1 mL of LaSR medium supplemented with A8301 to each well of the 6 well plate.
    d. Return the plate to the 37°C, 5% CO_2 incubator.

21. Epicardial cell passaging with Versene.
    a. Warm LaSR medium and Versene at 15°C–25°C for approximately 30 min.

b. Obtain a Matrigel-coated or gelatin-coated 6 well plate.
c. For passaging to a single 6 well plate, prepare a conical tube with 6.25 mL of LaSR medium supplemented with 0.31 μL of A83-01 (TGFβ inhibitor, final concentration 0.5 μM).

Note: We recommend splitting at a ratio of 1:3, however 1:2-1:12 is acceptable.

Note: If thawing cells, we suggest using 2 mL of media per well of a 6 well plate for better attachment.

Optional: Add 62.5 μL of FBS of to the medium (final concentration of 1% (vol/vol) FBS). This helps to increase viability and attachment of EpiCs.

d. When the cells are >90% confluent (usually about 5–8 days after passaging, daily monitoring is necessary), aspirate the medium and add 1 mL of warmed Versene to 2 wells. At this point, 300k–500k cells should be present in each well.
e. Incubate the plate at 37°C, 5% CO_2 for 8 min.

Note: Accutase incubation time should be adjusted based on visual observation of the detachment process. If cells do not easily detach from the plate after mechanical dissociation with a pipette tip, cells should be incubated longer the next time EpiCs are passaged. If cells are floating in the Versene before 8 min, EpiCs should be incubated for less time.

f. Aspirate the Versene. Using P1000, take 1 mL of medium out of the conical prepared in step 21c. Dispense the medium over the surface of the plate, with the tip perpendicular to the plate, until all of the colonies are detached (~2–3 washes). Vigorous pipetting is required to disperse the cells since the EpiCs tend to detach from the plate in clumps. After the cells are removed from the surface of the well, pool the contents of all wells into the conical tube containing medium.
g. Aspirate remaining liquid from a fresh Matrigel-coated plate or obtain a fresh gelatin-coated plate. Gently mix the conical tube contents 5–10 times using a 10 mL pipette. Seed 1 mL of the cell suspension into each well of a Matrigel-coated or gelatin-coated 6 well plate.
h. Return the plate to the 37°C, 5% CO_2 incubator after plating the cells. Move the plate in 3 quick, short, back-and-forth and side-to-side motions to disperse the cells across the surface of the wells. Troubleshooting 6, Troubleshooting 7, Troubleshooting 8.

Note: We expect passaged cells to look similar to those shown in Figure 4.

Note: hPSC-derived EpiCs can be passaged 15–20 times. If there are significant changes in morphology (e.g., emergence of mesenchymal-like cells), then discard the plate and perform a new differentiation. Passaging cells at a low density may induce mesenchymal transitions.

Differentiation of epicardial cells to cardiac fibroblasts

Timing: 10 days

Timing: 45 min (Hands-on time: 15 min) for step 22

This step describes differentiation of hPSC-derived EpiCs to CFBs. For detailed characterization of EpiC-FBs, please see (Floy et al., 2021). We will describe the differentiation in terms of a confluent 6 well plate of EpiCs, however the differentiation can be adapted for other well plate sizes as shown in Table 1.

22. Day EpiCs + 0–9.

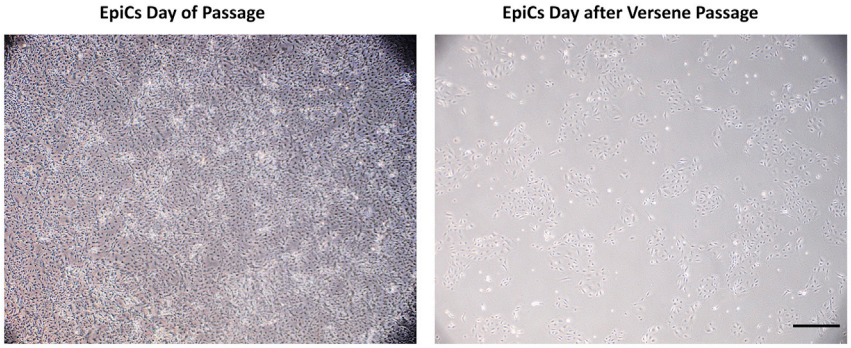

Figure 4. Brightfield images of passaging H9 hESC-derived EpiCs

Left image shows EpiCs immediately prior to Versene passaging and right image shows one day later passaged at a 1:3 split ratio. Scale bar is 200 μm.

a. When EpiCs are at ~100% confluency, based on visual observation, begin the fibroblast differentiation. The initiation of fibroblast differentiation is defined as Day EpiC + 0 in this part of the protocol.

Note: We have successfully differentiated EpiCs at a range of confluencies from 50%-100%.

b. Warm LaSR at 15°C–25°C for approximately 30 min.
c. Prepare medium by adding 5 μL of 0.1 mg/mL bFGF (final concentration of 10 ng/mL) to 50 mL of LaSR.

Note: LaSR containing bFGF can be stored at 4°C for 3–4 days. We do not recommend longer storage as bFGF degrades at 15°C–25°C.

d. Aspirate medium from EpiCs and treat them with 1 mL of LaSR medium containing 10 ng/mL bFGF daily.

Note: We optimized this differentiation protocol by treating EpiCs with 10 ng/mL bFGF or 75 ng/mL bFGF (Figure 5). We did not observe a difference in percentage cells staining positive with TE7 or mean TE7 fluorescence intensity by flow cytometry between treatment with 10 ng/mL bFGF and 75 ng/mL bFGF over 3–10 days of treatment. Additionally, we observed an increase in TE7 staining and VIM expression by flow cytometry across 10 days of 10 ng/mL bFGF treatment but additional treatment out to 14 days did not significantly increase TE7 staining. Therefore, we suggest differentiation with 10 ng/mL bFGF for 10 days.

e. Return the plate to the 37°C, 5% CO_2 incubator.

Maintenance of epicardial-derived cardiac fibroblasts

Timing: 1 h (Hands-on time: 30 min) for step 23

Timing: 45 min (Hands-on time: 15 min) for step 24

23. Passaging of EpiC-FBs (Day EpiC + 10, etc.).

Pause point: EpiC-FBs at Day EpiC + 10 or any passage afterward can be stored in liquid nitrogen for long periods of time.

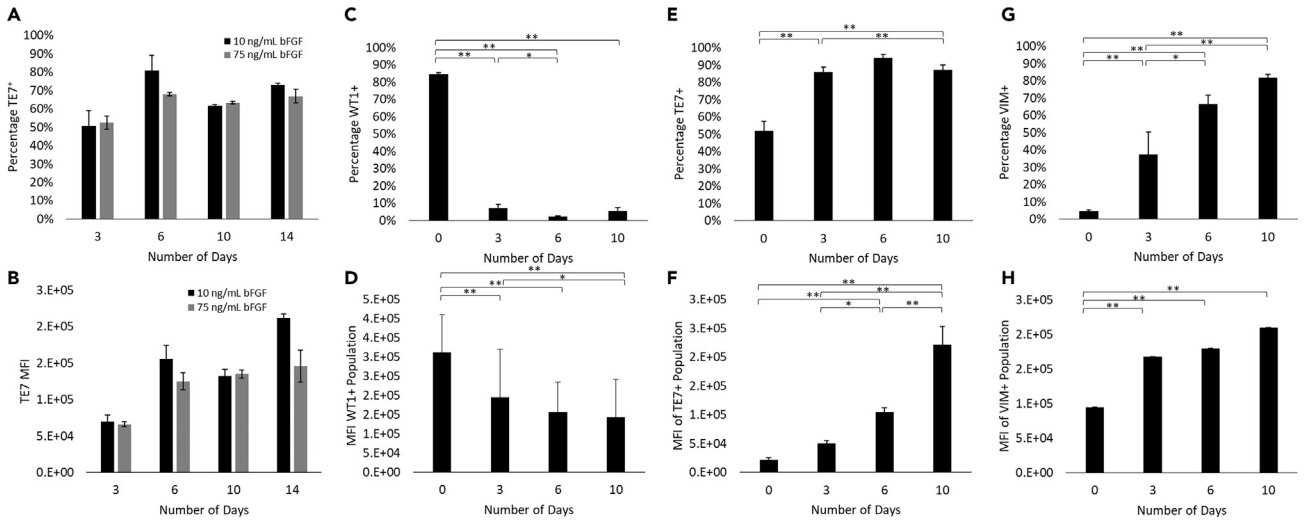

Figure 5. Optimization of EpiC-FB differentiation by flow cytometry

(A-B) Effect of bFGF concentration on TE7 (Cat#CBL271, RRID:AB_93449) expression. Bars represent the average of 3 wells across one differentiation and error bars represent the standard deviation. Black represents 10 ng/mL bFGF and gray represents 75 ng/mL bFGF. One representative differentiation of 3 independent differentiations is shown. Statistics are a two-way ANOVA with Tukey's post-hoc test where * is $p<0.05$.

(C–H) Effect of duration of 10 ng/mL bFGF concentration on WT1 (Cat#Ab89901, RRID:AB_2043201), TE7 (Cat#CBL271, RRID:AB_93449), and VIM (Cat#IC2105G, RRID:AB_2889353) expression. Bars represent the average of 3 wells across one differentiation and error bars represent the standard deviation. One representative differentiation of 3 independent differentiations is shown. Statistics are a one-way ANOVA with Tukey's post-hoc test where * is $p<0.05$ and ** is $p<0.01$.

Note: Passaging is usually required every 4–6 days if EpiC-FBs are passaged at a 1:6 split ratio. After thawing, EpiC-FBs typically take 8–10 days to reach confluence. In 3 independent differentiations, we observed senescence after ~60 days (15 passages).

Note: We expect passaged EpiC-FBs to exhibit morphology similar to the cells shown in Figure 6.

a. Warm FibroGRO medium containing 2% (vol/vol) FBS at 15°C–25°C for approximately 30 min.
b. Add 1 mL of Accutase to one well of a 6 well plate of day 10 EpiC-FB cells. Incubate at 37°C, 5% CO_2 for 10 min.

Note: Trypsin can also be used to singularize EpiC-FBs. We recommend using a shorter incubation time.

c. Prepare quenching medium by adding 1 mL of FibroGRO medium containing 2% (vol/vol) FBS to a conical tube.
d. Harvest Accutase cell suspensions using a P1000 and combine the Accutase cell suspension with the quench medium.

Note: Vigorous pipetting is required as cells tend to detach in clusters.

e. Centrifuge the cells at 200 × *g* for 5 min at 15°C–25°C.
f. Label a 6 well tissue culture plate (uncoated) with hPSC line, hPSC passage number, EpiC passage number, EpiC-FB passage number, date, and initials.
g. Aspirate the supernatant from the conical tube. Using 10 mL pipette, resuspend the cell pellet in 12 mL of FibroGRO medium containing 2% (vol/vol) FBS. Transfer 2 mL of cell solution to each well of the uncoated 6 well tissue culture plate.

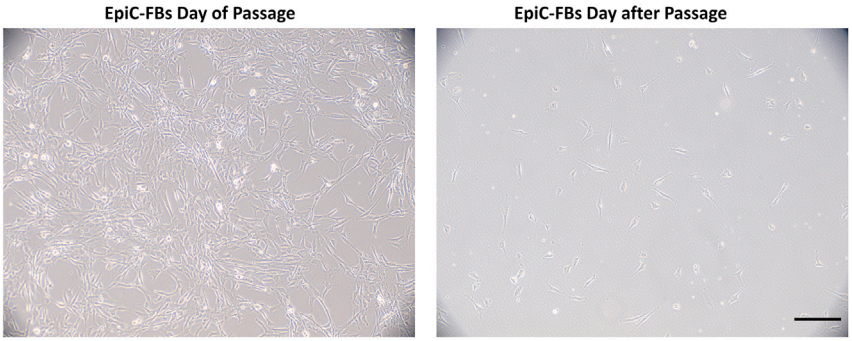

Figure 6. Brightfield images of H9 EpiC-FBs

Left image shows EpiC-FBs immediately prior to Accutase passaging and right image shows one day after passage at a 1:6 split ratio. Scale bar is 200 μm.

Note: We do not recommend a lower split ratio than 1:6 as the cells will quickly become confluent. A 1:12 split ratio is also acceptable.

Note: We recommend replating approximately 66k cells (approximately a 1:6 split ratio) in each well of a 6 well plate.

h. Return the plate to the 37°C, 5% CO_2 incubator after plating the cells. Move the plate in 3 quick, short, back-and-forth and side-to-side motions to disperse the cells across the surface of the wells. Troubleshooting 9.

24. Epicardial-derived fibroblast maintenance.
    a. Warm FibroGRO medium containing 2% (vol/vol) FBS at 15°C–25°C for approximately 30 min.
    b. If cells are below 70%–80% confluent, aspirate medium and add 2 mL of FibroGRO medium containing 2% (vol/vol) FBS to each well every 2 days. Otherwise, passage as described in the previous section.

Note: Maintenance of EpiC-FBs in medium containing higher percentages of serum increased cell size, a marker associated will FB stress. Removal of serum significantly reduced proliferation of EpiC-FBs (Figure 7). Hence, we recommend using FibroGRO medium containing 2% (vol/vol) FBS as the maintenance medium.

c. Return the plate to the 37°C, 5% CO_2 incubator. Troubleshooting 10.

Downstream assays

Timing: 2 days (Hands-on time: first day 1 h, second day 30 min with additional time for imaging) for step 25

Timing: 2 days (Hands-on time: first day 1 h, second day 1 h with additional time for analyzing flow cytometry samples) for step 26

Timing: 1–3 days (Hands-on time: 3 h) for step 27

These steps describe immunofluorescence microscopy, flow cytometry, and qPCR as quality control metrics for a successful EpiC-FB differentiation.

25. Immunofluorescence microscopy.

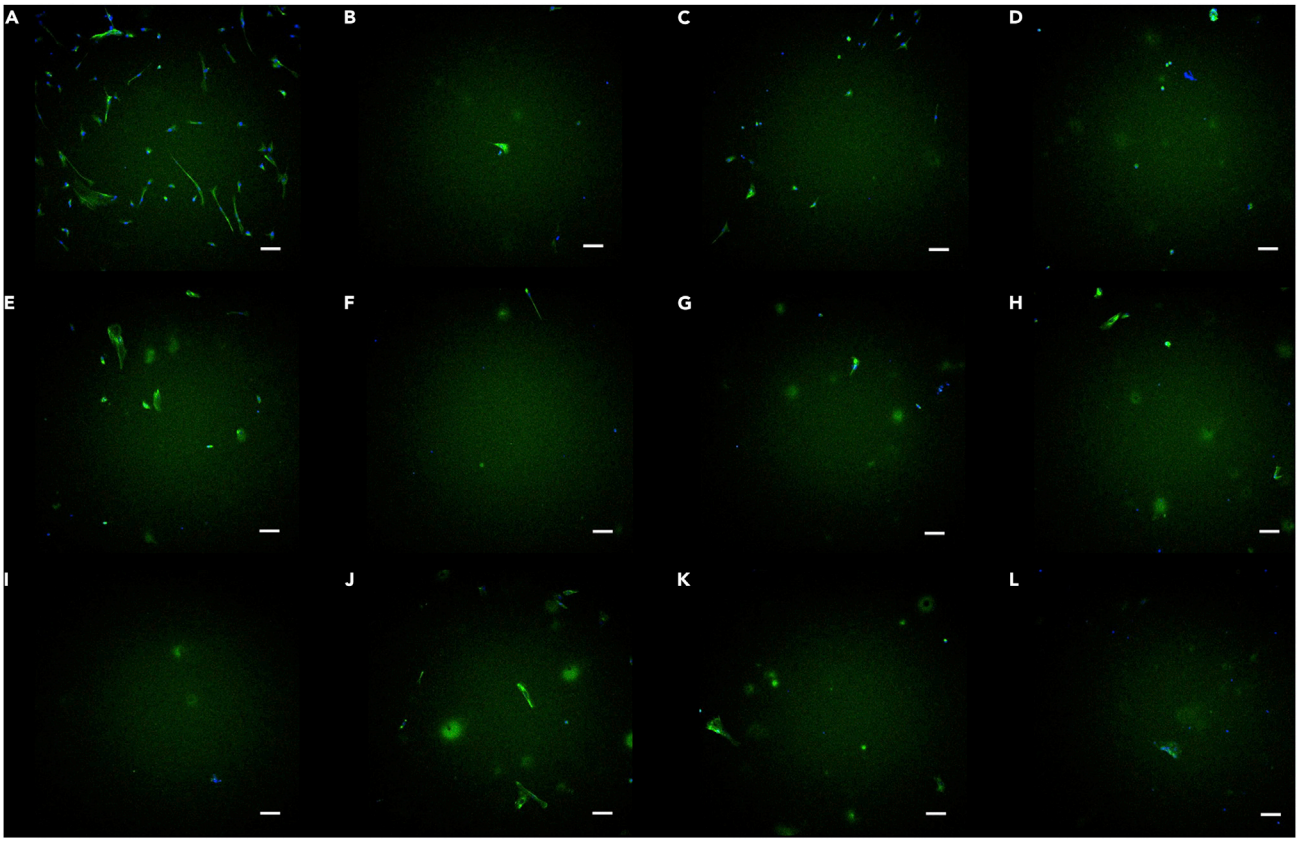

Figure 7. Testing media formulations for maintaining hPSC-FBs. H9 EpiC-FBs were seeded at 5,200 cells per well of a 96 well plate

Immunostaining of H9 EpiC-FBs for vimentin (green, Cat#IC2105G, RRID:AB_2889353) with Hoechst nuclear counterstain (blue) were taken 3 days after seeding cells from thaw are shown where a) FibroGRO containing 2% (vol.vol) FBS b) FibroGRO without FBS c) FibroGRO without FBS with 0.5 μM A83-01 d) FibroGRO without FBS, with 10% (vol/vol) knockout serum replacement e) RPMI f) RPMI with 2% (vol/vol) B27 with insulin supplement g) RPMI with 2% (vol/vol) B27 with insulin supplement and 10 ng/mL bFGF h) RPMI with 5 μg/mL insulin, 10 ng/mL bFGF and 3.75% (vol.vol) GlutaMAX i)RPMI with 2% (vol/vol) B27 with insulin supplement and 3.75% (vol/vol) GlutaMAX j) RPMI with 10 ng/mL bFGF and 5 μg/mL insulin k) RPMI with 10% (vol/vol) knockout serum l)RPMI+GlutaMAX. Examples of one representative differentiation out of 3 independent differentiations are shown in H9 or 19-9-11 hPSC lines. Scale bar is 100 μm.

Note: These steps describe immunofluorescent analysis of fibroblast marker expression via imaging. We recommend plating EpiC-FBs in a 96 well plate for immunofluorescence analysis with 3 well replicates for each staining condition. We suggest including EpiCs and undifferentiated hPSCs as staining controls. If staining for ECM proteins, we recommend using an empty well as a negative control as Matrigel contains ECM proteins that may be bound by the antibodies.

Note: Immunofluorescence can be performed on slides or other well sizes. For a 48 well plate, we recommend using a volume of 100 μL. For a 24 well plate, we recommend using a volume of 150 μL. For a 12 well plate, we recommend using a volume of 300 μL. We do not suggest using a larger plate size since this would require a large amount of antibody.

a. Aspirate medium from the 96 well immunofluorescence plate. Fix cells by adding 50 μL of 4% PFA to each well and incubate at 15°C–25°C for 10 min. Alternatively, fix by adding 50 μL of ice-cold methanol to each well and incubating for 5 min at 15°C–25°C.
b. Aspirate PFA or methanol solution from the cells.

Pause point: If desired, 50 μL/well DPBS can be added to the fixed plates and plates can be sealed with Parafilm for storage at 4°C. We recommend not storing plates more than a few days prior to staining and imaging.

c. Add 50 μL of blocking solution (0.4% (wt/vol) dry milk in 0.1% (vol/vol) Triton X-100 in DPBS or 0.5% (wt/vol) BSA in 0.1% (vol/vol) Triton X-100 in DPBS) to each well. Incubate for 1 h at 15°C–25°C or 8–24 h at 4°C.
d. Prepare primary antibody solutions by diluting antibodies in the blocking buffer according to the key resources table.

Note: We recommend preparing a slight excess in volume of primary and secondary antibody solution as it is important to treat all wells with the same amount of antibody. We also recommend preparing at least 500 μL of antibody solution.

e. After blocking, aspirate blocking solution and add 50 μL of primary antibody solution to each well. Incubate at 15°C–25°C for 1 h or 4°C for 8–24 h. We recommend staining with conjugated antibodies (e.g., VIM) at the same time as the secondary antibodies to prevent photodegradation of the fluorophore.
f. After incubation, aspirate the primary antibody solution from each well and wash each well 3 times with >50 μL of DPBS.
g. Prepare secondary antibody solutions by diluting antibodies in the blocking buffer according to the key resources table.
h. Aspirate the DPBS wash and add 50 μL of secondary antibody solution to each well. Incubate in the dark at 15°C–25°C for 1 h or 4°C for 8–24 h.
i. Aspirate antibody solution and wash each well with >50 μL of DPBS.
j. Aspirate the DPBS wash and add 50 μL of Hoechst solution to each well. Incubate in the dark at 15°C–25°C for 5 min.

Note: Incubating for 10–15 min is acceptable; it will increase the brightness of the Hoechst staining. For image quantification, we suggest that incubation time is kept consistent between experiments.

k. Aspirate Hoechst solution. Add 50 μL DPBS to each well to keep wells from drying out.
l. Image on a fluorescence microscope.
m. Processing of immunostaining results can be done in the Fuji or similar software.

Note: We suggest imaging on an epifluorescence or confocal microscope at 20× or higher magnification to be able to visualize antibody localization.

Note: Expected immunofluorescence results for selected fibroblast markers are shown in Figure 8.

Pause point: Immunostained plates sealed in Parafilm and wrapped in aluminum foil can be stored at 4°C for several days to several weeks depending on the brightness of the stain. We recommend imaging plates as soon as possible.

26. Flow cytometry.

Note: These steps describe flow cytometry to assess fibroblast differentiation efficiency. Flow cytometry can be performed at P1 or any subsequent passage. We suggest using between 0.3 M-0.5 M cells from each sample for a given staining condition and using 3 well replicates from a given differentiation.

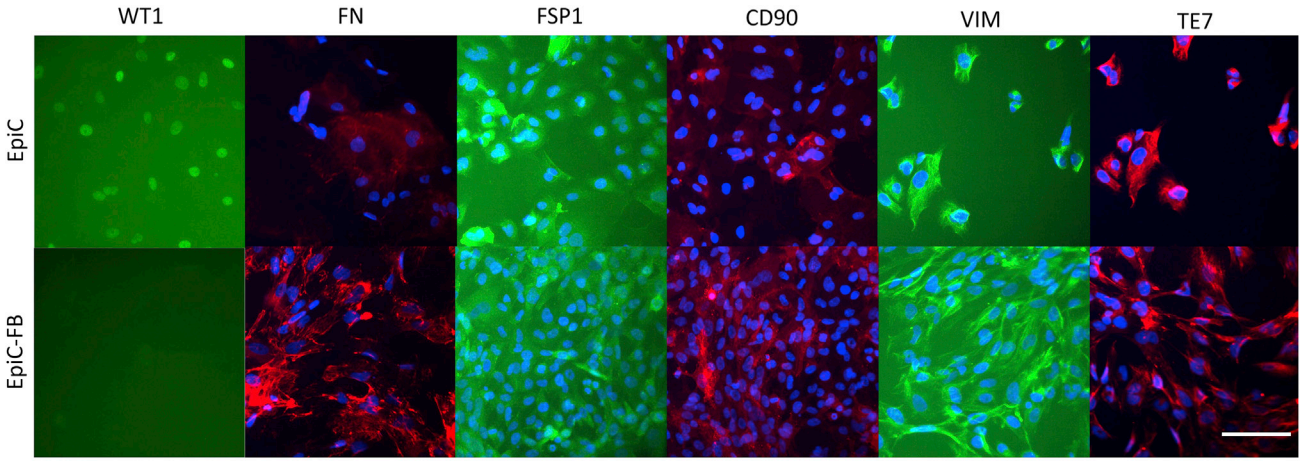

Figure 8. Example immunofluorescence results for EpiC and EpiC-FB differentiations

Example immunofluorescence results of H9 hESC-derived EpiC and H9 hESC-derived EpiC-FB differentiations for WT1 (green, Cat#Ab89901, RRID:AB_2043201), FN (red, Cat#Sc-8422, RRID:AB_627598), FSP1 (green, Cat#ABF32, RRID:AB_11203822), CD90 (red, Cat#328102, RRID:AB_940393), VIM (green, Cat#IC2105G, RRID:AB_2889353), and TE7 (red, Cat#CBL271, RRID:AB_93449). Blue represents nuclear Hoechst staining. Scale bar is 100 μm.

a. Singularize cells using Accutase to detach the cells from the substrate. Follow steps 9b–9h to singularize hPSCs, steps 16d–16i to singularize CPCs, steps 19d-19g to singularize EpiCs, and steps 23b–23e to singularize EpiC-FBs. We recommend using 1 mL/well (6 well plate) or 0.5 mL/well (12 well plate) of Accutase and incubating for 5 min (undifferentiated hPSCs), 8 min (EpiCs), or 10 min (EpiC-FBs) at 37°C, 5% CO_2. We recommend moving the plate and checking if cells are detaching; longer exposure to Accutase may be necessary if cells remain attached.
b. Prepare a 15 mL conical tube for each sample with equal volume D-MEM/F-12 to volume Accutase used for quenching. Label each tube.
c. Transfer Accutase cell suspension into the appropriate conical using a P1000.
d. Count the total number of cells in each conical using a hemocytometer.
e. Centrifuge the cells at 200 × *g* for 5 min at 15°C–25°C.
f. Aspirate the supernatant and add 1 mL of 1% (wt/vol) PFA to each conical and resuspend the pellet using a P1000. Incubate at 15°C–25°C for 20 min to fix the cells.

Note: Fixation for between 5-20 min is acceptable.

g. Centrifuge the cells at 200 × *g* for 5 min at 15°C–25°C.
h. Aspirate the supernatant and resuspend cells in 1 mL/tube of ice cold 90% (vol/vol) methanol in water to allow for permeabilization and analysis of intracellular targets.

Pause point: Store samples at −20°C prior to antibody staining if necessary. Samples can be store at least up to one month for highly expressed proteins.

i. To perform antibody staining and flow cytometry analysis, aliquot 0.3–0.5 M cells in a 15 mL conical. Label the conical tube appropriately.
j. Add 2 mL of Flow buffer 1 to each tube.
k. Centrifuge the cells at 200 × *g* for 5 min at 15°C–25°C.
l. Aspirate the supernatant and resuspend the pellet in 2 mL of Flow buffer 1. Centrifuge the cells at 200 × *g* for 5 min at 15°C–25°C. Repeat the Flow buffer 1 wash.
m. Prepare 100 μL/sample of primary antibody solution in Flow buffer 2 according to the key resources table.

n. Aspirate the supernatant and resuspend the pellet in 100 μL of primary antibody solution.
o. Incubate samples at 15°C–25°C for 1 h or 4°C for 8–24 h.
p. Following incubation, add 2 mL of Flow buffer 1 to each tube.
q. Centrifuge the cells at 200 × *g* for 5 min at 15°C–25°C.
r. Prepare 100 μL/sample of secondary antibody solution in Flow buffer 2 according to the key resources table.
s. Aspirate the supernatant and resuspend the pellet in 100 μL of secondary antibody solution.
t. Incubate samples in the dark for at least 30 min at 15°C–25°C.
u. Aspirate the supernatant then add 2 mL of Flow buffer 2 to resuspend cells. Centrifuge the cells at 200 × *g* for 5 min at 15°C–25°C. Repeat the Flow buffer 2 wash.
v. Aspirate the supernatant then resuspend the cell pellet in 300 μL Flow buffer 1. Transfer the samples to 5 mL flow cytometry round bottom tubes.

Note: We recommend using flow cytometry tubes that have a filter in the lid. This allows for easy filtration at this step immediately prior to flow analysis.

Note: The cell pellet can be resuspended in a volume range from 200-500 μL depending on the desired cell flowrate though the cytometer.

w. Keep samples on ice until performing flow cytometry.
x. Analyze by flow cytometry according to instrument instructions.
y. Processing of flow cytometry data can be done in the FlowJo or similar software.

Note: We recommend collecting at least 10,000 events. For controls, we recommend including samples that express and do not express the marker of interest such as primary cardiac fibroblasts and hPSCs. Isotype controls or no primary controls can be used in addition to validate gating strategies.

Note: Expected flow cytometry results are shown in Figure 9.

27. qPCR.

Note: These steps describe qPCR for cardiac markers (*GATA4*, *TBX18*, and *TBX20*) in the hPSC- derived fibroblast populations compared to housekeeping gene *GAPDH*. Primer sequences are shown in the key resources table. We recommend using at least one confluent well of a 6 well plate of hPSCs, EpiCs, or EpiC-FBs for qPCR analysis per sample. Additionally, we recommend using 3 well replicates from each differentiation. From mRNA extractions, expected yield of one confluent well of a 6 well plate is 40 μL at 100–200 ng/μL for hPSCs, 100–200 ng/μL for EpiCs, and 100–200 ng/μL for EpiC-FBs.

a. Singularize cells using an Accutase lift. Follow steps 9b–9h to singularize hPSCs, steps 16d–16i to singularize CPCs, steps 19d–19g to singularize EpiCs, and steps 23b–23e to singularize EpiC-FBs. We recommend using 1 mL/well (6 well plate) or 0.5 mL/well (12 well plate) and incubating for 5 min (undifferentiated hPSCs), 8 min (EpiCs), or 10 min (EpiC-FBs) at 37°C, 5% CO_2. We recommend shaking the plate and visually checking if cells are lifting; longer exposure to Accutase may be necessary if cells are attached.
b. Prepare a 15 mL conical tube for each sample with equal volume D-MEM/F-12 to volume Accutase used. Label each tube.
c. Transfer the Accutase cell solution to the appropriate conical tubes using a P1000.
d. Centrifuge the cells at 200 × *g* for 5 min at 15°C–25°C.
e. Aspirate the supernatant.

Optional: Freeze the cell pellet at −80°C prior to mRNA extraction for at least 1 h.

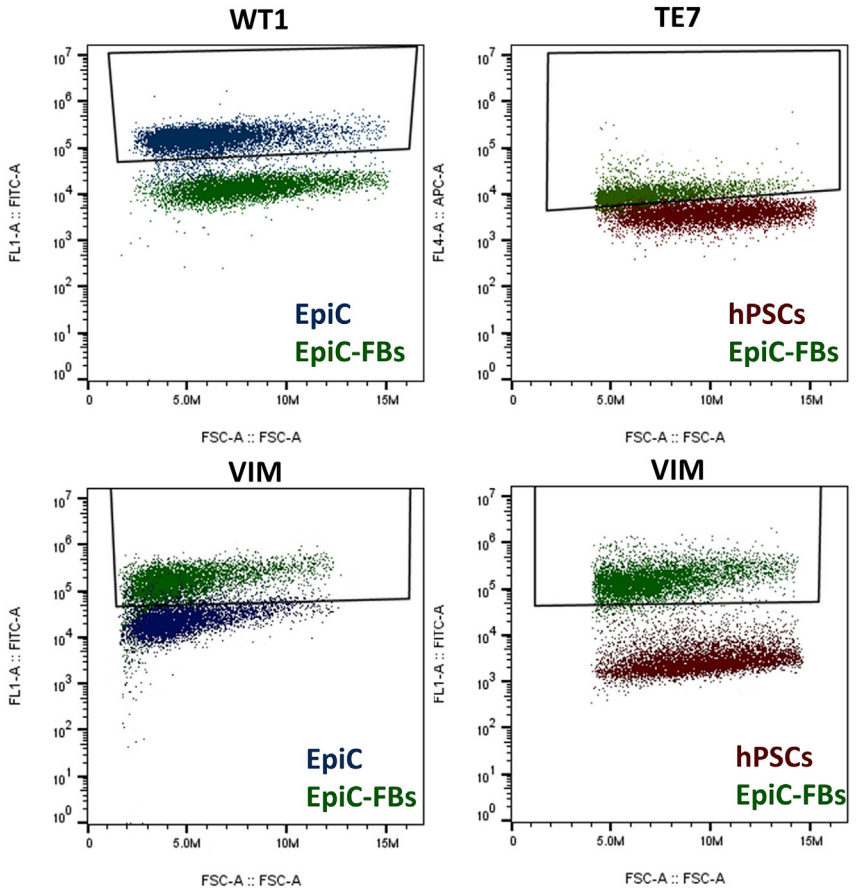

Figure 9. Example flow cytometry results for EpiC and EpiC-FB differentiations

For WT1 (Cat#Ab89901, RRID:AB_2043201), EpiCs were gated as positive compared to the EpiC-FB population. By these gates, 90% of the EpiCs and less than 1% of the EpiC-FBs expressed WT1. For VIM (Cat#IC2105G, RRID:AB_2889353) and TE7 (Cat#CBL271, RRID:AB_93449), undifferentiated hPSC samples were used as a negative control compared to EpiC-FBs to determine appropriate gating. By these gates, 99% of the EpiC-FBs and less than 2% of the undifferentiated samples expressed VIM. Additionally, 83% of the EpiC-FBs and less than 1% of the undifferentiated samples expressed TE7.

Pause point: Cell pellets can be stored at −80°C for several months prior to extraction. We recommend only storing cell pellets for about a week before RNA extraction to minimize RNA degradation.

f. Extract mRNA using a commercially available kit (e.g., Qiagen RNeasy kit) according to manufacturer's instructions, including the optional DNase treatment (Qiagen).

⚠ CRITICAL: At this stage the mRNA is very susceptible to degradation. Clean gloves well using ELIMINase decontaminant. Take care to not contaminate samples.

⚠ CRITICAL: Change pipette tips between every sample. Do not cross contaminate RNA samples or contaminate the stock reagents with RNA.

Note: Although we suggest using a Qiagen mRNA extraction kit and DNAse kit, other similar kits are acceptable. We strongly recommend DNAse treatment as this will remove genomic DNA.

g. Quantify mRNA using Nanodrop spectrophotometer. Ensure that A260/A280 peak ratio is approximately 2, and A230/A280 is approximately 2 to verify mRNA quality.

Note: A low 260/280 peak suggests that the sample has deteriorated or that there is DNA contamination and a 230/280 peak that is far from 2 is indicative of organic contaminants.

h. Store mRNA at −80°C.

Pause point: mRNA can be stored at −80°C for over a year if freeze-thaw cycles are avoided. We recommend thawing mRNA on ice.

i. Reverse transcribe 1,000 ng mRNA into cDNA using Omniscript Reverse Transcriptase kit (Qiagen) and Oligo(dT)20 Primers (Life Technologies) according to manufacturer's instructions.

⚠ CRITICAL: Clean gloves well using ELIMINase decontaminant. Take care to not contaminate samples.

Note: Although we suggest using a Qiagen Reverse Transcriptase kit, other similar kits are acceptable.

j. Store cDNA at −80°C until qPCR analysis.

Note: cDNA can be stored for over a year at −80°C. Try to avoid freeze-thaw cycles. If a significant change in Ct values is observed over time, then prepare new cDNA as this likely is due to degradation of the cDNA.

k. Dilute primers (sequences in the key resources table) to 100 nM in DNA/RNA free water. Prepare working aliquots of 10 μL 100 nM forward primer, 10 μL 100 nM reverse primer, and 180 μL DNA/RNA free water in a conical tube. We recommend storing primers at −20°C.

l. To perform qPCR, prepare reaction mixtures in a microcentrifuge tube with 1 μL primer mixture, 10.5 μL DNA/RNA free water, and 12.5 μL PowerUP Sybr Master Mix per desired reaction. We recommend performing technical replicates of each sample. A reference gene should be included; we recommend *GAPDH*.

qPCR reaction

Reagent	Amount
100 nM Forward and Reverse Primers (Primer Mixture)	1 μL
DNA/RNA free water	10.5 μL
PowerUP Sybr Master Mix	12.5 μL
cDNA	1 μL

m. Pipette 24 μL of reaction mix into each well and add 1 μL of cDNA. We suggest adding cDNA to the side of the well.

⚠ CRITICAL: It is critical to add exactly the same amount of primer mix and cDNA to every well.

⚠ CRITICAL: Change pipette tips between every well.

n. Carefully cover the qPCR plate with the cover seal. We recommend going over the cover with a scraper. When the cover secured, tear along edges of cover to remove excess flap.

Table 3. qPCR cycling conditions

Steps	Temperature	Time	Cycles
Start-up	4°C	2 min	
Initial Denaturation	95°C	15 min	1
Denaturation	95°C	15 s	40 cycles
Annealing	60°C	1 min	
Extension	95°C	30 s	
Melt Curve	65°C–95°C	0.5 °C/5 s	
Hold	25°C	5 min	

o. Centrifuge briefly so that the reaction mixture is pooled at the bottom of each well. We recommend 50 × g for 10 s. Turn on the qPCR machine and load the plate.

p. Run samples using the program shown in Table 3.

q. After performing qPCR, store the plate at −20°C. The products can be separated and visualized on an agarose gel if desired to verify product size and that there was only one product amplified.

r. To analyze, average technical replicate Ct values. There should be less than 0.5 Ct difference between technical replicates. Similar quality control can be performed by checking the melting curves. Quantify relative expression using ΔΔCt and perform appropriate statistical analysis.

$$2^{-\Delta\Delta Ct} = 2^{\frac{Ct_{control} - Ct_{interest}}{Ct_{housekeeper\ \ control} - Ct_{housekeeper\ \ interest}}}$$

Note: Expected qPCR results are shown in Figure 10.

Downstream assay: Fibroblast stress activation

Timing: 4 days

Timing: 1 h (Hands-on time: 30 min) for step 28

Timing: 45 min (Hands-on time: 15 min) for step 29

These steps detail how to analyze fibroblast stress fiber activation after treatment with serum and TGFβ1. Stress fiber activation is quantified by flow cytometry for SMA expression. Additional activation media such as activation by Angiotensin-II are described in (Floy et al., 2021). We recommend

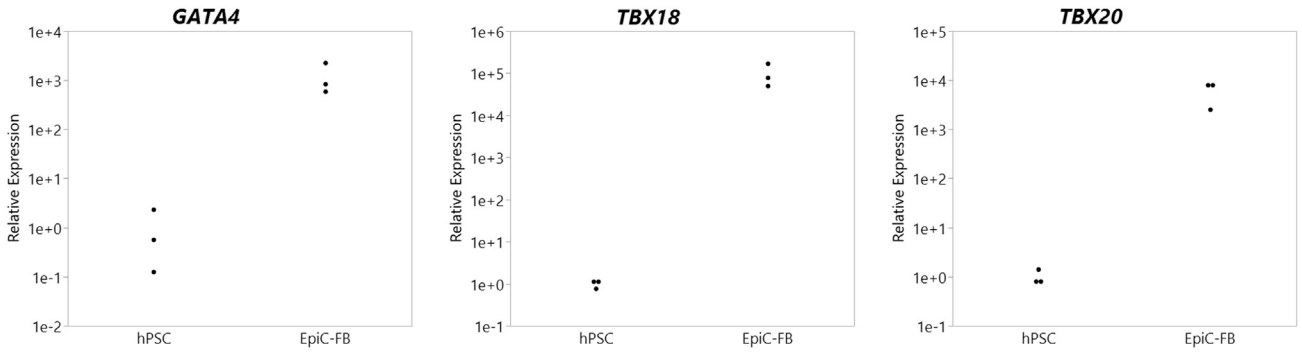

Figure 10. Example qPCR results for expression of *GATA4*, *TBX18*, and *TBX20* in an H9 EpiC-FB differentiation

Dots are the average of 2 technical replicates and each dot is representative of a well in the differentiation where relative expression is calculated compared to *GAPDH* using the ΔΔCt method.

performing this assay in a 6 well plate with at least 3 wells per condition as a smaller plate size may not yield a sufficient number of cells for flow cytometry analysis.

28. Fibroblast activation Day 0, seeding of EpiC-FBs for fibroblast activation assay.
    a. Warm FibroGRO medium containing 2% (vol/vol) FBS at 15°C–25°C for approximately 30 min.
    b. Add 1 mL of Accutase to one well of a 6 well plate of day 10 EpiC-FB cells. Incubate at 37°C, 5% CO_2 for 10 min.

    Note: Trypsin can also be used to singularize EpiC-FBs. We recommend using a shorter incubation time with Trypsin.

    c. Prepare quenching medium by adding 1 mL of FibroGRO medium containing 2% (vol/vol) FBS to a conical tube.
    d. Harvest Accutase cell suspensions using a P1000 and combine the Accutase cell suspension with the quench medium.

    Note: Vigorous pipetting is necessary as cells tend to detach in clusters.

    e. Centrifuge the cells at 200 × *g* for 5 min at 15°C–25°C.
    f. Label an uncoated 6 well tissue culture plate with hPSC line, hPSC passage number, EpiC passage number, EpiC-FB passage number, date, and initials.
    g. Aspirate the supernatant from the conical tube. Using a 10 mL pipette, resuspend the cell pellet in 12 mL of FibroGRO medium containing 2% (vol/vol) FBS. Transfer 2 mL of cell solution to each well of the labelled 6 well tissue culture plate.

    Note: We recommend replating approximately 66k cells (approximately a 1:6 split ratio) in each well of a 6 well plate.

    h. Return the plate to the 37°C, 5% CO_2 incubator after plating the cells. Move the plate in 3 quick, short, back-and-forth and side-to-side motions to disperse the cells across the surface of the wells.

29. Fibroblast activation Day 2.
    a. Two days after seeding the EpiC-FBs, warm FibroGRO medium containing 2% (vol/vol) FBS and D-MEM/F-12 containing 10% (vol/vol) FBS at 15°C–25°C for approximately 30 min.
    b. Prepare a conical tube containing 6 mL of D-MEM/F-12 containing 10% (vol/vol) FBS.
    c. Add 40 µL of 15 µg/mL TGFβ1 (final concentration of 100 ng/mL) to the conical tube.
    d. Aspirate the medium from the EpiC-FBs. Add 2 mL/well of FibroGRO to 3 wells. Add 2 mL/well of D-MEM/F-12 containing 10% FBS and 100 ng/mL TGFβ1 to the remaining 3 wells.
    e. Return the plate to the 37°C, 5% CO_2 incubator.
30. Fibroblast activation assay day 4.
    a. Two days later, analyze activation potential by flow cytometry analysis for SMA expression following step 26.

    Note: An example of flow cytometry data after EpiC-FB activation is shown in Figure 11. We typically see little variability between wells of a given differentiation. However, basal activation level and degree of activation between differentiations varies significantly as described in (Floy et al., 2021).

EXPECTED OUTCOMES

This protocol describes a method to differentiate hPSCs into EpiC-FBs. Before starting the differentiation, hPSCs should be in compact colonies with no morphological evidence of spontaneous

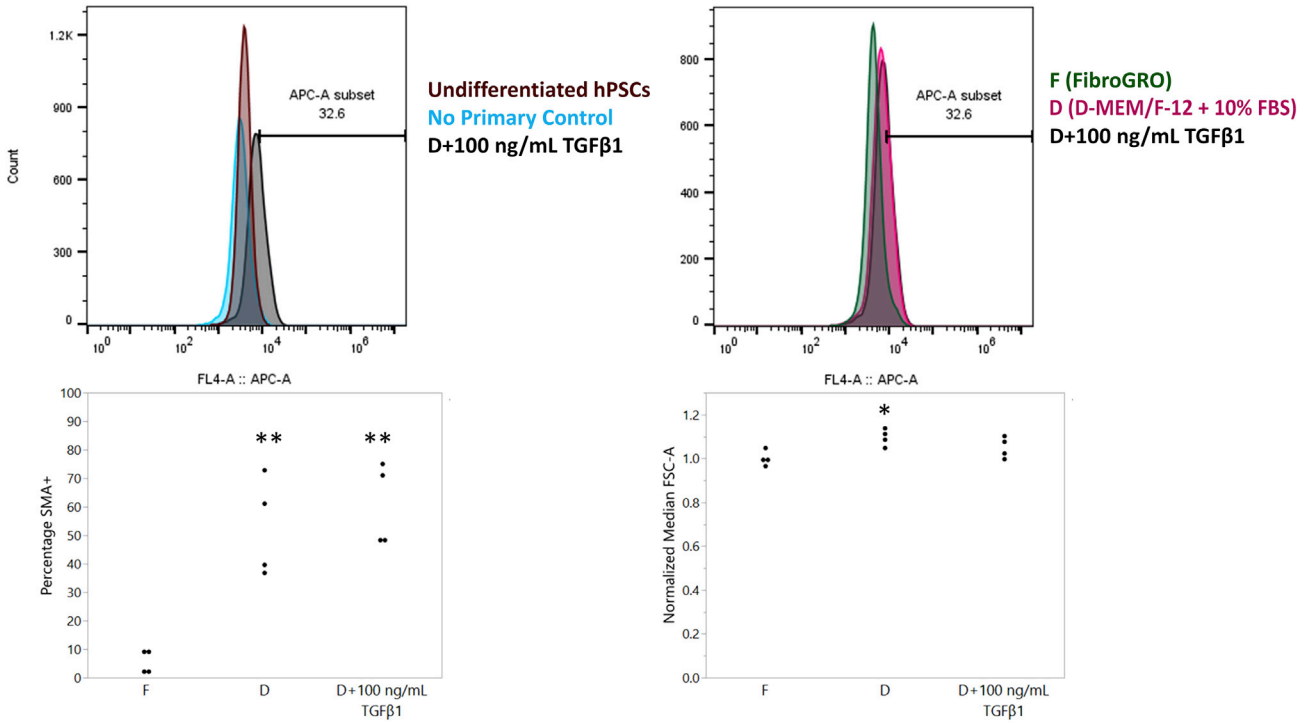

Figure 11. Example fibroblast activation flow cytometry results

19-9-11 EpiC-FBs were treated with FibroGRO maintenance medium (F), D-MEM/F-12 containing 10% (vol/vol) FBS (D), or D-MEM/F-12 containing 10% (vol/vol) FBS and 100 ng/mL TGFβ1 for two days prior to flow cytometry analysis for SMA (Cat#MA5-11544 RRID: AB_10981631) expression. Shown are the flow cytometry gating strategy using undifferentiated hPSCs and no primary negative controls, percentage SMA+ cells and normalized median FSC-A. Statistics are a one-way ANOVA with Dunnett's post-hoc test comparing to FibroGRO conditions where * is $p<0.05$ and ** is $p<0.01$.

differentiation and should exhibit high expression (>90%) of the pluripotency marker NANOG by flow cytometry. Differentiation to mesodermal progenitors is induced by a 24 h exposure to CHIR99021. Lack of brachyury expression at Day 1 may indicate poor quality of the initial hPSCs. At Day 6 of differentiation, CPCs which express ISL1 and NKX2.5 should arise.

Subsequently, to differentiate hPSC-derived CPCs to EpiCs, the CPCs are treated with CHIR99021. By Day 8–9, a cobblestone-like morphology can be observed. By Day 12, EpiCs arise and >80% of cells should express WT1, as assessed by flow cytometry. Passaging the hPSC-derived EpiCs in the presence of TGFβ inhibitor A83-01 allows for maintenance and expansion of $WT1^+$ cells for at least two months (15–20 passages).

To generate EpiC-FBs, confluent EpiCs are treated with bFGF for 10 days. As early as 3 days after bFGF treatment, EpiCs lose their cobblestone morphology as they undergo epithelial-to-mesenchymal transition. Differentiated EpiC-FBs can be maintained in FibroGRO medium containing 2% (vol/vol) FBS for more than 60 days (15 passages) before becoming quiescent. By flow cytometry, more than 80% of EpiC-FBs express VIM and TE7. Additionally, they express 10^3–10^5 times higher levels of *GATA4*, *TBX18*, and *TBX20* compared to undifferentiated hPSCs.

QUANTIFICATION AND STATISTICAL ANALYSIS

For applications of hPSC EpiC-FBs, we suggest using at least 3 well replicates and repeating the experiment at least 3 times using independent differentiations (starting at a new passage of hPSCs). Additionally, we recommend that these 3 independent differentiations are in at least 2 different hPSC lines. Finally, we recommend performing statistical analysis on the data across all 3

differentiations with "differentiation number" as a blinded variable. For example, if there was one treatment group, we suggest performing a two-way ANOVA with "treatment" and "differentiation number" as the 2 variables.

LIMITATIONS

We have successfully performed the EpiC-FB differentiation in the H9, H9-cTnT-eGFP, and H9-7TGP hESC lines, and the 19-9-11, WTC-CAAX-RFP, and WTC-LMNB1-eGFP hiPSC lines. However, the efficiency of the protocol may vary with other hPSC lines and will likely require optimization of undifferentiated hPSC density on Day -2, concentration of CHIR99021 on Day 0, length of CHIR99021 exposure starting on Day 0, and possibly IWP2 concentration on Day 3. Additionally, epicardial differentiation may require optimization of seeding density on Day 6 and CHIR99021 concentration on Days 7 and 8. The EpiC-FB differentiation may require optimization of the seeding density at Day 0.

TROUBLESHOOTING

Problem 1

At steps 3k or 9l, poor attachment of hPSCs to Matrigel-coated plates may result if ROCK inhibitor is not in the medium when replating singularized cells. Alternatively, the Matrigel coating may not support cell adhesion.

Potential solution

Include ROCK inhibitor in replating medium (steps 3d and 9i); use a qualified ECM substrate.

Problem 2

At step 11d, cell death or detachment on Day 1 after hPSC treatment with CHIR99021 may be due to nonoptimal seeding density or concentration of CHIR99021.

Potential solution

Optimize initial seeding density (step 9l) and Day 0 CHIR99021 concentration (step 11c). Optimization is especially important for new lots of CHIR99021 and new hPSC lines.

Problem 3

At step 4k or 16m, detachment of CPCs may be due to poor quality differentiation to CPCs or low viability of thawed CPCs.

Potential solution

Optimize initial seeding density (step 9l) and Day 0 CHIR99021 concentration (step 11c) to improve CPC yield. Try to minimize pipetting of CPCs and thaw cells quickly. Minimize time thawed cells spend at 15°C–25°C. Seed cells and transfer to the incubator quickly after thawing.

Problem 4

At step 4k, detachment of EpiCs after thawing from cryopreservation may be due to replating singularized EpiCs without ROCK inhibitor.

Potential solution

Replate singularized EpiCs with ROCK inhibitor or include 1% (vol/vol) FBS in the replating medium (step 4d).

Problem 5

At step 19j, a low percentage of $WT1^+$ cells on Day 12 is possibly due to a low purity of CPCs, spontaneous differentiation in hPSC lines, or suboptimal seeding density of Day 6 CPCs.

Potential solution

Optimize initial hPSC seeding density (step 9l) and Day 0 CHIR99021 concentration (step 11c), remove spontaneously differentiated hPSCs from hPSC lines prior to differentiation using colony picking, thaw a new cryotube of hPSCs, optimize seeding density of Day 6 CPCs (step 16l) or decrease Day 7 and 8 CHIR99021 concentration to 2 μM (step 17c).

Problem 6

At step 21h, slow proliferation of $WT1^+$ cells after passage could be due to a low initial cell seeding density or replating singularized cells.

Potential solution

Increase seeding density of EpiCs (step 21f), passage EpiCs as colonies with Versene (step 21) instead of Accutase, or passage with 1% (vol/vol) FBS in the replating medium (step 21c).

Problem 7

At step 21h, slow proliferation of $WT1^+$ cells after thawing (step 4k) could be due to a low initial seeding density or low viability of cells after thawing.

Potential solution

Increase EpiC seeding density (step 4j), replate with 1% (vol/vol) FBS in the medium (step 4d), or freeze EpiCs at Day 12 or P1 instead of higher passages.

Problem 8

At step 21h, unwanted differentiation of WT1+ cells during passaging may be due to a nonoptimal concentration of TGFβ inhibitor, cold medium, mechanical stresses during passaging, or passaging singularized cells.

Potential solution

Increase concentration of TGFβ inhibitor (steps 20b and 21c), warm medium (steps 20a and 21a), passage EpiCs as colonies with Versene instead of Accutase (step 21), or passage with 1% (vol/vol) FBS in medium (step 21c).

Problem 9

At step 23h, low yields of EpiC-FBs after bFGF treatment may possibly be due to suboptimal seeding density of EpiCs.

Potential solution

Increase confluence of EpiCs prior to initiating differentiation with bFGF (step 22a).

Problem 10

At steps 5j or 24b, slow proliferation of EpiC-FBs after differentiation may result from lack of media supplements in FibroGRO medium, degraded bFGF in FibroGRO medium, batch-to-batch variability in FBS, or EpiC-FB low seeding density.

Potential solution

Add fresh media supplements to fresh FibroGRO basal medium, try a new batch of FBS, or increase EpiC-FB seeding density (step 23g).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sean Palecek (sppalecek@wisc.edu).

Materials availability

This study did not generate any new reagents. hPSC lines can be obtained from WiCell (https://www.wicell.org) or Coriell Institute (https://www.allencell.org/cell-catalog.html) upon completion of a material transfer agreement. H9-7TGP line can be obtained by contacting Sean Palecek upon completion of a material transfer agreement with the University of Wisconsin-Madison.

Data and code availability

This study did not generate/analyze any datasets or code that were submitted to any repositories.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.E.F., F.S., and S.P.P.; methodology, M.E.F.; investigation, M.E.F. and F.S.; validation, F.S.; writing – original draft, M.E.F.; writing – review and editing, F.S. and S.P.P.; visualization, M.E.F. and F.S.; supervision, S.P.P.; funding acquisition, S.P.P.

DECLARATION OF INTERESTS

M.E.F. and S.P.P. are inventors on provisional US patent applications related to differentiation of hPSCs to EpiC-FBs.

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