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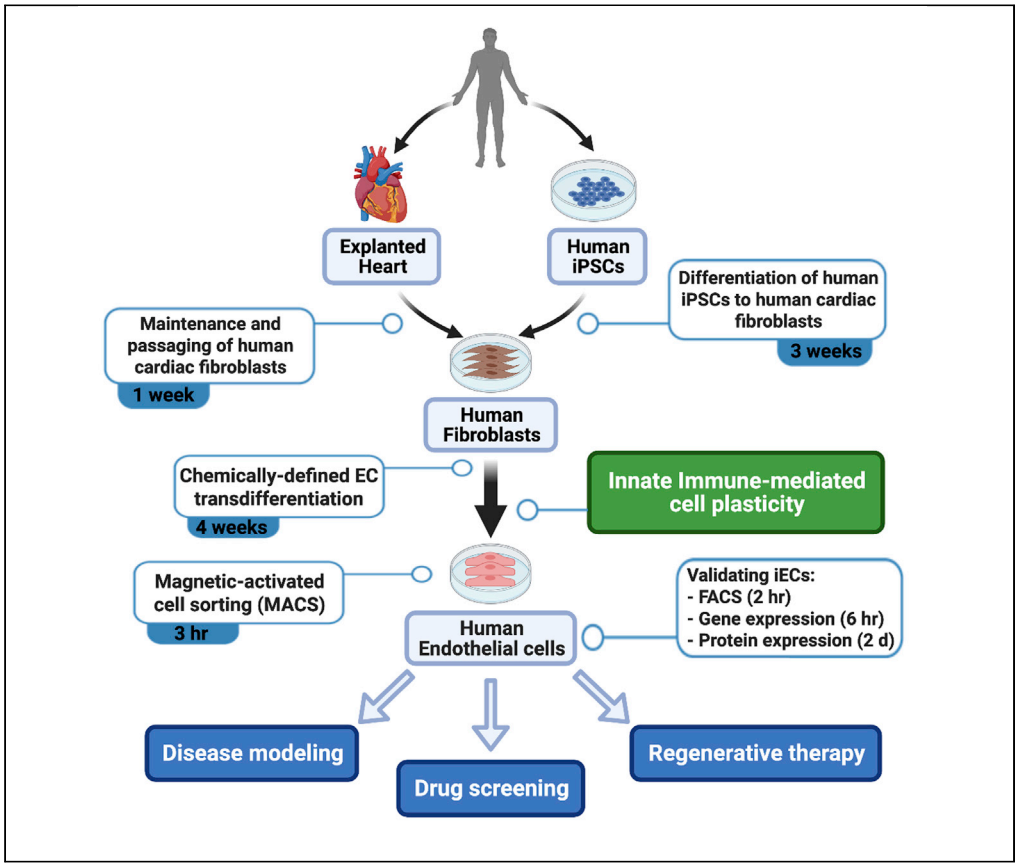
Protocol

A protocol for transdifferentiation of human cardiac fibroblasts into endothelial cells via activation of innate immunity



Endothelial cells (ECs) have emerged as key pathogenic players in cardiac disease due to their proximity with cardiomyocytes. iPSCs have been employed to generate ECs. However, it may be more clinically relevant to transdifferentiate fibroblasts into ECs directly without introducing pluripotent or virally driven transcription factors. Here, we present a protocol that describes the direct conversion of human cardiac fibroblasts into ECs by leveraging the innate immune system. Our protocol produces bona fide human ECs with 95%–98% purity by first passage.

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Highlights

An optimized protocol for direct reprogramming of human endothelial cells (ECs)

A clinically relevant protocol devoid of the use of exogenous transcription factors

The protocol leverages the innate immune pathway for EC transdifferentiation

Induced human ECs can be generated with a purity of 95%–98% by first passage

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A protocol for transdifferentiation of human cardiac fibroblasts into endothelial cells via activation of innate immunity

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SUMMARY

Endothelial cells (ECs) have emerged as key pathogenic players in cardiac disease due to their proximity with cardiomyocytes. Induced pluripotent stem cells (iPSCs) have been employed to generate ECs. However, it may be more clinically relevant to transdifferentiate fibroblasts into ECs directly without introducing pluripotent or virally driven transcription factors. Here, we present a protocol that describes the direct conversion of human cardiac fibroblasts into ECs by leveraging the innate immune system. Our protocol produces bona fide human ECs with 95%–98% purity by first passage.

For complete details on the use and execution of this protocol, please refer to Liu et al. (2020) and Sayed et al. (2015).

BEFORE YOU BEGIN

Human fibroblasts are commercially obtained and used according to ethical guidelines. All experiments are conducted in Class II biosafety cabinets using standard sterile techniques. All cells are cultured and propagated in a humidified 37°C incubator and 5% CO_2. This transdifferentiation protocol can be applied to other types of fibroblasts including skin fibroblasts. Prior to initiation of the protocol, prepare the below media, solutions and matrigel-coated plates. Similarly, the media should be pre-warmed at 37°C for 30 min before the start of the protocol. Please refer to Key Resources Table for a comprehensive list of reagents and resources. Please refer to Materials and Equipment for complete recipe tables.

1. Human Fibroblast Medium: Dulbecco's modified Eagle's medium (DMEM, Gibco, catalog number: 11320033), supplemented with 20% fetal bovine serum (FBS), and 1% penicillin/streptomycin, filter-sterilized.
2. Human iPSC medium: Essential 8™ Medium (Gibco, catalog number: A1517001).
3. Differentiation medium: RPMI 1640 medium, no glutamine (Gibco, catalog number: 21870076).
4. Human Endothelial medium: EGM™ Endothelial Cell Growth Medium BulletKit™ (Lonza, catalog number: CC-3124).
5. Freezing medium: Bambanker (Fisher Scientific, catalog number: NC9582225).

Matrigel-coated culture plates

6. Thaw growth factor-reduced Matrigel on ice. Once thawed, aliquot the Matrigel (250 μL, each) in 1.5 mL Eppendorf tubes and store at −20°C freezer for future use.
7. Thaw one Matrigel aliquot on ice and resuspend in cold DMEM/F12 at 1:250 dilution.
8. For a 6-well plate, add 2 mL of diluted Matrigel solution per well.
9. Coat the plates for minimum of 30 min in 37°C cell culture incubator and 5% CO_2 before use. Additional plates can be prepared and stored in 37°C cell culture incubator for up to 1 week or 4°C for up to 1 month after sealing it with parafilm to avoid drying of the plates.

Gelatin-coated culture plates

10. Prepare 0.2% gelatin solution in DPBS, filter-sterilized. Store at 4°C for future use.
11. For a 6-well plate, add 2 mL 0.2% gelatin to ensure entire well is covered.
12. Incubate the plate for minimum of 30 min in 37°C cell culture incubator.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
PE-conjugated anti-human CD31 (1:400)	Miltenyi Biotec	130-110-669
Anti-human CD31 (1:100)	Thermo Fisher	BBA7
Anti-human CD144 (1:500)	Abcam	ab33168
Alexa Fluor 488 donkey anti-mouse IgG (1:1000)	Thermo Fisher	A21202
Alexa Fluor 594 donkey anti-mouse IgG (1:1000)	Thermo Fisher	A21203
Chemicals, peptides, and recombinant proteins		
DPBS	Gibco	14190
DMEM/F12	Gibco	11320033
Essential 8 medium and supplement	Gibco	A1517001
RPMI 1640 medium	Gibco	21870076
B27 Supplement, minus insulin	Gibco	A1895601
EGM2 medium	Lonza	CC-3124
Fibroblast Growth Medium 3	PromoCell	C23025
Growth Medium 3 SupplementMix	PromoCell	C39345
BD Matrigel	Fisher Scientific	08-774-552
Gelatin	Sigma	G1393
TrypLE Express dissociation reagent	Gibco	12605010
FBS	Life Technologies	26140079
Bambanker	Fisher Scientific	NC9582225
GlutaMax	Thermo Fisher	35050-038
Penicillin/streptomycin	Life Technologies	15140-122
DMSO	Sigma	D2650
Rock inhibitor Y27632	Selleckchem	S1049
CHIR 99021	Selleckchem	S2924
IWR-1	Selleckchem	S7086
Retinoic acid	Sigma	R2625
KnockOut™ Serum	Thermo Fisher	10828010
Polyinosine-polycytidylic [Poly(I:C)]	InvivoGen	tlrl-pic
VEGF	PeproTech	100-20-250UG
BMP4	PeproTech	120-05ET
FGF-basic	Fisher Scientific	233-FB-025
8-Bromo-cAMP	Fisher Scientific	11-401-0
SB431542	Selleck Chemicals	S1067

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Paraformaldehyde	Fisher Scientific	50-980-487
Bovine serum albumin (BSA)	Cell Signaling Technology	9998S
Triton X-100	Sigma	T8787
Normal donkey serum	Sigma	D9663
Permount™ Mounting Medium	Fisher Scientific	SP15-100
Matrigel Basement Membrane Matrix	Fisher Scientific	CB-40234
Acetylcholine	Sigma-Aldrich	A6625-25G
Critical commercial assays		
MACS® LS Columns	Miltenyi Biotec	130-042-401
MACS® BSA Stock Solution	Miltenyi Biotec	130-091-376
MACS® autoMACS Rinsing Solution	Miltenyi Biotec	130-091-222
MACS® Strainers 30 µM	Miltenyi Biotec	130-098-458
MACS® CD144 MicroBeads	Miltenyi Biotec	130-097-857
Nitrate/Nitrite Fluorometric Assay Kit	Cayman Chemicals	780051
LDL Uptake Assay Kit (Fluorometric)	BioVision	K585-100
RNeasy Plus Mini Kit	QIAGEN	74136
qScript® cDNA Synthesis Kit	VWR	89168-788
qScript cDNA SuperMix	VWR	101414-106
TaqMan Universal PCR Master Mix	Fisher Scientific	4364340
Deposited data		
RNA sequencing data sets	Gene Expression Omnibus (GEO)	GSE142217
RNA sequencing data sets	Gene Expression Omnibus (GEO)	GSE45176
Experimental models: Cell lines		
Human cardiac fibroblasts	ScienCell Research Lab	6330
Healthy control iPSCs	Stanford CVI Biobank	N/A
Oligonucleotides		
Real-time qPCR probes – CD31	Life Technologies	Hs00169777_m1
Real-time qPCR probes – CD144	Life Technologies	Hs00901465_m1
Real-time qPCR probes – KDR	Life Technologies	Hs00911700_m1
Real-time qPCR probes – vWF	Life Technologies	Hs01109446_m1
Real-time qPCR probes – NOS3	Life Technologies	Hs01574659_m1
Real-time qPCR probes – GAPDH	Life Technologies	Hs02758991_g1
Other		
Nalgene™ vacuum filter unit	Fisher Scientific	09-741-04
6-Well cell culture plates	Fisher Scientific	08-772-1B
10 cm Cell culture plates	Fisher Scientific	08-772-E
15 mL Polypropylene conical tubes	Fisher Scientific	14-959-53A
Cryovials	Fisher Scientific	13-700-500
CoolCell® freezing container	Corning	CLS432002-1EA
FACSAria II cell sorter	BD Biosciences	N/A
Software and algorithms		
FlowJo 8.7 software	BD Biosciences	N/A

MATERIALS AND EQUIPMENT

All media and solutions are prepared using sterile techniques.

Matrigel 1:250

Add 200 µL of matrigel thawed on ice into 50 mL cold DMEM/F12 medium to make a stock solution. Add 2 mL diluted matrigel solution to each well of a 6-well cell culture plate.

Gelatin 0.2%

Add 50 mL 2% gelatin into 450 mL DPBS to obtain a 0.2% gelatin stock solution and filter with 0.22 µm filter.

Y27632 10 mM
Dissolve 10 mg of Y27632 in 3.12 mL DMSO to make a 10 mM stock solution. A working concentration of 5 μM Y27632 would be needed per well. Make 100 μl aliquots and store in −20°C for up to 6 months.

CHIR99021 10 mM
Dissolve 5 mg of CHIR99021 in 1.08 mL DMSO to make a 10 mM stock solution. A working concentration of 6 μM CHIR99021 would be needed per well. Make 100 μl aliquots and store in −20°C for up to 6 months.

IWR-1 10 mM
Dissolve 10 mg of IWR-1 in 2.44 mL DMSO to make a 10 mM stock solution. A working concentration of 5 μM IWR-1 would be needed per well. Make 100 μl aliquots and store in −20°C for up to 6 months.

Retinoic acid 100 mM
Dissolve 50 mg of Retinoic acid in 1.66 mL DMSO to make a 100 mM stock solution. A working concentration of 2 μM Retinoic acid would be needed per well. Make 100 μl aliquots and store in −20°C for up to 12 months.

Poly(I:C) 1 mg/mL
Dissolve 10 mg of poly(I:C) in 10 mL of sterile endotoxin-free water to make a 1 mg/mL stock solution. Make serial dilutions of poly(I:C) to make a working solution of 1 μg/mL. A working concentration of 30 ng/mL poly(I:C) would be needed per well. Make 100 μl aliquots and store in −20°C for up to 12 months.

VEGF 250 μg/mL
Dissolve 250 μg of VEGF in 1 mL DPBS and 0.1% MACS BSA solution to make a 250 μg/mL stock solution. A working concentration of 50 ng/mL VEGF would be needed per well. Make 100 μl aliquots and store in −20°C for up to 6 months.

BMP4 100 μg/mL
Dissolve 100 μg of BMP4 in 1 mL sterile water and 0.1% MACS BSA solution to make a 100 μg/mL stock solution. A working concentration of 20 ng/mL BMP4 would be needed per well. Make 100 μl aliquots and store in −80°C for up to 3 months.

FGF-basic 100 μg/mL
Dissolve 100 μg of FGF-basic in 1 mL sterile water and 0.1% MACS BSA solution to make a 100 μg/mL stock solution. A working concentration of 20 ng/mL FGF-basic would be needed per well. Make 100 μl aliquots and store in −20°C for up to 3 months.

8-Br-cAMP 100 mM
Dissolve 50 mg of 8-Br-cAMP in 1.15 mL sterile water to make a 100 mM stock solution. A working concentration of 100 μM 8-Br-cAMP would be needed per well. Make 100 μl aliquots and store in −20°C for up to 6 months.

SB431542 50 mM
Dissolve 10 mg of SB431542 in 520 μL of DMSO to make a 50 mM stock solution. A working concentration of 10 μM SB431542 would be needed per well. Make 100 μl aliquots and store in −80°C for up to 6 months.

Human fibroblast medium		
Reagent	Final concentration	Amount
DMEM/F12	n/a	399 mL
FBS	20%	100 mL
GlutaMax	1%	0.5 mL
Penicillin/streptomycin	1%	0.5 mL
Total	**n/a**	**500 mL**
Once prepared, store at 4°C for up to 1 month		

Human iPSC medium		
Reagent	Final concentration	Amount
Essential 8 Media	n/a	490 mL
E8 Supplement	2%	10 mL
Total	**n/a**	**500 mL**
Once prepared, store at 4°C for up to 1 month		

Human differentiation medium		
Reagent	Final concentration	Amount
RPMI 1640 medium	n/a	490 mL
B27 supplement minus insulin	2%	10 mL
Total	**n/a**	**500 mL**
Once prepared, store at 4°C for up to 1 month		

Fibroblast growth medium		
Reagent	Final concentration	Amount
Fibroblast Growth Medium 3	n/a	495 mL
SupplementMix	n/a	5 mL
Total	**n/a**	**500 mL**
Once prepared, store at 4°C for up to 6 weeks		

Human endothelial (EGM-2) medium		
Reagent	Final concentration	Amount
Endothelial Cell Growth Basal Medium 2	n/a	500 mL
Epidermal Growth Factor	5 ng/mL	Kit aliquot
bFGF	10 ng/mL	Kit aliquot
Insulin-like Growth Factor (R3 IGF-1)	20 ng/mL	Kit aliquot
VEGF 165	0.5 ng/mL	Kit aliquot
Ascorbic Acid	1 µg/mL	Kit aliquot
Heparin	22.5 µg/mL	Kit aliquot
Hydrocortisone	0.2 µg/mL	Kit aliquot
Total	**n/a**	**500 mL**
Once prepared, store at 4°C for up to 1 month		

Induction medium		
Reagent	Final concentration	Amount
DMEM/F12	n/a	425 mL
FBS	7.5%	37.5 mL
Knockout serum	7.5%	37.5 mL
Poly(I:C)	30 ng/mL	15 µL
Total	**n/a**	**500 mL**
Once prepared, store at 4°C for up to 2 weeks.		

Transdifferentiation medium		
Reagent	Final concentration	Amount
EGM2 medium	n/a	500 mL
VEGF	50 ng/mL	100 μL
bFGF	20 ng/mL	100 μL
BMP4	20 ng/mL	100 μL
Total	**n/a**	**500 mL**
Once prepared, store at 4°C for up to 2 weeks.		

Maintenance medium		
Reagent	Final concentration	Amount
EGM2 medium	n/a	500 mL
VEGF	50 ng/mL	100 μL
bFGF	20 ng/mL	100 μL
8-Br-cAMP	100 μM	500 μL
Total	**n/a**	**500 mL**
Once prepared, store at 4°C for up to 2 weeks.		

Expansion medium		
Reagent	Final concentration	Amount
EGM2 medium	n/a	500 mL
SB431542	10 μM	100 μL
Total	**n/a**	**500 mL**
Once prepared, store at 4°C for up to 2 weeks.		

Cryopreservation medium		
Reagent	Final concentration	Amount
Bambanker	n/a	1 mL/vial
Store at 4°C for up to 12 months.		

MACS buffer		
Reagent	Final concentration	Amount
MACS® AutoMACS Rinsing Solution	95%	95 mL
MACS® BSA Stock Solution	5%	5 mL
Total	**n/a**	**100 mL**
Once prepared, use the same day.		

Immunofluorescence blocking buffer		
Reagent	Final concentration	Amount
DPBS	88.75%	88.75 mL
BSA	1%	1 mL
Normal Donkey Serum	10%	10 mL
Triton X	0.25%	250 μL
Total	**n/a**	**100 mL**
Once prepared, aliquot and store at −20°C for up to 2 months.		

Immunofluorescence antibody buffer		
Reagent	Final concentration	Amount
DPBS	97.75%	9.775 mL
BSA	1%	0.1 mL
Normal Donkey Serum	1%	0.1 mL
Triton X	0.25%	25 µL
Total	n/a	10 mL

Once prepared, aliquot and store at −20°C for up to 2 months.

STEP-BY-STEP METHOD DETAILS

Human fibroblasts

Cardiac fibroblasts are an essential cell type of the human heart that not only provide structural support to the cardiomyocytes but are also an important source of paracrine growth factors (Tallquist and Molkentin, 2017). Under pathophysiological conditions, FBs become proliferative and synthesize extracellular matrix that result in scar formation and fibrosis (Fu et al., 2018; Sayed et al., 2013). Human FBs can be obtained from commercial vendors (who isolate FBs from human heart tissue) or can be obtained by differentiating human iPSCs (Figure 1). The acquisition of human heart tissue is rare and expensive, and thus iPSC-derived FBs pose as an ideal alternative. In this protocol we obtained human FBs from commercial vendor, ScienCell Research Laboratories. They were passaged and maintained as described below. We obtained human iPSC-derived cardiac fibroblasts (iPSC-CFs) from the laboratory of Dr. Joseph Wu following their established protocol (Zhang et al., 2020). Healthy control iPSCs were obtained from Stanford CVI Biobank (https://med.stanford.edu/scvibiobank.html) using an Institutional Review Board (IRB)–approved protocol. A brief summary is provided below.

Maintenance and passaging of human cardiac fibroblasts

Timing: 1 week

1. Prepare a 6-well matrigel coated plate for the recovery of human cardiac fibroblasts. Incubate the plate for 30 min in a 37°C cell culture incubator.
2. Thaw and quickly recover one cryovial of commercial human fibroblasts in 1-well of the matrigel coated 6-well plate using 2 mL fibroblast medium.
3. Following 24 h incubation, replace the medium with fresh 2 mL fibroblast medium.
4. Change medium every 2 days till confluent.
5. Once confluent, passage the human fibroblasts 1:6 split by incubating with 1 mL TrypLE per well for 5 min in a 37°C cell culture incubator and plate onto a new 6-well matrigel coated plate to achieve 70%–80% confluency.

⚠ CRITICAL: We recommend a confluency of 70%–80% before starting the transdifferentiation protocol and a confluency of 100% before cryopreserving them.

Pause point: At this stage of the protocol, the cells can be cryopreserved for future use by resuspending them in Bambanker (freezing medium) and placing in CoolCell® at −80°C for 24 h followed by long-term storage in liquid nitrogen. Commercial human cardiac fibroblasts are usually received from the vendor at passage one and can be expanded for 15 population doublings as per the vendor's recommendations.

Alternate method to obtain human cardiac fibroblasts: Differentiation of human iPSCs into human cardiac fibroblasts

Timing: 3 weeks

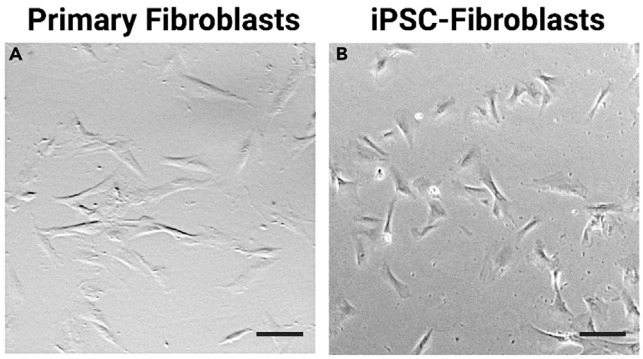

Figure 1. Obtaining human fibroblasts

(A) Representative brightfield image of commercially obtained primary human cardiac fibroblasts.

(B) Representative brightfield image of iPSC-derived human cardiac fibroblasts. Image is of day 21 iPSC-CFs since start of differentiation. Scale bars, 50 μm.

Human induced pluripotent stem cells (iPSCs) have served as an ideal platform to generate patient-specific cardiovascular cells to model human diseases (Sayed et al., 2016; Sayed and Wu, 2017) or assess cardiotoxicity (Sayed et al., 2019; Stack et al., 2019; Thomas et al., 2021). In this protocol, we used healthy control iPSCs cultured in human iPSC medium (E8 medium) to generate cardiac fibroblasts. We include an overview of the protocol:

6. Seed iPSCs in matrigel coated 6-well plate till 80%–85% confluency.
7. Once confluent, initiate the differentiation protocol by replacing the E8 medium with 2 mL RPMI/B27 minus insulin (RPMI/B27-insulin) per well supplemented with 6 μM CHIR99021 for 2 days.
8. On day 2, replace the medium with fresh 2 mL RPMI/B27-insulin per well for 24 h. After the initiation of the protocol, cells are not washed with 1× PBS.
9. On day 3, replace the medium with freshly prepared 2 mL RPMI/B27-insulin per well supplemented with 5 μM IWR-1 for 2 days.
10. On day 5, replace the medium with fresh 2 mL RPMI/B27-insulin per well for 24 h.
11. On day 6, passage the differentiating cells with 1 mL TrypLE per well and seed them in new matrigel coated 6-well plates (at 1:12 split ratio) in freshly prepared 2 mL Advanced DMEM/GlutaMAX medium per well with 1% FBS supplemented with 5 μM CHIR99021, 2 μM retinoic acid, and 5 μM of Y27632.
12. On day 7, replace the medium with freshly prepared 2 mL Advanced DMEM/GlutaMAX medium per well supplemented with 5 μM CHIR99021 and 2 μM retinoic acid for 2 days.
13. On day 9, replace the medium with fresh 2 mL Advanced DMEM/GlutaMAX medium per well for 2 days.
14. On day 11, passage the cells with 1 mL TrypLE per well and seed them in new matrigel coated 6-well plates (at 1:3 or 1:6 split ratio) in freshly prepared 2 mL Advanced DMEM/GlutaMAX medium per well supplemented with 2 μM SB431542 for 24 h.
15. On day 12, replace the medium with fresh 2 mL Advanced DMEM/GlutaMAX medium per well supplemented with 2 μM SB431542, and change medium till the cells are confluent.
16. On day 14, passage the cells with 1 mL TrypLE per well and seed them in new matrigel coated 10 cm cell culture dish (at 1:3 split ratio or 2 full 6-well plates to one 10 cm dish) in freshly prepared 10 mL fibroblast growth medium supplemented with 20 ng/mL bFGF and 10 μM SB431542.
17. On day 16, replace the medium with fresh 10 mL fibroblast growth medium per dish supplemented with 20 ng/mL bFGF and 10 μM SB431542.
18. On day 18, replace the medium with fresh 10 mL fibroblast growth medium per dish supplemented with 20 ng/mL bFGF and 10 μM SB431542.

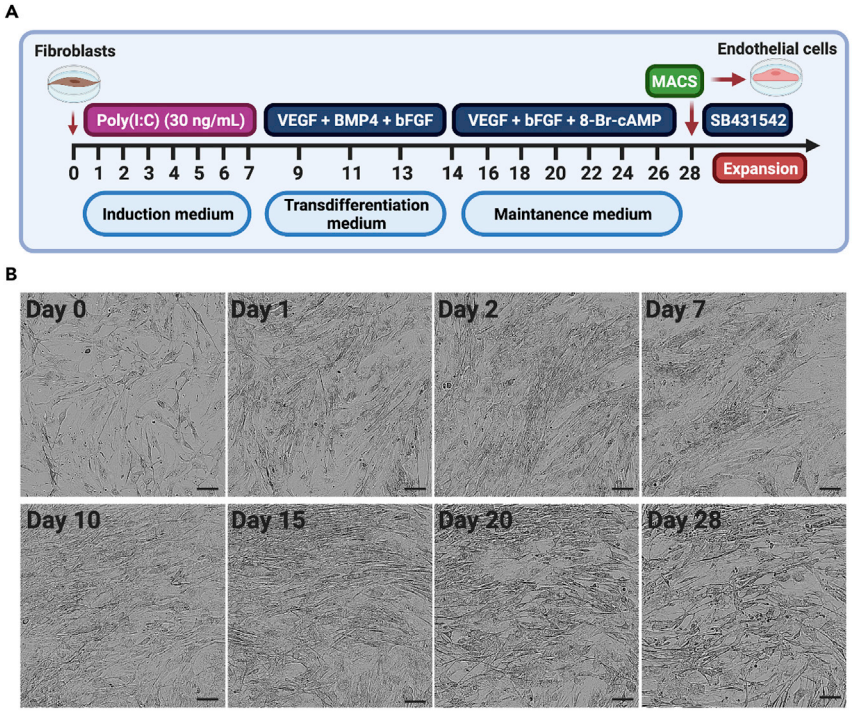

Figure 2. Protocol to transdifferentiate human fibroblasts into human endothelial cells

(A) Schematic of protocol to transdifferentiate human fibroblasts into endothelial cells. The schematic outlines the time course and treatments of growth factors and small molecules for the transdifferentiation of fibroblasts.

(B) Representative phase-contrast images of human fibroblasts undergoing transdifferentiation at different time-points (days) using Incucyte® Live-Cell Imaging system. Scale bars, 100 μm.

19. By day 20, the resulting cardiac fibroblasts are confluent and can be passaged for transdifferentiation.

⚠ CRITICAL: By day 18, the purity of cardiac fibroblasts differentiated from iPSCs should be >95% (Zhang et al., 2019a). However, we recommend checking the purity of iPSC-CFs before initiating their transdifferentiation to ECs by assessing the expression of CF markers such as COL-I, DDR2, and TCF21 via fluorescence-activated cell sorting (FACS).

Pause point: At this stage the iPSC-CFs can be cryopreserved for future use by resuspending them in Bambanker (freezing medium) and placing in CoolCell® at −80°C for 24 h followed by long-term storage in liquid nitrogen.

Transdifferentiating human fibroblasts to endothelial cells

Timing: 4 weeks

Activation of innate immune pathways have been shown to play an important role in nuclear reprogramming (Lee et al., 2012; Liu et al., 2021; Liu et al., 2017; Sayed et al., 2017) and transdifferentiation of one cell type to another (Sayed et al., 2015; Zhou et al., 2019). The protocol described below employs a safe and efficient way to directly differentiate human fibroblasts to endothelial cells without the use of any exogenous transcription factors (Sayed et al., 2015) (Figures 2A and 2B). Transdifferentiated ECs (iECs) attain an efficiency of 95%–98% by passage 1 and can be cryopreserved for future research purposes.

Note: Human fibroblasts obtained either commercially or derived using human iPSCs should be cultured at least 3–4 days before initiating the transdifferentiating protocol. The protocol was optimized using a 6-well plate.

20. On day 0 (the day before transdifferentiation), prepare 6-well gelatin-coated plates 30 min prior to seeding the human fibroblasts.
21. Following 30 mins, remove the excess gelatin and seed human fibroblasts in human fibroblast medium at a density of 1×10^5 cells per well of a 6-well plate for 24 h.
22. On day 1, aspirate the spent medium and replace with induction medium (see recipe) containing DMEM/F12 supplemented with 7.5% FBS, 7.5% knockout serum (KSR), and 30 ng/mL poly(I:C).
23. Treat cells daily with fresh induction medium (day 2–6).
24. On day 7, switch the induced human fibroblasts over to transdifferentiation medium (see recipe) containing EGM2 medium supplemented with 50 ng/mL vascular endothelial growth factor (VEGF), 20 ng/mL bone morphogenetic protein 4 (BMP4), and 20 ng/mL basic fibroblast growth factor (bFGF) for 2 days.
25. On day 9, replace the spent medium with fresh transdifferentiation medium for 2 days.
26. On day 11, replace the spent medium with fresh transdifferentiation medium for 2 days.
27. On day 13, replace the spent medium with fresh transdifferentiation medium for 1 day.
28. On day 14, switch the transdifferentiated human fibroblasts over to maintenance medium (see recipe) containing EGM2 medium supplemented with 50 ng/mL VEGF, 20 ng/mL bFGF, and 100 μM 8-bromoadenosine-3′:5′-cyclic monophosphate sodium salt (8-Br-cAMP) for the next 2 weeks, changing medium every 2 days.
29. On day 28, the cells are ready for CD144 MACS sorting.

⚠ CRITICAL: We recommend observing the cells throughout the protocol as distinct morphological changes can be seen, especially after the addition of the transdifferentiation medium (Figure 2).

CD144 magnetic-activated cell sorting (MACS)

Timing: 3 h

This step sorts the iECs from the transdifferentiation protocol. CD144, also known as VE-Cadherin has been widely used for the isolation of ECs derived from embryonic stem cells (ESCs) and iPSCs (Liu et al., 2020; Sayed et al., 2020; Sayed et al., 2015).

30. Aspirate the spent medium and dissociate the cells using 1 mL TrypLE express per well for 10 mins at 37°C.
31. Pipette the cells repeatedly to ensure single cell suspension and collect the cells in 1 mL basal EGM2 medium to neutralize the TrypLE express.
32. Strain the cell suspension through a pre-rinsed 40 μm strainer into a 50 mL conical tube, centrifuge at 300 × *g* for 5 min, and resuspend 1×10^7 cells in 160 μL ice-cold MACS buffer.

⚠ CRITICAL: Pre-rinse the strainer with MACS buffer before use. Typically, one strainer is used for every 6-well plate, however, replace the strainer with a new one if any clogging is observed.

33. Incubate the cell suspension with 40 μL of CD144 magnetic microbeads at 4°C for 15 min in dark.
34. Wash the cells with 1 mL MACS buffer and centrifuge at 300 × *g* for 5 min.
35. During the centrifugation step, setup the LS columns on the magnetic MACS sorting apparatus and pre-rinse the columns with 3 mL MACS buffer.

⚠ CRITICAL: Ensure the cells remain on ice before and after sorting to avoid cell death.

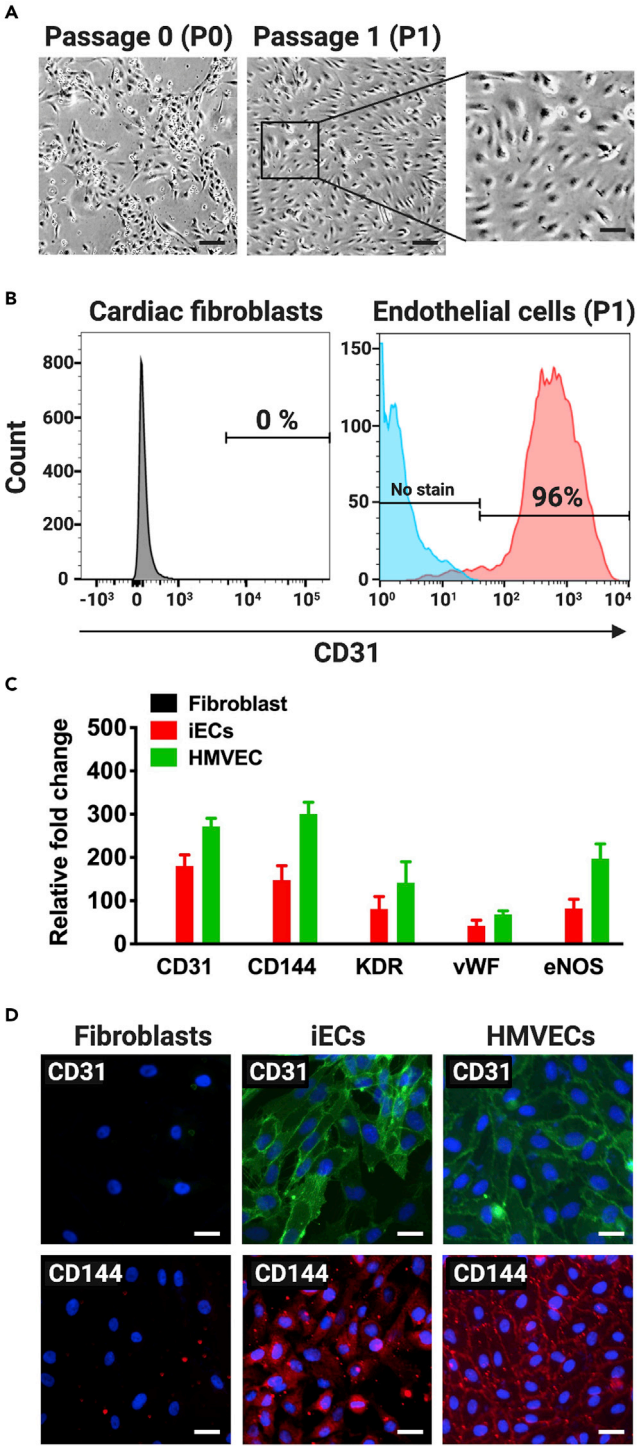

Figure 3. Isolation and validation of induced endothelial cells (iECs)

(A) Representative brightfield images of iECs transdifferentiated from human fibroblasts at passage 0 (P0, *left panel*) and passage 1 (P1, *right panel*) show typical cobblestone appearance. Scale bars, 100 μm. *Inset* shows high magnification of P1 iECs. Scale bars, 50 μm.

(B) FACS analysis of cardiac fibroblasts (*left panel*) and iECs (*right panel*) with CD31 antibody shows ~96% purity at P1.

Figure 3. *Continued*
(C) Quantitative PCR (qPCR) data of transdifferentiated iECs show expression of EC markers, CD31, CD144, KDR, vWF, and eNOS as relative fold change when compared to human fibroblasts and normalized to GAPDH, a housekeeping gene. Human microvascular endothelial cells (HMVEC) were used as positive control.
(D) Representative immunofluorescent images of transdifferentiated iECs show expression of CD31 and CD144 protein levels compared to human fibroblasts. Scale bars, 25 μm.

36. After centrifugation, remove supernatant and resuspend the cell pellet with 1 mL MACS buffer.
37. Pass the cell suspension through the LS column.
38. Wash the column with 3 mL MACS buffer, repeating it 3 times to remove unlabeled cells.
39. After washes, remove the column from the magnetic separator and place on a 15 mL conical tube.
40. Add 5 mL MACS buffer to the column and firmly push the column plunger to elute CD144+ labeled cells.

Pause point: CD144+ iECs can be cryopreserved using Bambanker in liquid nitrogen at this stage.

⚠ CRITICAL: Follow the steps listed in the Miltenyi Biotech MACS sorting protocol (https://www.miltenyibiotec.com/US-en/products/macs-cell-separation.html). Prewash the LS columns with MACS buffer to ensure removal of any bubbles. When washing the columns, wait till all the previous wash volume has passed through the column before adding the next volume. When plunging the cells from the column ensure steady and even pressure is applied on the column plunger.

Re-plating iECs

Timing: 30 min

⚠ CRITICAL: As the yield of transdifferentiated iECs at passage 0 (P0) is ~ 2.5×10^5 cells for each 6-well plate of human fibroblasts, seed the iECs in 1-well of a 6-well plate.

41. Centrifuge the CD144+ labeled cells at 300 × *g* for 5 min. Resuspend the cell pellet in expansion medium (see recipe) containing EGM2 and 10 μM SB431542 and seed the cells in 1-well of a 6-well plate precoated with 0.2% gelatin.
42. Ensure cells are evenly dispersed and place them in 37°C incubator.

Maintaining and passaging iECs

Timing: up to passage 8

Transdifferentiated iECs can be maintained with medium changes every 2 days and expanded up to 8 passages (Figure 3A).

43. Once 100% confluent, dissociate the P0 iECs with 1 mL TrypLE for 5 min at 37°C. Neutralize the cell suspension with basal EGM2 medium, centrifuge at 300 × *g* for 5 min, and resuspend the cell pellet with expansion medium.

⚠ CRITICAL: Passage P0 iECs on gelatin-coated plates at a 1:3 split ratio.

Cryopreserving iECs

Timing: 30 mins

Pause point: Passage 1 (P1) iECs can be cryopreserved for future use.

44. Aspirate the spent medium from cells and dissociate them with 1 mL TrypLE for 5 min at 37°C. Neutralize the cells with media and centrifuge at 300 × *g* for 5 min.
45. Aspirate the supernatant and resuspend the cells gently with 1 mL Bambanker.
46. Transfer the cell suspension into cryogenic storage vials and place them in CoolCell® freezing container to be placed in −80°C for 24 h.
47. Transfer the cryogenic vials to liquid nitrogen for long-term storage.

Validating iECs: Fluorescence-activated cell sorting (FACS)

Timing: 2 h

Once the iECs have been expanded they can be characterized and assessed for their purity using FACS (Figure 3B). CD31, also known as platelet endothelial cell adhesion molecule (PECAM-1), is highly expressed at the intercellular junctions of ECs and plays an important role in maintaining vascular permeability. It is a ubiquitous surface marker of ECs.

48. Dissociate iECs from gelatin-coated plates with 1 mL TrypLE for 5 min at 37°C. Neutralize TrypLE with media and centrifuge at 300 × *g* for 5 min.
49. Aspirate the supernatant and resuspend the cell pellet gently in 1× PBS containing 2% FBS at 5 × 10^5 cells per 100 µL volume per tube.
50. Incubate the cells with PE-conjugated antibody against CD31 at 1:400 dilution for 30 min on ice.
51. Wash cells to remove excess antibody, centrifuge and resuspend in 1× PBS containing 2% FBS.
52. Run FACS.

Note: A FACSAria II cell sorter was used for sorting and data was analyzed using FlowJo 8.7 software (TreeStar).

Validating iECs: Characterizing gene expression

Timing: 6 h

The gene expression profile of iECs using quantitative PCR can be used to confirm the identity of the transdifferentiated cells (Figure 3C). CD31 has been primarily used to demonstrate the presence of ECs. Similarly, CD144, also known as vascular endothelial cadherin (VE-cadherin) is a marker for ECs (Liu et al., 2020; Sayed et al., 2015).

53. RNA extraction
    a. Dissociate iECs from 1-well of a 6-well plate using 1 mL TrypLE for 5 min at 37°C. Neutralize the cells with basal EGM2 media and centrifuge at 300 × *g* for 5 min.
    b. Extract RNA using an RNA extraction kit such as Qiagen RNeasy Plus Mini Kit.
    c. Resuspend the pellet with lysis buffer (buffer RLT) to disrupt them by pipetting up and down.
    d. Add equal volume of 70% ethanol to the homogenized cell lysate.
    e. Transfer the cell lysate to RNeasy spin column® to capture the RNA, followed by washes with RW1 and RPE buffers.
    f. Elute RNA with RNAse-free water.
    g. Determine the RNA concentration using a Nanodrop.

54. cDNA preparation
    a. Use qScript® cDNA Synthesis Kit for cDNA synthesis.
    b. Thaw the RNA and qScript® cDNA SuperMix on ice and prepare 0.2 mL thin-walled PCR tube for the PCR reaction.

Reagent	1× (volume)
RNA (1 μg to 10 pg total RNA)	variable
Nuclease-free water	variable
qScript Reaction Mix (5×)	4.0 μL
qScript RT	1.0 μL
Final volume	20.0 μL

c. Once mixed, vortex gently and centrifuge to pool the reaction volume.
d. Place the tubes in a thermal cycler and follow the recommended PCR program.

Number of cycles	Temperature	Time
1 cycle	22°C	5 min
1 cycle	42°C	30 min
1 cycle	85°C	5 min
Hold	4°C	5 min

55. Quantitative PCR
    a. Dilute the cDNA samples (1:5 ratio) with RNase/DNase-free water.
    b. Thaw the gene-specific primers (CD31, CD144, KDR, vWF, and NOS3) on ice and prepare the reaction mix.

Reagent	1× (volume)
cDNA sample	3 μL
TaqMan Universal PCR Master Mix	10 μL
Gene-specific primer	1 μL
Water	6 μL
Final volume	20.0 μL

c. Turn on the ABI Real time PCR machine (ABI 7300) 30 mins prior to running the qPCR.
d. Follow the recommended qPCR program.

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial denaturation	94°C	2 min	1
Denaturation	94°C	30 s	40
Annealing	58°C	30 s	
Extension	68°C	1 min	
Final extension	N/A	N/A	
Hold	4°C	Forever	

56. Analyze the expression of EC genes by normalizing the data to a housekeeping gene (GAPDH) and express the data as relative fold changes with the ΔCt method of analysis.

Validating iECs: Characterizing protein expression

Timing: 2 days

In addition to gene expression profiling, the protein expression of endothelial markers such as CD31 and CD144 can also be used to characterize the transdifferentiated iECs (Figure 3D).

57. Aspirate the media from 1-well of a 6-well plate and wash cells with DPBS.
58. Fix cells with 4% paraformaldehyde (PFA) for 1 h at RT.
59. Wash cells 3 times with DPBS.
60. Block cells with blocking buffer containing 10% normal donkey serum and 1% BSA for 1 h at RT.
61. Apply CD31 (1:100) and CD144 (1:500) primary antibodies diluted in antibody buffer containing 1% normal donkey serum and 1% BSA for 16 h at 4°C.
62. Wash cells 3 times with DPBS.
63. Apply secondary antibodies (1:1000) diluted in antibody buffer containing 1% normal donkey serum and 1% BSA for 1 h at RT. Add DAPI at 5 μg/mL during this incubation.
64. Wash cells 5 times with DPBS.
65. Capture images using a fluorescent microscope.
66. Cells can be stored in PBS at 4°C for future imaging. Seal the edges of the plate with parafilm to prevent the cells from drying out.

EXPECTED OUTCOMES

Induced endothelial cells (iECs) transdifferentiated from human fibroblasts can be available for downstream phenotypic characterization within 4 weeks. Importantly, these transdifferentiated iECs can be generated from different fibroblasts including cardiac, skin, and iPSC-derived fibroblasts and are devoid of virally-driven transcription factors. To maximize the output of iECs, we recommend starting with three 6-well plates of human fibroblasts. In this scenario, the expected outcome would be collection of at least two 6-well plates of iECs by passage 2. We recommend dedicating 2-wells for each downstream phenotypic characterization. The most widely used assays to assess the function of ECs are listed here: a) *In vitro* angiogenesis assay to assess the capacity to form network of tubular structures; b) Nitric oxide assay to assess the capacity to produce NO; c) Acetylated LDL-uptake assay to assess the capacity to uptake Ac-LDL; d) *In vitro* scratch assay to assess the migration capacity; e) *In vivo* angiogenesis assay to assess the capacity to from capillaries (Huang et al., 2021; Liu et al., 2018; Paik et al., 2018; Sayed et al., 2020; Zhang et al., 2019b).

LIMITATIONS

Despite being a simple, safe protocol, which is devoid of the use of exogenous transcription factors, to directly differentiate human fibroblasts to endothelial cells, there are a few limitations and concerns. First, the efficiency of generating iECs using this protocol is low. Every 6-well plate of human fibroblasts that are transdifferentiated yields about 4%–-5% of iECs. Second, the differentiation efficiency could vary between which human fibroblasts are being used for transdifferentiation. Lastly, the isolation of iECs from the transdifferentiated cell suspension is heavily dependent on endothelial cell surface markers.

TROUBLESHOOTING

Problem 1

Poor proliferative capacity of human fibroblasts before initiating the transdifferentiation protocol (step 5).

Potential solution

Culturing human fibroblasts long-term in tissue culture plates might lead to cellular senescence, which can render the cells non-proliferative. We recommend seeding the human fibroblasts at a much higher seeding density so that the transdifferentiation protocol can be initiated within a day.

Problem 2

Extensive iPSC detachment during iPSC-CF differentiation (step 7).

Potential solution

For most iPSC lines, 6 μM CHIR99021 for 2 days is sufficient to induce differentiation to cardiac progenitor cells. However, if massive cell death is observed after addition of CHIR99021, the concentration and time of exposure for CHIR99021 should be adjusted to ensure successful differentiation of iPSC-CFs.

Problem 3

Low yield of iPSC-CFs after differentiation (step 19).

Potential solution

The protocol to generate iPSC-CFs involves three stages, which include: i) differentiation of iPSCs into cardiac progenitors, ii) differentiation of cardiac progenitors into proepicardial cells, and iii) differentiation of iPSC-derived epicardial cells into cardiac fibroblasts. Passing through these stages, it is possible to obtain a low yield of iPSC-CFs. This could be due to low quality of iPSC-derived epicardial cells. We recommend to carefully review the morphology of iPSC-derived epicardial cells before differentiating them to iPSC-CFs. In case, the cells fail to exhibit the typical cobblestone-like morphology, we recommend discarding the cells and stop the differentiation process to iPSC-CFs.

Problem 4

Low yield of transdifferentiated iECs after MACS sorting (step 40).

Potential solution

There are a few steps that need to be followed to avoid a very low yield of iECs. 1) Before MACS sorting, an important step is to ensure complete and total dissociation of the cell suspension. For this, we recommend adding TrypLE for 10 min at 37°C and vigorously pipetting the cell suspension for 5 mins. 2) During MACS sorting, avoid clogging of the LS columns by ensuring the cells remain in single cell suspension throughout the MACS protocol. 3) Additional washes of the LS columns can also increase the yield on iECs.

Problem 5

Low maturity of transdifferentiated iECs (step 41).

Potential solution

Primary human endothelial cells are significantly more mature when compared to transdifferentiated iECs, especially with respect to their gene and protein expression (Sayed et al., 2015). Even though, it is unlikely that transdifferentiated iECs could fully mimic primary ECs, mainly due to differences in their enivornmental mileu, we recommend two steps that can improve some aspects of iEC maturity. These include: i) culturing iECs on specific substrates such as fibronectin, and ii) passaging iECs to passage 2 before subjecting them to *in vitro* characterization.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Nazish Sayed (sayedns@stanford.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The datasets (RNA sequencing) generated during this study are available at Gene Expression Omnibus (GEO) under accession numbers GEO: GSE142217 and GSE45176.

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AUTHOR CONTRIBUTIONS

C.L., P.M., D.T., and I.Y.C. performed the experiments and analyzed the data. C.L., K.S., D.S., and N.S. wrote the manuscript.

DECLARATION OF INTERESTS

Dr. Sayed is an inventor of the intellectual property, assigned to Stanford University, related to the use of innate immune signaling for nuclear reprogramming and transdifferentiation.

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