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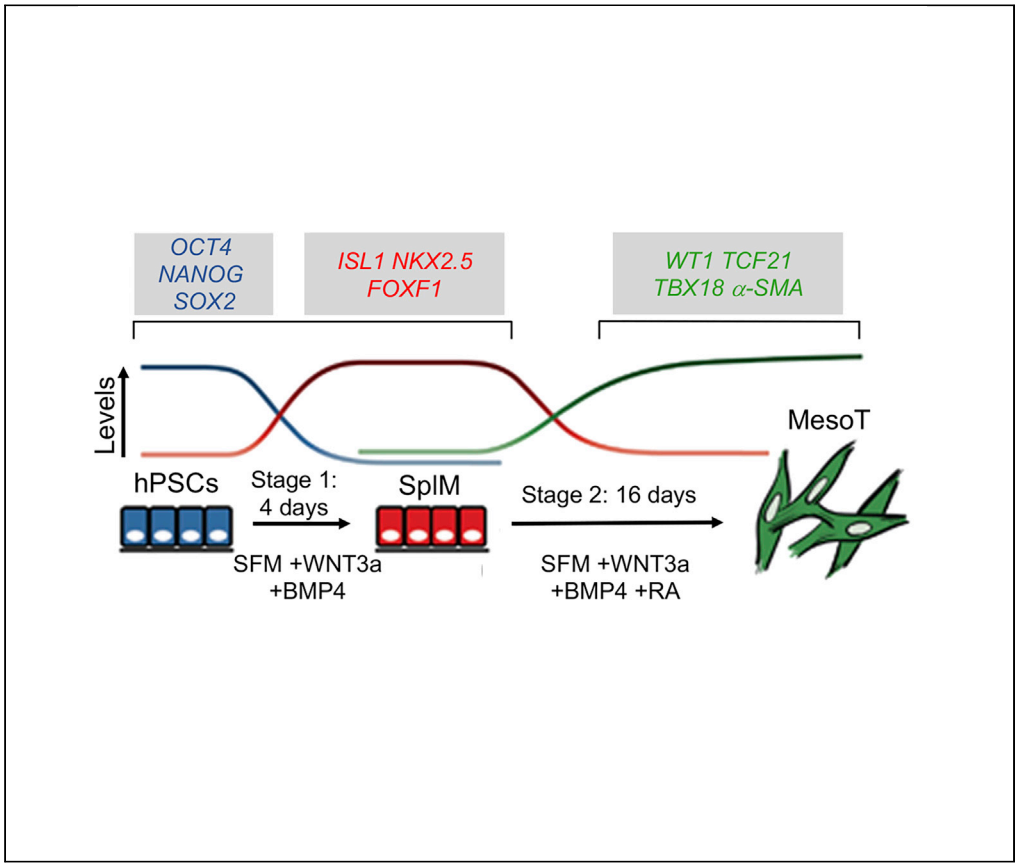
Multipotent Vascular Progenitor Cells of the Mesothelium Lineage Generated from Human Pluripotent Stem Cells



Vascularization is critical for organ homeostasis and function, but cell-based technologies that promote vascular regeneration are limited. This protocol describes steps to generate human pluripotent stem cell (hPSC)-derived vascular progenitors of the mesothelium lineage. This technology has several advantages for the generation of vascular cells. First and foremost, MesoT cells are multipotent progenitors that can generate smooth muscle cells and endothelial cells. MesoT cells therefore have potential utility in tissue repair, tissue engineering, and in vascularization of laboratory grown organs.

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HIGHLIGHTS

Generation of vascular progenitors (MesoT) from human pluripotent cells (hPSCs)

MesoT cells are equivalent to *in vivo* vascular progenitor cells in the embryonic heart

hPSC-derived MesoT cells are multipotent and derived from the mesothelium lineage

Human MesoT cells have potential utility in regenerative medicine

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Multipotent Vascular Progenitor Cells of the Mesothelium Lineage Generated from Human Pluripotent Stem Cells

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SUMMARY

Vascularization is critical for organ homeostasis and function, but cell-based technologies that promote vascular regeneration are limited. This protocol describes steps to generate human pluripotent stem cell (hPSC)-derived vascular progenitors of the mesothelium lineage. This technology has several advantages for the generation of vascular cells. First and foremost, MesoT cells are multipotent progenitors that can generate smooth muscle cells and endothelial cells. MesoT cells therefore have potential utility in tissue repair, tissue engineering, and in vascularization of laboratory grown organs.

For complete details on the use and execution of this protocol, please refer to Colunga et al. (2019).

BEFORE YOU BEGIN

General Laboratory Preparations

Timing: 30–60 min

1. Prepare media and solutions.
2. Set bead bath to 37°C.
3. Warm medium to 37°C.
4. All procedures are performed in a Class II biological hood with standard aseptic technique and cells are cultured in a humidified 37°C incubator with 5% CO_2.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse Monoclonal anti-TBX18 (Clone 635305)	R&D Systems	Cat#MAB63371; RRID: AB_10892533
Rabbit Monoclonal anti-Wilms Tumor Protein (Clone CAN-R9(IHC)-56-2)	Abcam	Cat#ab89901; RRID: AB_2043201
Goat Polyclonal anti-E-cadherin	R&D Systems	Cat#AF648; RRID: AB_355504

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse Monoclonal anti-alpha Smooth Muscle Actin (Clone 1A4)	Abcam	Cat#ab7817; RRID: AB_262054
Rabbit Polyclonal anti-CD31	Abcam	Cat#ab28364; RRID: AB_726362
ALDH1A2	Sigma	Cat#HPA010022
Vimentin	Abcam	Cat#ab92547
Donkey Polyclonal anti-Mouse IgG (H+L) Alexa Fluor 488 Conjugated	Thermo Fisher	Cat#:A21202; RRID: AB_141607
Donkey Polyclonal anti-Rabbit IgG (H+L) Alexa Fluor 488 Conjugated	Thermo Fisher	Cat#A21206; RRID: AB_141708
Donkey Polyclonal anti-Goat IgG (H+L) Alexa Fluor 555 Conjugated	Thermo Fisher	Cat#A21432; RRID: AB_141788
Donkey Polyclonal anti-Mouse IgG (H+L) Alexa Fluor 555 Conjugated	Thermo Fisher	Cat#A31570; RRID: AB_2536180
Donkey Polyclonal anti-Rabbit IgG (H+L) Alexa Fluor 555 Conjugated	Thermo Fisher	Cat#A31572; RRID: AB_162543
Donkey Polyclonal anti-Sheep IgG (H+L) Alexa Fluor 647 Conjugated	Thermo Fisher	Cat#A21448; RRID: AB_2535865
Donkey Polyclonal anti-Goat IgG (H+L) Alexa Fluor 647 Conjugated	Thermo Fisher	Cat#A21447; RRID: AB_141844
Donkey Polyclonal anti-Rabbit IgG (H+L) Alexa Fluor 647 Conjugated	Thermo Fisher	Cat#A31573; RRID: AB_2536183
Chemicals, Peptides, and Recombinant Proteins		
Probumin Life Science Grade	EMD Millipore	Cat#821005
Animal-Free Recombinant Human Heregulinβ-1	Peprotech	Cat#AF-100-03
LONG® R3 IGF-I human	Sigma-Aldrich	Cat#85580C
Recombinant Human FGF basic, 145 aa (TC Grade) Protein, CF	R&D Systems	Cat#4114-TC
Recombinant Human/Mouse/Rat Activin A Protein, CF	R&D Systems	Cat#338-AC
Recombinant Human Wnt-3a Protein	R&D Systems	Cat#5036-WN
Recombinant Human BMP-4 Protein, CF	R&D Systems	Cat#314-BP
Recombinant Human VEGF 165 Protein	R&D Systems	Cat#293-VE
Recombinant Human PDGF-BB Protein, CF	R&D Systems	Cat#220-BB
SB 431542	Tocris	Cat#1614
L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate ≥95%	Sigma-Aldrich	Cat#A8960
Transferrin (HOLO), Human Plasma, Tissue Culture Grade	Athens Research & Technology	Cat#16-16-032001-LEL
Glutagro supplement (200mM L-alanyl-L-Glutamine)	Corning	Cat#25-015-CI
Antibiotic Antimyotic Solution 100X	Corning	Cat#30-004-CI
MEM Nonessential Amino Acids	Corning	Cat#25-025-CI
Trace Elements A	Corning	Cat#25-021-CI
Trace Elements B	Corning	Cat#25-022-CI
Trace Elements C	Corning	Cat#25-023-CI
2-Mercaptoethanol	Gibco	Cat#21985023

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Dulbecco's Modification of Eagle's Medium/ Ham's F-12 50/50 Mix	Corning	Cat#15-090-CM
Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix	Thermo Fisher	Cat#A1413302
Matrigel	VWR	Cat#47743-715
Collagenase, Type IV, powder	Thermo Fisher	Cat#17104-019
TrypLE™ Select Enzyme (10X), no phenol red	Thermo Fisher	Cat#A1217701
Retinoic acid, ≥98% (HPLC), powder	Sigma-Aldrich	Cat#R2625
DPBS, 1X without calcium or magnesium	Corning	Cat#21-031
Accutase	Innovative Cell Technologies	Cat#AT104
Donkey Serum Sterile Filtered	Equitech-Bio	Cat#SD30
Triton X-100	Fisher Scientific	Cat#BP151
Glycine	Sigma-Aldrich	Cat#G8898
4′,6-Diamidino-2-phenylindole dihydrochloride	Sigma-Aldrich	Cat#D9542
ProLong Diamond Antifade Reagent	Invitrogen	Cat#P36961
EDTA (0.5 M), pH 8.0	Thermo Fisher	Cat#AM9260G
Sodium Chloride	Fisher Scientific	Cat#S640
Paraformaldehyde, 16% Solution, EM Grade.	VWR	Cat#15170
Experimental Models: Cell Lines		
WA09 hESCs (NIH approval number NIHhESC-10-0062)	WiCell	RRID: CVCL_9773
Other		
Stericup-GP, 0.22 μm, polyethersulfone, 500 mL, radio-sterilized	EMD Millipore	Cat#SCGPU05RE
Steriflip™ Sterile Disposable Vacuum Filter Units	EMD Millipore	Cat#SE1M 179 M6
Fisherbrand™ Sterile Cell Strainers	Fisher Scientific	Cat#22-363-547
p20 Pipet tips	Rainin	Cat#30389226
p200 Pipet tips	Rainin	Cat#30389240
p1000 Pipet tips	Rainin	Cat#30389213
5mL/10mL/25mL/50mL Sterile polystyrene Disposable Serological Pipets	Fisher Sci	Cat#1367811/11D11/E/11F
CELLTREAT 9IN PP Pasteur Pipet	Fisher Sci	Cat#50-828-726
Corning centrifuge tubes, 15 mL	Fisher Sci	Cat#0553859A
Corning centrifuge tubes, 50 mL	Fisher Sci	Cat#05526B
Tissue culture dishes, 60mm	Fisher Sci	Cat#08772F
Tissue culture dishes, 100mm	Fisher Sci	Cat#12556002
Lab-Tek chamber slides	Thermo Scientific	Cat#177437
Microscope Cover Glass: Rectangles 24*60	Fisher Sci	Cat#12545G

MATERIALS AND EQUIPMENT

Growth Factor Stock Solution

Reconstitute growth factors as per the manufacturer's recommendations and aliquot into small volume.

⚠ CRITICAL: Store aliquoted stock solutions at −20°C. Keep working stocks at 4°C for a week.

L-Ascorbic Acid 2-Phosphate Sesqui-Magnesium Salt Hydrate Stock Solution

Reagent	Final Concentration	Mass/Volume
L-Ascorbic acid 2-phosphate sesqui-magnesium salt hydrate	50 mg/mL	5 g
H_2O (Sterile)	n/a	100 mL
Total	**n/a**	**100 mL**

Store at 4°C for 2 weeks.

Probumin Stock Solution

Reagent	Final Concentration	Mass/Volume
Probumin	20%	1 kg
Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 (F12) (DMEM/F12)	n/a	5 L
Total	**n/a**	**5 L**

Dissolve Probumin with constant stirring (6–8 h) and filter the solution in Stericup™ sterile vacuum filter units. Store at 4°C for up to 6 months.

Transferrin Stock Solution

Reagent	Final Concentration	Mass/Volume
Transferrin	20 mg/mL	100 mg
DMEM/F12	n/a	5 L
Total	**n/a**	**5 L**

Store at 4°C for 2 weeks.

Retinoic Acid Stock Solution

Reagent	Final Concentration	Mass/Volume
All-trans retinoic acid (RA)	10 mM	100 mg
Dimethyl sulfoxide	n/a	33.28 mL
Total	**n/a**	**33.28 mL**

Aliquot and store at −20°C in dark.

⚠ CRITICAL: Aliquot RA for single-use only and discard after one thaw.

Serum-free Medium (SFM)

Reagent	Final Concentration	Volume (mL)
DMEM/F12	n/a	430.89
Probumin	2%	50
Nonessential amino acids	1X	5
Antimycotic/antibiotic	1X	5

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Reagent	Final Concentration	Volume (mL)
L-Alanyl-L-glutamine	1X	5
Trace element A	1X	0.5
Trace element B	1X	0.5
Trace element C	1X	0.5
β-Mercaptoethanol	0.1 mM	0.9
Transferrin	10 μg /mL	0.25
L-Ascorbic acid 2-phosphate sesqui-magnesium salt	50 μg/mL	0.5
Recombinant human heregulin β-1	10 ng/mL	0.5
LONG® R3 IGF-I	200 ng/mL	0.1
Recombinant human Activin A (rhActivin A)	10 ng/mL	0.2
Recombinant human basic fibroblast growth factor	8 ng/mL	0.16
Total	**n/a**	**500**

Filter medium in Stericup™ sterile vacuum filter units. Store at 4°C for up to 1 week.

⚠ CRITICAL: Aliquot medium (50 mL) without growth factors into small volume if it will not be used up in a week and add growth factors before use.

Splanchnic Mesoderm (SplM) Medium

Reagent	Final Concentration	Volume (mL)
SFM	n/a	49.9
Recombinant human Wingless 3a protein (rhWNT3a)	25 ng/mL	0.05
Recombinant human bone morphogenic protein 4 (rhBMP4)	100 ng/mL	0.05
Total	**n/a**	**50**

Store at 4°C for up to 1 week.

MesoT Medium

Reagent	Final Concentration	Volume (mL)
SFM	n/a	9.985
Recombinant human Wingless 3a protein (rhWNT3a)	25 ng/mL	0.01
Recombinant human bone morphogenic protein 4 (rhBMP4)	50 ng/mL	0.005
RA	4 μM	0.004
Total	**n/a**	**10**

Store medium without RA at 4°C for up to 1 week.

⚠ CRITICAL: Only add RA after medium is warmed up and before use.

Dissociation Solution I

Reagent	Final Concentration
DMEM/F12	n/a
Collagenase type IV	800 units/mL

Adjust volume based on plate size (i.e. 3–5 mL for a 60 mm dish) and filter the solution with Steriflip™ sterile vacuum filter units.

⚠ CRITICAL: Do not store this solution. Make fresh as needed.

Dissociation Solution II

Reagent	Final Concentration	Mass/Volume
Dulbecco's Phosphate Buffered Saline (DPBS)	n/a	499.5 mL
Sodium chloride	1.85 g/L	0.925 g
0.5 M EDTA (pH 8.0)	0.5 mM	0.5 mL
Total	n/a	500 mL

Filter the solution through Stericup™ sterile vacuum filter units. Keep it sterile at 25–37°C.

Geltrex/Matrigel Coated Plates/Slides

DMEM/F12
1:200 Geltrex/Matrigel

Add enough volume of final dilution to tissue culture dishes/plates/slides (i.e. 2 mL for a 60 mm dish) and incubate at 37°C, 5% CO_2 for at least 30 min.

⚠ CRITICAL: Chill DMEM/F12 and tubes before use. Work quickly and perform the process on ice.

Coated plates can be kept in the incubator for 1 week, but dispose the plates if they dry out.

STEP-BY-STEP METHOD DETAILS

Generating SplM from hPSCs

Timing: 4 days

This step provides directions about how to perform the first stage of differentiation, involving conversion of hPSCs to SplM.

1. Culture hPSCs on Geltrex-coated plates in SFM from our previous protocol (Menendez et al., 2013). hPSCs should be >95% positive for pluripotency markers OCT4 and NANOG and be of normal karyotype. Once cell density reaches 80%–90% of the plate surface area, aspirate medium and add enough Accutase® (25°C–37°C) to cover the plate surface (i.e. 2 mL to a 60 mm plate). Incubate the plate in a 37°C, 5% CO_2 incubator for 5–7 min.

   Note: Monitor dissociation under a bright-field microscope (10X) to ensure that cells are dissociated into singlets. Extend the dissociation time to 10 min if needed.

2. Pool all cells in a 15 mL conical tube. Wash the plate with 2 mL of SFM and collect into the same conical tube. Centrifuge at 200 × *g* for 4 min at 25°C–37°C.
3. Aspirate the supernatant and fully resuspend cell pellets in 5 mL of SplM medium then, plate at a density of 5 × 10^4–1 × 10^5 cells per cm^2 onto Geltrex-coated plates to initiate differentiation.

   Note: Seeding density for SplM differentiation may vary for different cell lines. Adjust seeding density if required. For WA09, seed cells at a density of 5 × 10^4 cells per cm^2. Other hPSC lines such as WA01, WA07, TE03 (https://grants.nih.gov/stem_cells/registry/current.htm) and K3 (hiPSC) (Si-Tayeb et al., 2010) are routinely seeded at a density of 1 × 10^5 cm^2. A suggested density range of ±1.25, 1.5 and 2.0-fold that of the seeding density described in the main protocol.

4. Culture the cells at 37°C in a 5% CO_2 incubator.
5. Replace SplM medium each day until day 4. Warm medium to 37°C before use.

Alternatives: Several protocols have been described for the generation of SplM from hPSCs. We recommend using SFM described in this protocol supplemented with differentiation factors although other media formulations support SplM differentiation (Lindsley et al., 2006; Sumi et al., 2008). So long as >90% of cells express ISL1, NKX2.5 and FOXF1 after 4 days these cultures should be suitable to use for next stage of differentiation (MesoTs). In some alternative approaches, GSK3β inhibitors are used in place of WNT ligands to the same effect. Base media such as RPMI can also be used instead of DMEM/F12. In addition to Geltrex, low growth factor Matrigel can also be used as a matrix onto which cells are seeded. Different cell lines respond to different concentrations of differentiation factors. It may be necessary to titrate these factors if desired results are not obtained but it is recommended that conditions specified in the main protocol are used initially.

Making MesoT Vascular Progenitor Cells from Splanchnic Mesoderm

Timing: 16 days

Here, directions are provided for conversion of SplM to MesoT cells.

6. After 4 days in SplM differentiation medium, cells are ready for culture in MesoT medium. First, aspirate the medium and wash the plate with DPBS twice.

Note: No re-plating of cells is required during the transition from SplM to MesoT.

7. Pre-warm MesoT medium (-RA) in a 37°C bath and thaw out one vial of aliquot RA. Add RA into warm medium at final concentration of 4 μM to make complete MesoT medium.

⚠ CRITICAL: After reconstitution, RA should be aliquoted into 1.5 mL tubes for single use. RA should be protected from light so working quickly is crucial. Do not re-freeze or re-use RA.

8. Aspirate DPBS and add complete MesoT medium onto the plate.
9. Culture cells at 37°C, 5% CO_2. Change MesoT medium daily for 16 days. See Figure 1 for expected results.

Alternatives: Generation of MesoT cells from SplM is best performed with DMEM/F12 basal media supplemented with fraction V BSA that is depleted of fatty acids, growth factor and endotoxin activity. Probumin (Life Science Grade) or equivalent products from other vendors (LifeCycle Biotechnology, MP Biomedical) are suitable for this application. Replacement of BSA with other stabilizers such as polyvinyl alcohol (PVA) and amino acids are also an option (Biggers et al., 1997). Batch testing of BSA is always recommended. Media is supplemented with FGF2, WNT3a, BMP4 and all-trans retinoic acid. Optimal concentrations of these compounds may vary depending on the cell line used but a useful starting point is defined in the main protocol. Depending on the hPSC line used, MesoT differentiation ranges from 14–18 days.

Generation of Smooth Muscle Cells from MesoT Cells

Timing: 12 days

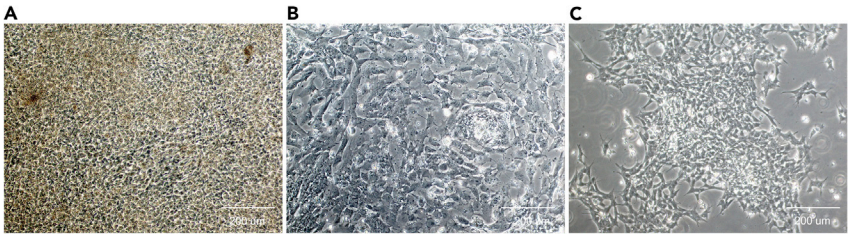

Figure 1. Successful Differentiation versus Failed Differentiation of MesoT Cells

Successful differentiation (A) versus failed differentiation (B and C) of MesoT cells. (A) A layer of collagen forms beneath densely packed MesoTs after 20 days of differentiation- seen as white areas between cells. (B and C) Cells are sub-confluent and lack a definable collagen layer, indicative of sub-optimal differentiation. All images are at 10X. Micron bars, 200 μm.

This step involves the generation of smooth muscle cells from MesoT cells.

10. On day 16 of MesoT differentiation, aspirate medium and add warm dissociation solution I onto the plate. Incubate cells with dissociation solution I in a 37°C, 5% CO_2 incubator for 30–45 min.
11. Monitor cell detachment every 15 min and stop dissociation once the edge of cell layer detaches from the plate by aspirating dissociation solution I. See Figure 2 for expected results.

12. Add dissociation solution II with TrypLE™ at 9:1 to plate and incubate at 37°C, 5% CO_2 for 15 min. Check cells under microscope until cell are fully dissociated into single cells.
13. Once in a single cell suspension, transfer all cells into a 50 mL conical tube and centrifuge cells at 200 g for 4 min at 25°C–37°C.
14. Seed at 1.5 × 10^5 cells per cm^2 in SMC differentiation medium comprising SFM depleted of rhActivin A, supplemented with 50 ng/mL PDGF-BB on Geltrex-coated plates or chamber slides.
15. Change SMC differentiation medium every other day until day 12.

Generation of Endothelial Cells from MesoT Cells

Timing: 12 days

Here, steps for the generation of endothelial cells from MesoT cells are described.

16. On day 16 of MesoT differentiation, aspirate medium and add warm dissociation solution I onto the plate. Incubate cells with dissociation solution I in a 37°C, 5% CO_2 incubator for 30–45 min.
17. Monitor cell detachment every 15 min and stop dissociation once the edge of cell layer detaches from the plate by aspirating dissociation solution I (Figure 2).
18. Add dissociation solution II with TrypLE™ at 9:1 to plate and incubate at 37°C, 5% CO_2 for 15 min. Check cells under microscope until cell are fully dissociated into single cells.

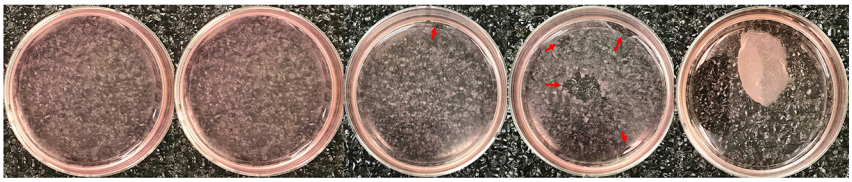

Figure 2. Monitoring MesoT Dissociation

Step 1: 3 mL of dissociation solution I (Col IV) is added to a 60 mm plate followed by incubation at 37°C. Images shown (from left to right) indicate incubation of cells in Col IV for 0, 10, 20 and 30 min, respectively. Optimal time of dispersal is 30–45 min. The far-right image represents excessive dispersal. Red arrows indicate areas where the cell sheet is lifting from the culture dish. When the edge of the cell sheet starts to detach, cells are ready to proceed to the next step of dissociation (addition of TrypLE).

19. Once in a single cell suspension, transfer all cells into a 50 mL conical tube and centrifuge cells at 200 g for 4 min at 25°C–37°C.
20. Seed at 1.5 × 10^5 cells per cm^2 in endothelial cell (EC) differentiation medium made by removing rhActivin A from SFM and supplementing with 50 ng/mL rhVEGF-A_{165} and 20 μM SB 431542.
21. Change differentiation medium every other day until day 12.

⚠ CRITICAL: We recommend filtering dissociated cells through a sterile 40 μm cell strainer before counting. This additional step can ensure cells in singlets and also eliminate collagens.

Generation of Mixed Populations of Smooth Muscle Cells and Endothelial Cells from MesoT Cells

Timing: 12 days

22. On day 16 of MesoT differentiation, aspirate medium and add warm dissociation solution I onto the plate. Incubate cells with dissociation solution I in a 37°C, 5% CO_2 incubator for 30–45 min.
23. Monitor cell detachment every 15 min and stop dissociation once the edge of cell layer detaches from the plate by aspirating dissociation solution I.
24. Add dissociation solution II with TrypLE™ at 9:1 to plate and incubate at 37°C, 5% CO_2 for 15 min. Check cells under microscope until cell are fully dissociated into single cells.
25. Once in a single cell suspension, transfer all cells into a 50 mL conical tube and centrifuge cells at 200 g for 4 min at 25°C–37°C.
26. Seed at 1.5 × 10^5 cells per cm^2 in 'mixture' differentiation medium made by removing rhActivin A from SFM and supplementing with 50 ng/mL rhVEGF-A_{165} on Geltrex-coated plates or chamber slides.
27. Change medium every other day until day 12.

EXPECTED OUTCOMES

This protocol describes the generation of multipotent vascular progenitors that have significant potential utility in regenerative medicine (Figure 3). The first stage of differentiation towards SplM is initiated by supplementing SFM with rhBMP4 (days 0–4) and rhWNT3a protein (day 0–1). These conditions promote the exit from pluripotency and progression towards SplM through a mesendoderm (primitive streak equivalent) intermediate. This is indicated by cells undergoing an epithelial to mesenchymal (EMT), down-regulation of pluripotency markers (OCT4, NANOG, SOX2) and the transient upregulation of primitive streak markers such as T/BRACHYURY (Colunga et al., 2019). At 2–3 days of differentiation, mesendoderm markers decline and are replaced by the expression of *ISL1*, *NKX2.5* and *FOXF1*, indicative of SplM cell identity (Colunga et al., 2019). SplM itself is a multipotent progenitor with broad-range cardiovascular differentiation potential, including the generation of cardiomyocytes (Birket et al., 2015) and blood lineages (Lancrin et al., 2009; Zovein et al., 2010). Next stage of differentiation involves the conversion of SplM into MesoT progenitor cells. This uses the same SFM as for SplM but is supplemented with all-trans RA for a further 16 days. At this stage, cells can be collected or fixed for downstream analysis (i.e. qRT-PCR, flow cytometry or immunofluorescence staining) to validate cell identity (Table 1). ~ 90% of cells in cultures can be expected to express WT1, TBX18, ALDH1A2, vimentin and α-smooth muscle actin while being negative for epithelial cell markers such as E-cadherin (Figure 4). Passaging and other mechanical manipulation of cells is not required during differentiation towards SplM and MesoT, making the protocol straight-forward and reproducible. On day 16, cells are ready for downstream analysis (i.e. immunofluorescence staining). MesoT cells are multipotent and give rise to SMCs and ECs at high-efficiency. Over 90% of cells exposed to SMC medium for 12 days express typical SMC markers such as calponin and α-SMA. These cells do not express MesoT markers such as WT1, TBX18 and TCF21 or endothelium markers such as vWF, VE-cadherin and CD31 (Figure 5). Cells that are cultured in endothelium

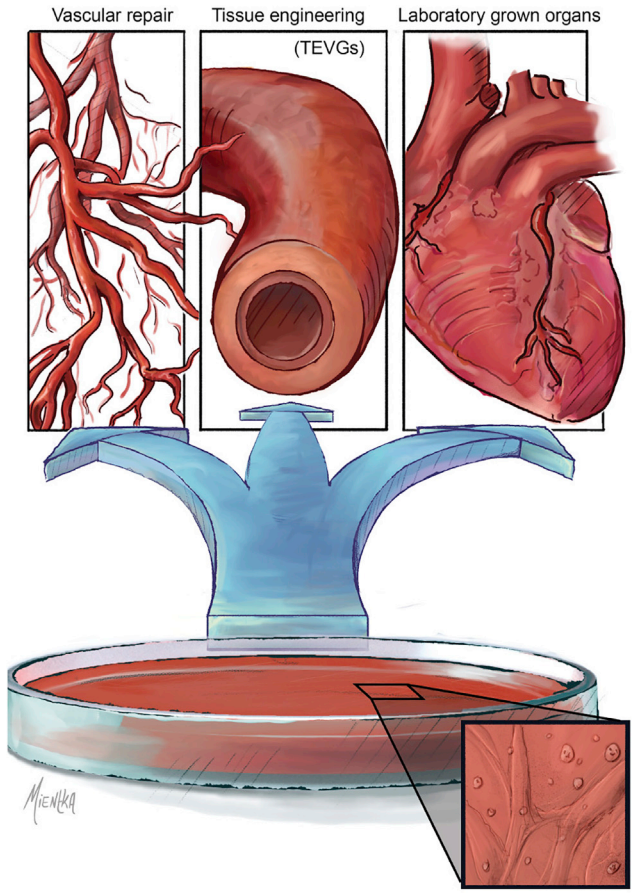

Figure 3. Applications for MesoT Cells

First, in vascular repair where cells can be used to repair existing vasculature or to establish new vasculature in disease or damaged tissue. Second, incorporation of MesoT cells and their derivatives into tissue engineered vascular grafts (TEVGs). Finally, MesoT cells can potentially be used as a source of vascular progenitors to seed the vasculature in laboratory constructed organs.

medium expresses endothelium markers of vWF and CD31 but are negative for MesoTs marker (WT1) and α-SMA expression. Cells cultured in 'mixture' differentiation medium generate SMCs and ECs; ~40% of cells express endothelium markers (CD31 and vWF) and ~ 60% of cells express SMC markers (α-SMA and Calponin) under the prescribed conditions.

LIMITATIONS

For clinical use, a completely defined media system should be used. This will involve omitting Probumin and matrices, such as Matrigel.

Table 1. Human Cell Characterization

Cell Type	Markers	Assays
PSCs	*OCT4, SOX2, NANOG*	qRT-PCR, IF
SplMs	*ISL1, NKX2-5, GATA4, FOXF1*	qRT-PCR, flow cytometry, IF
MesoTs	*WT1, TBX18, TCF21, ALDH1A2, PDGFR-β, VIM, ACTA2*	qRT-PCR, flow cytometry, IF
SMCs	*ACTA2, CNN1, MYH11*	IF
ECs	*CD31, CDH5, vWF*	IF

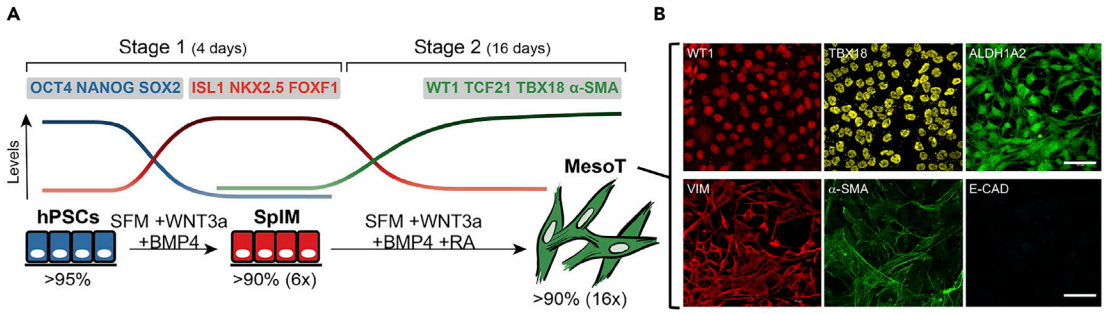

Figure 4. Generation of MesoTs from Human Pluripotent Stem Cells

(A) 2-step generation of MesoT cells from human pluripotent stem cells (hPSCs) in serum-free media (SFM). Stage 1 is driven by WNT3a and BMP4 which converts hPSCS to splanchnic mesoderm (SpIM). Stage 2 specifies SpIM to become MesoT vascular progenitors by addition of retinoic acid. The purity of cells at each stage, indicated by expression of characteristic markers, and the fold-amplification in cell number are indicated.

(B) Characteristic markers of MesoT vascular progenitors (WT1, TBX18, ALDH1A2) and those illustrating their mesenchymal status (vimentin^{+}, α-SMA^{+}, E-CAD^{-}). Micron bar, 50 μm.

TROUBLESHOOTING

Problem

Step 1 & 12 Cells are in clumps after dissociation

Potential Solutions

- Warm up dissociation solutions
- Monitor cells under bright field microscope to ensure cells are fully dissociated into singlets

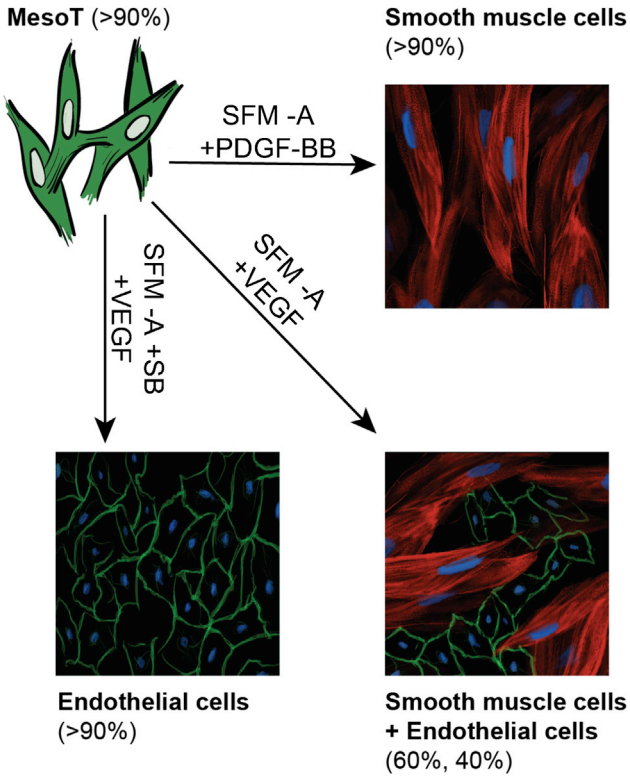

Figure 5. Schematic Illustrating the Differentiation of MesoT Cells into Vascular Lineages

Conditions for differentiation of MesoT cells to endothelial cells, smooth muscle cells and mixtures of endothelial and smooth muscle cells are indicated together with the purity of resulting cell populations.

- In Step 12, 19 and 25 ensure that cell sheets are detached from plates before changing into 1X TrypLE solution

Problem

Failed/heterogeneous differentiation

Potential Solution

- SplM differentiation: Seeding density is cell line dependent. Optimize seeding density when using different cell lines. A suggested density range is ±1.25, 1.5 and 2.0-fold that of the seeding density recommended in the main protocol.
- SplM and MesoT differentiation: Expired/invalid growth factors. Make sure all the growth factors are fresh reconstituted (<3 months, -20°C).
- SplM and MesoT differentiation: Modification of growth factor concentrations. Starting with the amounts of growth factors and small molecules in the main protocol, it is recommended that a range of ±1.5 and 2.0-fold be used if the differentiation efficiency is sub-optimal (<90% marker^{+}).

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AUTHOR CONTRIBUTIONS

L.C. and S.D. contributed equally to the preparation of the manuscript.

DECLARATION OF INTERESTS

Patents have been awarded or are pending for intellectual property described in this report (S.D.).

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