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
Protocol

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% CO2.

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#ab9901; 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

| PROTEIN | SOURCE | IDENTIFIER |
|---------|--------|------------|
| 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#AB960 |
| 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-Cl |
| Antibiotic Antimyotic Solution 100X | Corning | Cat#30-004-Cl |
| MEM Nonessential Amino Acids | Corning | Cat#25-025-Cl |
| Trace Elements A | Corning | Cat#25-021-Cl |
| Trace Elements B | Corning | Cat#25-022-Cl |
| Trace Elements C | Corning | Cat#25-023-Cl |
| 2-Mercaptoethanol | Gibco | Cat#21985023 |

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## Protocol

| REAGENT or RESOURCE                          | SOURCE                          | IDENTIFIER        |
|----------------------------------------------|---------------------------------|-------------------|
| Dulbecco’s Modification of Eagle’s Medium/   | Corning                         | Cat#15-090-CM     |
| Ham’s F-12 50/50 Mix                        |                                 |                   |
| Geltrex™ LDEV-Free, hESC-Qualified, Reduced| Thermo Fisher                    | Cat#A1413302      |
| Growth Factor Basement Membrane Matrix       |                                 |                   |
| 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  |
| Coming centrifuge tubes, 15 mL                                   | Fisher Sci                     | Cat#0553859A    |
| Coming 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         |
| H₂O (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%                 | 2%                  | 50          |
| Nonessential amino acids    | 1X                  | 5           |
| Antimycotic/antibiotic      | 1X                  | 5           |

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Continued

| 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 sesquimagnesium 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.
Δ 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

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% CO₂ for at least 30 min.

Δ 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% CO₂ 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 × 10⁴–1 × 10⁵ cells per cm² 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 × 10⁴ cells per cm². 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 × 10⁵ cm². 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% CO₂ 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% CO₂. 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
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% CO₂ 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% CO₂ 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 x 10⁵ cells per cm² 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% CO₂ 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% CO₂ for 15 min. Check cells under microscope until cell are fully dissociated into single cells.

**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.

**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 x 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-A165 and 20 μM SB 431542.

21. Change differentiation medium every other day until day 12.

Δ 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 collagen.

### 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% CO2 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% CO2 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 x 10^5 cells per cm^2 in ‘mixture’ differentiation medium made by removing rhActivin A from SFM and supplementing with 50 ng/mL rhVEGF-A165 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 straightforward 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
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, N intellectuals, 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 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.
**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

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**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.

**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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