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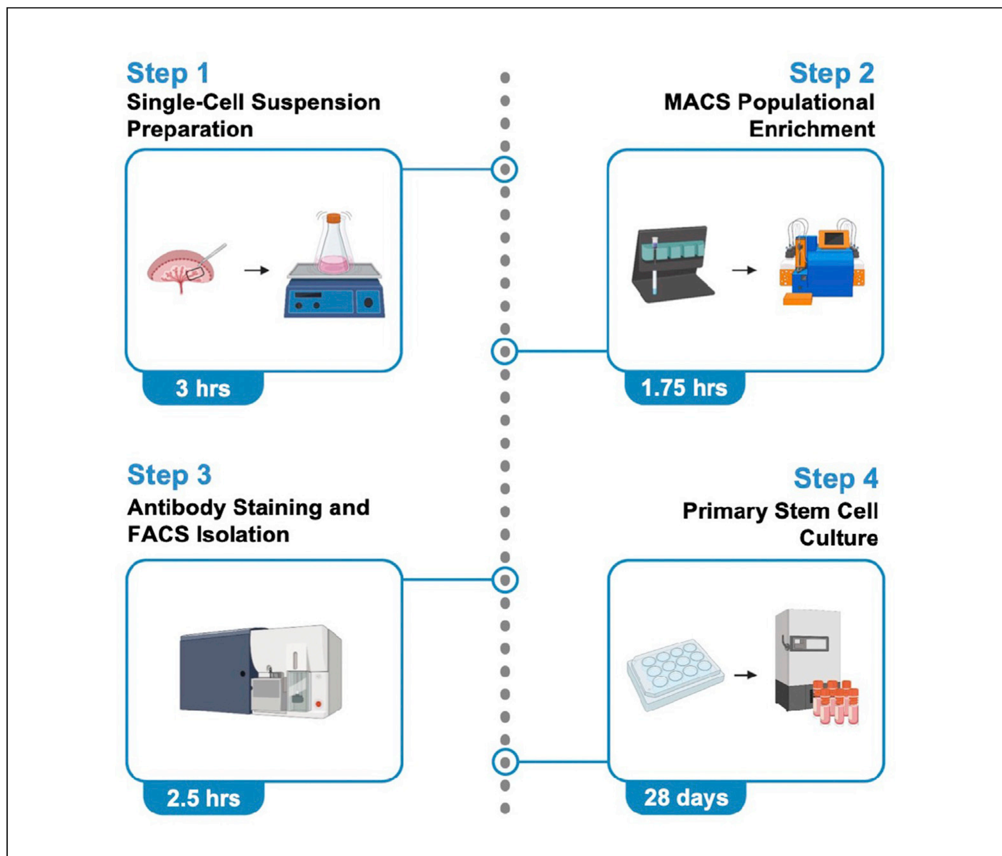
Protocol

High-yield isolation of pure fetal endothelial colony forming cells and mesenchymal stem cells from the human full-term placenta



A need to identify a stem cell source for human endothelial colony forming cells (ECFCs) and mesenchymal stem cells (MSCs) that is high yield is crucial for their implementation in ischemia. Our lab has developed an isolation protocol to do this using full-term human villous placental tissue. This protocol describes enzymatic tissue digestion followed by MACS and FACS, achieving an 8 times greater yield versus traditional isolation techniques and delivering pure fetal stem cell colonies within 21–28 days cell culture.

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Highlights

A detailed protocol to isolate pure fetal stem cells from human full-term placenta

Deriving fetal stem cells in clinically relevant quantities

Assessing the therapeutic capacity of ECFC and MSC in treating ischemic injury

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Protocol

High-yield isolation of pure fetal endothelial colony forming cells and mesenchymal stem cells from the human full-term placenta

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SUMMARY

A need to identify a stem cell source for human endothelial colony forming cells (ECFCs) and mesenchymal stem cells (MSCs) that is high yield is crucial for their implementation in ischemia. Our lab has developed an isolation protocol to do this using full-term human villous placental tissue. This protocol describes enzymatic tissue digestion followed by MACS and FACS, achieving an 8 times greater yield versus traditional isolation techniques and delivering pure fetal stem cell colonies within 21–28 days cell culture.

For complete details on the use and execution of this protocol, please refer to Patel et al. (2013) and Patel et al. (2014).

BEFORE YOU BEGIN

Timing: Varies

1. Obtain the relevant and appropriate institutional permissions for the use of human samples. All placental tissue samples referenced here were obtained with written and informed consent from the Royal Brisbane and Women's Hospital as approved by both the University of Queensland and the Royal Brisbane and Women's Hospital human research ethics committees.
2. Receive training on all relevant machinery and equipment including:
   a. The Miltenyi AutoMACS® Pro Separator. Please note that the MidiMACS™ Separator Starting Kit may be used in its place.
   b. A flow cytometer with FACS capabilities including 640 nm, 561 nm, 488 nm, 405 nm, and 355 nm lasers such as BD FACSAria™ Fusion Sorter.
3. Prepare all reagents according to their guidelines below, taking into account storage time and conditions.

⚠ CRITICAL: Reagent preparation should be performed inside a sterile biological safety cabinet (BSC).

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4. Prepare collagen coated plates according to the following guidelines:
   a. Add 298 μL of Acetic Acid to 50 mL UltraPure DNase/RNase-Free Distilled Water and filter using a 0.2 μm sterile filter.
   b. Add 600 μL of pre-sterilized Rat Tail Collagen Type 1 and gently homogenize.
   c. Add 200 μL of Collagen Type 1 Solution to a 24-well plate for ECFCs and a 96-well plate for MSCs, ensuring the bottom is thoroughly covered. You will only need to prepare 2–4 wells per plate type.
   d. Leave this to set for 1–24 h at room temperature (RT, 20°C–25°C) and run 2 × 20-min UV cycles within this time.
   e. Remove any remaining collagen solution via pipetting and wash 2 times with DPBS. Leave the flask or plate to dry completely and use immediately for cell culture or store in clingfilm at 4°C for up to 2 weeks.

⚠ CRITICAL: Collagen coated plate preparation should be performed inside a sterile BSC.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Dynabeads™CD45 Dilution: 250 μL/ 50 g starting tissue.	Life Technologies	Cat#11153D, https://www.thermofisher.com/order/catalog/product/11153D
CD34 Microbead Kit Dilution: 100 μL/ 50 g starting tissue.	Miltenyi Biotec	Cat#130-046-702, https://www.miltenyibiotec.com/AU-en/products/cd34-microbead-kit-human.html
Mouse anti-Human CD34 Clone: 581 Sample dilution: 15 μL/ 50 g starting tissue. Staining control volume: 3 μL	Bio-Rad	Cat# MCA1578, RRID: AB_1125259, https://www.bio-rad-antibodies.com/monoclonal/human-cd34-antibody-581-mca1578.html?f=rpe
Mouse anti-Human CD45 Clone: HI30 Sample dilution: 15 μL/ 50 g starting tissue. Staining control volume: 3 μL	BioLegend	Cat#304006, RRID: AB_314394, https://www.biolegend.com/en-us/products/fitc-anti-human-cd45-antibody-707?GroupID=BLG5926
Mouse anti-Human CD31 Clone: WM59 Sample dilution: 15 μL/ 50 g starting tissue. Staining control volume: 3 μL	BD Biosciences	Cat#561653, RRID: AB_10896326, https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/v450-mouse-anti-human-cd31.561653
Live/ Dead 7'AAD Dilution: 20 μL / 50 g starting tissue.	BD Biosciences	Cat#559925, RRID: AB_2869266
Biological samples		
Full-term (38–39 weeks) human placenta, isolated via cesarean section	Your relevant institution	N/A
Chemicals, peptides, and recombinant proteins		
HBSS, 1×, no calcium, no magnesium, no phenol red	Life Technologies	Cat#14175095
Penicillin-Streptomycin-Glutamine, 100×	Life Technologies	Cat#10378016
Gentamicin, 50 mg/mL	Life Technologies	Cat#15750-060
DPBS, 1×, no calcium, no magnesium	Life Technologies	Cat#14190136
Collagenase, Type I, powder	Life Technologies	Cat#17100017
Dispase II, powder	Life Technologies	Cat#17105041
DNase I, bovine pancreas	Sigma-Aldrich	Cat#D5025-375KU
Ammonium Chloride	Sigma-Aldrich	Cat#A9434-500G
Potassium Bicarbonate	Sigma-Aldrich	Cat#P9144-500G
UltraPure™ 0.5 M EDTA, pH 8.0	Invitrogen	Cat#15575020
DPBS, 1×, no calcium, no magnesium	Life Technologies	Cat#14190136
Bovine Serum Albumin	Sigma-Aldrich	Cat# A9418-100G
Dynabeads CD45	Life Technologies	Cat#11153D

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
UltraPure DNase/RNase-Free Distilled Water	Life Technologies	Cat#10977015
Acetic Acid, Molecular Weight: 60.05	Sigma-Aldrich	Cat#A6283-2.5L
Rat Tail Collagen, Type I	Sigma-Aldrich	Cat#C3867-1VL
Fetal Bovine Serum, Australian origin	Life Technologies	Cat#10099141
Endothelial Cell Growth Basal Medium-2, EBM-2	Lonza	Cat#CC-3156
Endothelial SingleQuots Kit	Lonza	Cat#CC-4176
TrypLe Express Enzyme, 1×, no phenol red	Life Technologies	Cat#12604021
DMSO	Sigma-Aldrich	Cat#D2650-100ML
Other		
Stainless steel tray	N/A	N/A
Anatomical scissors, scalpel, forceps	N/A	N/A
Petri dish, 100 mm × 15 mm	Corning	Cat#351029
Cell culture grade plates and flasks; 96-well, 24-well, T25, T75, T175	N/A	N/A
250 mL conical glass flask	N/A	N/A
Falcon™ 100 µm cell strainer	Fisher Scientific	Cat#352360
Falcon™ 40 µm cell strainer	Fisher Scientific	Cat#352340
Falcon™ 50 mL conical centrifuge tubes	Fisher Scientific	Cat#14-432-22
Falcon™ 15 mL conical centrifuge tubes	Fisher Scientific	Cat#05-527-90
1.5 mL Protein LoBind Tubes	Eppendorf	Cat#0030108116
FACS collection tubes, 5 mL Round-Bottom Polystyrene, with Cell Strainer Snap Cap	Corning, Falcon®	Cat#352235
2 mL Cryovials	Nunc	Cat#375418
10 mL serological pipettes	Interpath Services Pty Ltd	Cat#607180
25 mL serological pipettes	Interpath Services Pty Ltd	Cat#760180
Cool Cell™ Freezing Container	Corning	Cat#432001
P1000, P200, P20 pipettes and matching sterile filtered pipette tips	N/A	N/A
Cell Counting Slides for TC20™ with Trypan Blue	Bio-Rad	Cat#1450015
Bioshaker	Taitec	Cat# BR-22FH
Benchtop Centrifuge, equipped with rotor S-24-11-AT	Eppendorf	model no. 5427R
Water Bath, SDminiN	Taitec	Cat#0068750-000
Pipette gun	Thermo Scientific™	Cat#9501
TC20™ Cell Counter	Bio-Rad	Cat#1450102
DynaMag™-15 Magnet	Invitrogen™	Cat#12301D
Miltenyi AutoMACS® Pro Separator	Miltenyi Biotec	Cat#130-092-545
Miltenyi AutoMACS® Columns	Miltenyi Biotec	Cat#130-021-101
MidiMACS™ Separator Starting Kit (optional)	Miltenyi Biotec	Cat#130-042-501
Flow cytometer with FACS capabilities including 640 nm, 561 nm, 488 nm, 405 nm, and 355 nm lasers such as BD FACSAria™ Fusion Sorter	BD Biosciences	N/A
Original Research Publication	Patel et al. (2013).	https://doi.org/10.5966/sctm.2013-0092
Original Research Publication	Patel et al. (2014).	https://doi.org/10.1016/j.placenta.2014.09.001

MATERIALS AND EQUIPMENT

Stock solutions for isolation

Timing: 2 h

⚠ CRITICAL: Reagent preparation and storage should be performed under sterile conditions where possible.

Stock Collagenase		
Reagent	Final concentration	Amount
Collagenase type 1 powder	50 mg/mL	1 g
HBSS (w/o Ca, Mg)	–	20 mL
Total	N/A	**20 mL**

Filter this solution with a 0.2 μm sterile syringe filter. This solution can be stored at −20°C for up to 2 years.

Stock Dispase II		
Reagent	Final concentration	Amount
Dispase II powder	50 mg/mL	1 g
PBS (w/o Ca, Mg)	–	20 mL
Total	N/A	**20 mL**

Filter this solution with a 0.2 μm sterile syringe filter. This solution can be stored at −20°C for up to 2 years.

Stock DNase		
Reagent	Final concentration	Amount
DNase 1 powder	10,000 U/mL	375 KU
PBS (w/o Ca, Mg)	–	37.5 mL
Total	N/A	**37.5 mL**

This solution can be stored at −20°C for up to 3 years.

Working buffers for isolation

Timing: 2 h

⚠ CRITICAL: Working buffers should be prepared in a sterile BSC.

⚠ CRITICAL: All buffers listed here are optimized for 50 g starting tissue. Scale as appropriate.

Digestion Buffer		
Reagent	Final concentration	Amount
Stock Collagenase	1 mg/mL	1 mL
Stock Dispase II	1 mg/mL	1 mL
Stock DNase I	10 U/mL	1 mL
DPBS (w/o Ca, Mg) (warmed 37°C)	–	47 mL
Total	N/A	**50 mL**

Prepare this buffer directly before use. Do not store long-term.

Washing Buffer		
Reagent	Final concentration	Amount
Gentamicin (50 mg/mL)	0.1 mg/mL	200 μL
100× Penicillin	1×	5 mL
100× Streptomycin	1×	5 mL
HBSS (warmed 37°C)	–	500 mL
Total	N/A	**510.2 mL**

Prepare this buffer directly before use. Do not store long-term.

Red Blood Cell Lysis Buffer		
Reagent	Final concentration	Amount
Ammonium Chloride	8.26 mg/mL	4.13 g
Potassium Bicarbonate	1 mg/mL	0.5 g
EDTA 0.5 M	0.38 mM	383 µL
UltraPure DNase/Rnase-Free Distilled Water	–	500 mL
Total	N/A	**~500 mL**

This buffer can be stored at RT for 6 months, out of light.

MACS Buffer		
Reagent	Final concentration	Amount
BSA	10 mg/mL	5 g
EDTA 0.5 M	3 mM	3 mL
DPBS (w/o Ca, Mg)	–	500 mL
Total	N/A	**500 mL**

Filter this buffer with a 0.2 µm sterile filter. This can be stored at 4°C for 1 month. It is critical to keep this buffer at 4°C throughout the protocol.

Stock solutions for cell culture

Timing: 1.5 h

CRITICAL: Stock solutions should be prepared in a sterile BSC.

Heat-Inactivated FBS		
Reagent	Final concentration	Amount
FBS- Australia	100%	500 mL
Total	N/A	**500 mL**

Defrost 500 mL of FBS overnight at 4°C. Once defrosted, place the unopened FBS into a 55°C water-bath and heat for 60 min to inactivate the FBS. It is critical that the FBS remains sterile but note that the lid may need to be loosened prior to heating. Once heat-inactivated, in a BSC, filter the FBS through a 0.2 µm syringe filter and aliquot into 50 mL Falcon tubes. This solution can be stored at 4°C for 4 weeks or −20°C for 3 years. Thaw heat-inactivated FBS for use in this protocol, filtering with a 0.2 µm filter and keeping cool (4°C) for use as a sorting solution, but warm (37°C) when used to supplement basal culture medium.

Basal Culture Medium		
Reagent	Final concentration	Amount
Endothelial SingleQuots Kit	–	N/A
Endothelial Cell Growth Basal Medium-2 (EGM2)	–	500 mL
Total	N/A	**~500 mL**

Empty all Endothelial SingleQuots Kit vials into 500 mL of Endothelial Cell Growth Basal Medium-2. This can be stored at 4°C for up to 1 month.

Working solutions for cell culture

Timing: 1–2 h

CRITICAL: Working solutions should be prepared in a sterile BSC.

Complete Culture Medium (EGM2/10% Heat-Inactivated FBS)		
Reagent	Final concentration	Amount
Heat-Inactivated FBS	10%	5 mL
Basal Culture Medium	90%	45 mL
Total	N/A	**50 mL**

This solution can be stored at 4°C up to 1 month but is best to prepare fresh before each use. It is likely only one 50 mL tube will be required to begin. Warm to 37°C for use in stem cell culture.

Cryopreserving Solution		
Reagent	Final concentration	Amount
DMSO	10%	100 μL
Heat-Inactivated FBS	90%	900 μL
Total	N/A	**1,000 μL**

Prepare this solution directly before use. Note that 1 mL of cryopreserving solution is required per 1 million stem cells being cryogenically stored. Prepare appropriately.

Collagen Type 1 Solution		
Reagent	Final concentration	Amount
Acetic Acid	–	298 μL
Rat Tail Collagen Type 1	1.2%	600 μL
UltraPure DNase/RNase-Free Distilled Water	–	50 mL
Total	N/A	**~50 mL**

Both the collagen type 1 solution and prepared plates can be stored at 4°C up to 1 week. See Collagen Coated Plate Preparation for set-up.

DynaMag™-15 Magnet set-up

To use, remove the magnet with its stand from the box, sanitize, and place into the biological safety cabinet. Flip to have the 15 mL falcon tube right side up. To use, slide a 15 mL falcon tube into the corresponding slot, wait the appropriate time, and with the 15 mL falcon tube in place, tip the magnet forward to collect the supernatant. Refer here for use in the context of stem cell isolation.

Miltenyi AutoMACS® Pro Separator set-up

Caution: The use of this equipment requires a trained individual. Please refer to your institution for induction and training purposes.

> ***Alternatives:*** MidiMACS™ Separator Starting Kit. While the Miltenyi AutoMACS® Pro Separator is recommended due to advantages pertaining to purity, the MidiMACS™ Separator Starting Kit can be used in its place as a cheaper and more accessible alternative. To setup, remove the MACS™ Multi-stand with attached MACS™ Separator magnet from the box, sanitize, and place into the biological safety cabinet. Place a MACS™ column into the MACS™ Separator magnet field with the sample reservoir facing upwards and place a collection tube directly below the separator. Refer here for use in the context of stem cell isolation.

BD FACSAria™ Fusion Sorter

Caution: The use of this equipment requires a trained individual. Please refer to your institution for induction and training purposes.

STEP-BY-STEP METHOD DETAILS

> ⚠ CRITICAL: Placentas utilized in this protocol must be obtained with written and informed consent in accordance with the appropriate ethics guidelines, committees, and institutions.

⚠ CRITICAL: All steps should be completed under sterile conditions in a BSC free from contamination unless otherwise stated.

Placental tissue collection and preparation

⏲ Timing: 0.5 h

This describes the aseptic preparation of the placental tissue and includes dissection specifications to optimize the following section in which the tissue is enzymatically and mechanically digested.

1. Healthy human full-term placenta is donated via cesarean section and transferred to an autoclaved stainless-steel tray for dissection and processing within an hour immediately after surgery.
2. In a cell culture hood under sterile conditions, use forceps and scissors to remove any calcified tissue on the placenta and remove the thin layer of the maternal placental decidua (top layer) to avoid any maternal cell contamination.
3. Using forceps and scissors, dissect out 50 g of placental villi, which is enriched with fetal vasculature, and transfer onto a petri dish with washing buffer. Avoid collecting tissue from any clotted regions.
4. After placing the placenta villi section onto a petri dish, rinse the tissue with washing buffer until blood residue is removed. The tissue is now ready for digestion.

Placental tissue digestion

⏲ Timing: 2 h

This section specifies the steps for optimal placental tissue digestion, enabling creation of a placental single-cell suspension.

5. To aid enzymatic digestion efficiency, cut the villi into smaller pieces using surgical scissors, ensuring the vessel structures are homogenized.
6. Transfer the tissue into a 250 mL conical flask for digestion and add 75–80 mL of digestion buffer to the flask.
7. Digest the tissue for 2 h at 37°C in an incubated, preferably sterile, shaker, providing constant agitation at 65 rpm.

Preparation of single cell suspension

⏲ Timing: 1 h

Described here is the preparation of the placental single-cell suspension to allow for optimal downstream sub-populational enrichment. This section includes a pause point in which the placental single-cell suspension can be cryopreserved for use at a later date.

8. After 2 h of digestion, back in a BSC, filter the cell suspension to remove undigested aggregations using a 100 μm cell strainer, collecting the single cell suspension into an appropriate number of 50 mL falcon tubes at approximately 20 mL/ tube. Discard any undigested tissue. Troubleshooting 1.
9. Dilute the single-cell suspension by adding 20 mL of ice-cold DPBS per 50 mL falcon tube.
10. Pellet the cell suspension via centrifugation using 350 × *g* with (break speed 5) for 5 min at 4°C.
11. Discard 30–35 mL of the supernatant carefully using a 25 mL serological pipette. The cell pellet is soft, pipette slow to avoid loss of sample.
12. Resuspend the cell pellet in 30 mL of red blood cell (RBC) lysis buffer per 50 mL falcon tube, and incubate for 10 min on ice at 4°C.

⚠ CRITICAL: The suspension will appear pinkish-clear when lysis is successful. Incubation longer than 10 min will result in excessive cell death and loss of stem cell yield. It is critical to remove the remaining RBC lysis buffer. Troubleshooting 2.

13. To remove the RBC lysis buffer, wash the pellet 2 times. To wash the first time, centrifuge the cell suspension at 350 × *g* (break speed 5) for 5 min at 4°C. Discard 35 mL of the supernatant using a 25 mL serological pipette. The cell pellet is soft, pipette slow to avoid loss of sample.
14. To wash the second time, resuspend the cell pellet in 35 mL ice-cold MACS buffer per 50 mL falcon tube centrifuge at 350 × *g* (break speed 5) for 5 min at 4°C to re-pellet. Again, discard the supernatant using a 25 mL serological pipette. The cell pellet is soft pipette slow to avoid loss of sample.
15. Resuspend the cell suspension in 20 mL ice-cold MACS buffer to perform a cell count.

Pause point: Here it is possible to continue ECFC isolation (continue to step 16) or prepare samples for long-term cryogenic storage. Alternatively, cryogenic storage can be performed following CD45 depletion or CD34 enrichment to reduce the quantity of vials needed. See *Option a: cryogenic storage of sample* for cryogenic storage and see *Option b: thawing of samples from cryogenic storage* for thawing procedure.

a. Cryogenic storage of sample.
   i. Resuspend cell pellet at 1–5 million cells per mL of cryopreserving solution. Quickly aliquot 1 mL of the cell suspension per cryovial.
   ii. Place cryovials into a Cool Cell™ Freezing Container and transfer to a −80°C freezer or liquid nitrogen after 24 h.
b. Thawing of samples from cryogenic storage.

⚠ CRITICAL: To use cells from long term cryopreservation storage, the following steps should be completed rapidly to remove cells from DMSO and maximize cell viability.

   i. Add an appropriate amount of pre-warmed (37°C) Complete Culture Medium to a single cryovial of frozen cells. Gently pipette up and down until thawed.
   ii. Transfer the thawed cell suspension to a 15- or 50-mL falcon tube. If it is a pre-depletion/enrichment sample being thawed, 5 cryovials should be thawed per 50 mL falcon tube. Repeat until all cryovials are thawed.
   iii. Top up each falcon tube to 20 mL with Complete Culture Medium and wash the cells via centrifugation at 350 × *g* (break speed 5) for 5 min at 4°C.
   iv. Carefully discard the supernatant using a 25 mL serological pipette. The cell pellet is soft pipette slow to avoid loss of sample.
   v. Resuspend in 20 mL ice-cold MACS buffer and perform a cell count.

16. Resuspend the cell pellet in ice-cold MACS buffer at 1×10^7 cells/mL. Separate 50 mL falcon tubes can be consolidated at this step if appropriate.
17. Set aside a 100 μL aliquot of placental cell suspension (approximately 1×10^6 cells) into a sterile 1.5 mL Eppendorf tube and keep on ice until step 33 for preparation of flow cytometry with FACS antibody staining controls. This is referred to as the antibody control aliquot.

⚠ CRITICAL: Due to the low frequency of MSCs and ECFCs in the total placenta cell suspension, it is essential not to waste the enriched CD45 negative ($CD45^{NEG}$) and CD34 positive ($CD34^{POS}$) cell fraction.

Dynabeads™ CD45 depletion

Timing: 0.75 h

Here the placental single-cell suspension undergoes the first round of sub-populational enrichment via Dynabead™ CD45 depletion to remove any hematopoietic lineage contamination. The subsequent isolate is referred to as the CD45^{NEG} cell fraction. This enrichment enables time and cost reduction in later FACS steps, while increasing stem cell populational purity.

18. Prime 1 × 15 mL falcon tube of Dynabeads™ CD45 per 2.5 mL of placental cell suspension or less. To prime the beads, place 250 μL of Dynabeads™ CD45 in 3 mL of MACS buffer and homogenize.
19. Slot the Dynabeads™ CD45 suspension into the DynaMag™-15 Magnet placed inside the biological safety cabinet for 1 min to wash (See here for DynaMag Setup). Tip the magnet forward, keeping the 15 mL falcon tube containing Dynabeads™ CD45 suspension secure in the magnet to collect and subsequently discard the supernatant.
20. Remove the falcon tube containing the primed Dynabeads™ CD45 from the DynaMag™-15 Magnet and resuspend the Dynabeads™ CD45 in 250 μL of MACS buffer.
21. Add 2.5 mL of the placental cell suspension (1 × 10^7 cells/mL) to the washed Dynabeads™ CD45 suspension to make a Dynabeads™ CD45 cell suspension, gently mix well, and incubate for 20 min on a roller at 4°C.
22. Once incubated add 10 mL of MACS buffer to the Dynabeads™ CD45 cell suspension and place into the DynaMag™-15 Magnet for 2 min.
23. Collect the non-bound CD45^{NEG} cells in suspension in a new sterile 15 mL Falcon tube by tipping the magnet forward as previously described. This is the CD45^{NEG} cell suspension. Save this suspension.

⚠ CRITICAL: Label collection tubes clearly and adhere to the protocol to avoid the collection of the wrong fraction.

24. Pellet the CD45^{NEG} cell suspension at 350 × *g* (break speed 5) for 5 min at 4°C to collect the cell pellet. Carefully remove the supernatant using a 10 mL stripette.
25. Consolidate any CD45^{NEG} samples from separate falcon tubes together at 1 × 10^7 cells/mL in ice-cold MACS buffer. Proceed to step 26.

CD34 MACS microbead enrichment

Timing: 1 h

Here the placental single-cell suspension, now referred to as the CD45^{NEG} cell-suspension, undergoes its second round of sub-populational enrichment via selection for CD34^{POS} cells. This enrichment selects for endothelial lineage cells and, again, enables time and cost reduction in later FACS steps while increasing stem cell populational purity.

Alternatives: CD34^{POS} MACS™ Microbead enrichment can be achieved via 2 methods: *Option a: Automatic*, using the Miltenyi AutoMACS™ Pro Separator or *Option b: Manual*, using the MidiMACS™ Separator. Proceed to step 26 regardless and choose an alternative method at step 29.

26. Add 100 μL of CD34 microbeads to the CD45^{NEG} cell suspension for up to 1 × 10^8 cells. Mix well and incubate on ice for 20 min at 4°C.
27. After incubation, top up the CD45^{NEG} cell suspension with 10 mL MACS buffer.
28. Centrifuge the cell suspension at 350 × *g* for 5 min (break speed 5) at 4°C to collect the cell pellet. Carefully remove the supernatant using a 10 mL stripette.

29. CD34 enrichment.
    a. Automatic.

i. Resuspend the $CD45^{NEG}$ cell pellet in 5 mL of autoMACS running buffer and label 2 × 15 mL falcon tubes "CD34 negative" ($CD34^{NEG}$) and $CD34^{POS}$ for fraction collection. Pre-chill tubes by placing on ice for transport to the Miltenyi AutoMACS Pro Separator.
ii. Transport the sample, AutoMACS buffer, and chilled collection tubes to the Miltenyi AutoMACS Pro Separator.

⚠ CRITICAL: The Miltenyi AutoMACS Pro Separator should be operated by a trained individual only (See Miltenyi AutoMACS Pro Separator Setup). An overview of operation can be found below but is NOT a comprehensive guide. Please seek training where appropriate.

iii. Load the autoMACS running buffer into the corresponding position, switch on the Miltenyi AutoMACS Pro Separator and allow the initialisation program to run.
iv. Prime the Miltenyi AutoMACS Pro Separator.
v. Place the chill rack onto the autoMACS MiniSampler and load the $CD45^{NEG}$ cell suspension, $CD34^{NEG}$ collection tube, and $CD34^{POS}$ collection tube into their corresponding positions.
vi. Under the "Separation" menu, fill in the sample separation template field and select the "POSSEL" selection program with a "Rinse" wash cycle between samples if appropriate.
vii. Run program.
viii. Once complete, transfer the $CD45^{NEG}CD34^{POS}$ cell suspension to ice. Save this population.

⚠ CRITICAL: Label collection tubes clearly and adhere to the protocol closely to avoid collection of the wrong fraction.

ix. Ensure the correct shutdown functions are performed on the Miltenyi AutoMACS Pro Separator. Proceed to step 30.

b. Manual.
i. An LS column can load up to 2×10^9 total cells. Place the LS column in the magnetic field of the MidiMACS™ Separator (see here for MidiMACS™ Separator Setup).
ii. To prepare the LS column, pre-wash the column by loading 3 mL of MACS buffer and discard the flow-through.

⚠ CRITICAL: Make sure the column reservoir is empty before proceeding to the next step.

⚠ CRITICAL: Label collection tubes clearly and adhere to the protocol to avoid collection of the wrong fraction.

iii. Load the labeled $CD45^{NEG}$ cell suspension onto the pre-washed column. Collect the flow-through in a new sterile 15 mL Falcon tube. This is the $CD34^{NEG}$ fraction. It is not necessary to keep this fraction.

⚠ CRITICAL: Make sure the column reservoir is empty before proceeding to the next step.

iv. Wash the column with 3 mL of MACS buffer. Collect and combine with the $CD34^{NEG}$ flow-through. Repeat this wash 3 times.

⚠ CRITICAL: Make sure the column reservoir is empty before proceeding to the next step.

v. Remove column from the MidiMACS™ Separator and place it on a new sterile 50 mL falcon tube.
vi. Pipette 5 mL of MACS buffer onto the column and flush out the magnetic labeled cells by pushing the plunger into the column. This is the $CD45^{NEG}CD34^{POS}$ cell suspension. Save this suspension. Proceed to step 30.

⚠ CRITICAL: Label collection tubes clearly and adhere to protocol to avoid collection of the wrong fraction.

30. Back in the sterile BSC, top up the $CD45^{NEG}CD34^{POS}$ cell suspension with 10 mL of ice-cold MACS buffer. Pellet the cell suspension via centrifugation at 350 × *g* (break speed 5) for 5 min at 4°C.
31. Remove the supernatant and resuspend the cell pellet in 200 μL ice cold MACS buffer. Transfer cell suspension to a 1.5 mL Eppendorf tube labeled "Sample" and place on ice for FACS antibody staining.

Antibody staining

⏲ Timing: 1 h

Here the now enriched $CD45^{NEG}CD34^{POS}$ cell-suspension undergoes antibody staining in preparation for FACS. Re-staining for CD45 and CD34 again ensures populational purity upon isolation, while the addition of CD31 staining, a marker for mature endothelium, enables identification of the MSC (CD31 negative, $CD31^{NEG}$) and ECFC (CD31 low, $CD31^{LO}$) populations. It is essential that these steps are followed as specified to ensure accurate cell sorting outcomes, including the preparation of unstained and single-stain staining controls for compensation set-up. Fluorescence-minus-one (FMO) staining controls are also critical to correctly identifying both MSC and ECFC populations.

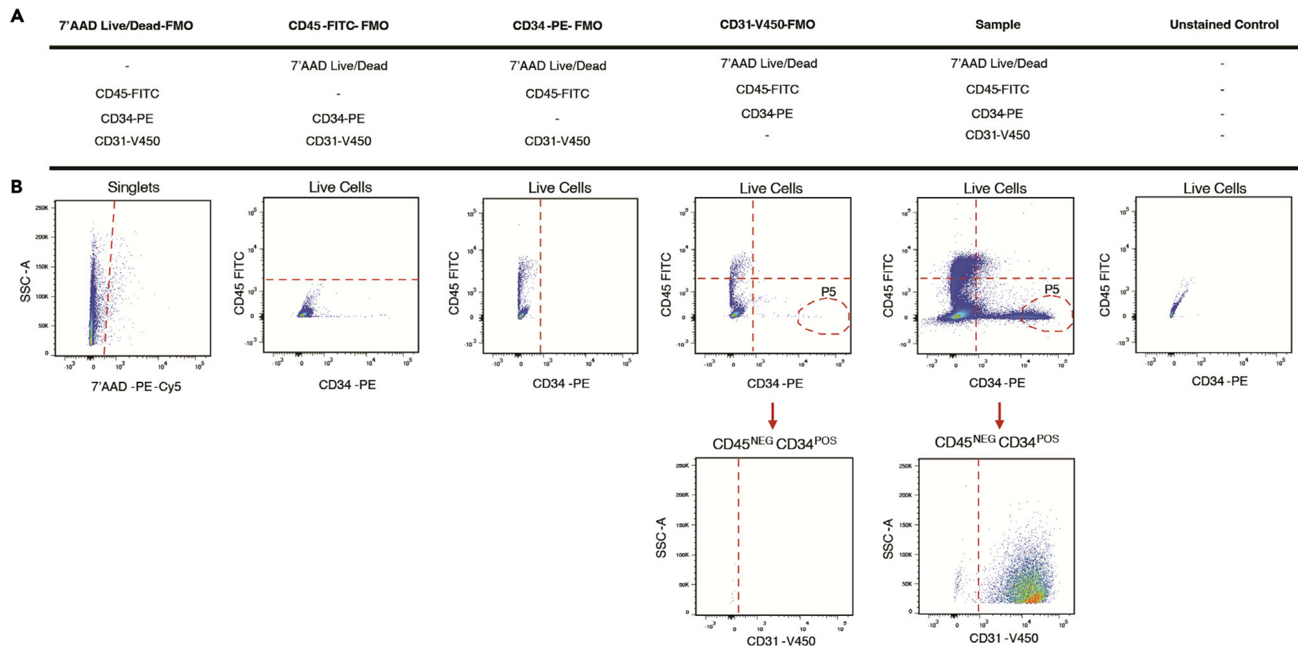
32. Prepare 9 × 1.5 mL Eppendorf tubes for FACS staining controls, placing 50 μL of ice-cold MACS buffer in each. Label each tube as a single stain (7′AAD, CD45, CD34, and CD31), an FMO (7′AAD-FMO, CD45-FMO, CD34-FMO, and CD31-FMO), and unstained control.
33. Dilute the 100 μL antibody control aliquot (approximately 1×10^6 cells) from step 17 into 450 μL total volume using ice cold MACS buffer and place 50 μL into each of the staining control tubes on ice for a total volume of 100 μL per control.
34. Add the appropriate volumes of CD45, CD34, and CD31 antibodies to their corresponding tubes (see: key resources table). See Figure 1A for sample, FMO, and unstained control antibody groupings. Homogenize thoroughly and incubate on ice at 4°C out of the light for 20 min, on a roller.

⚠ CRITICAL: Do not add any antibodies to the unstained control and do not add 7′AAD Live/Dead until 15 min prior to sorting. This stain does not require washing out of the sample.

35. Once incubation is complete, remove excess antibody by washing. To do so, add 1 mL of MACS buffer to each tube and centrifuge the cell suspensions at 350 × *g* (break speed 5) for 5 min at 4°C to pellet.
36. Aspirate the supernatant completely. To resuspend the pellet, add 200 μL of ice-cold MACS buffer to each staining control and add 500 μL of ice-cold MACS buffer to the sample.
37. To ensure a single-cell suspension for FACS, filter each cell suspension using a 40 μm cell strainer into a respectively labeled 5 mL FACS tube with its lid and place on ice for transport to a sterile flow cytometry facility.
38. For sorting collection, prepare sterile 5 mL FACS tubes or 1.5 mL Eppendorf tubes filled with 500 μL -1 mL filtered heat-inactivated FBS, labeled ECFC ($CD45^{NEG}CD34^{POS}CD31^{LO}$) and MSC ($CD45^{NEG}CD34^{POS}CD31^{NEG}$).

Fluorescence-activated cell sorting

⏲ Timing: 2 h



A

7'AAD Live/Dead-FMO	CD45-FITC- FMO	CD34-PE- FMO	CD31-V450-FMO	Sample	Unstained Control
-	7'AAD Live/Dead	7'AAD Live/Dead	7'AAD Live/Dead	7'AAD Live/Dead	-
CD45-FITC	-	CD45-FITC	CD45-FITC	CD45-FITC	-
CD34-PE	CD34-PE	-	CD34-PE	CD34-PE	-
CD31-V450	CD31-V450	CD31-V450	-	CD31-V450	-

Figure 1. FMO, sample, and unstained control antibody specifications with FMO gating strategies

(A) Antibody staining specifications for 7′AAD Live/Dead, CD45-FITC, CD34-PE, CD31-V450 fluorescent-minus-one (FMO) controls, Sample and Unstained Control.

(B) Representative flow plots demonstrating FMO gating strategy for 7′AAD Live/Dead, CD45-FITC, CD34-PE and CD31-V450 FMOs. Subsequent Sample gating is shown, as well as the Unstained Control.

This section details the steps required for the isolation of both MSC (CD45^{NEG}CD34^{POS}CD31^{NEG}) and ECFC (CD45^{NEG}CD34^{POS}CD31^{LO}) stem cell populations via FACS. It is critical to refer to the sample flow plots provided to ensure accuracy of population selection.

39. On the flow cytometer use a 100 μm nozzle to achieve low pressure (approx. 20 psi) and run the samples at a medium flow rate (<2.0) with an event rate of approximately 1,000 events/ second.

Warning: Flow cytometry with FACS should be completed on an appropriate machine operated by a trained individual. Please seek assistance or training where required.

40. Use the unstained control and single-stained controls to set up compensation. FMOs should be used to set the appropriate gates (Figures 1A and 1B).
41. Generate a forward scatter area (FSC-A) versus side scatter area (SSC-A) to select the total cell population (P1) (Figure 2A, Sample).
42. Generate an FSC-A versus forward scatter height (FSC-H) to select for singlets, named P2 (Figure 2A, P1). SSC-A versus side scatter height (SSC-H) will confirm this, named P3 (Figure 2A, P2).
43. To select for the live population, generate 7′AAD PE-Cy5A versus SSC-A and gate on the negative fraction, termed P4 (Figure 2B, P3). Troubleshooting 3.
44. Following this, generate CD34 PE-A versus CD45 FITC-A and create a gate on the CD45^{NEG}CD31^{POS} population, named P5 (Figure 2B, P4).
45. Generate a CD31 BV421-A versus SSC-A dot plot panel to identify the CD31^{NEG} MSC population (P6) and CD31^{LO} ECFC subset of endothelial cells (P7) (Figures 2B and 2C, P5).
46. Sort these populations directly into ice-cold filtered heat-inactivated FBS.

⚠ CRITICAL: Obtain cell population numbers from the sort for the purpose of cell culture. Troubleshooting 4.

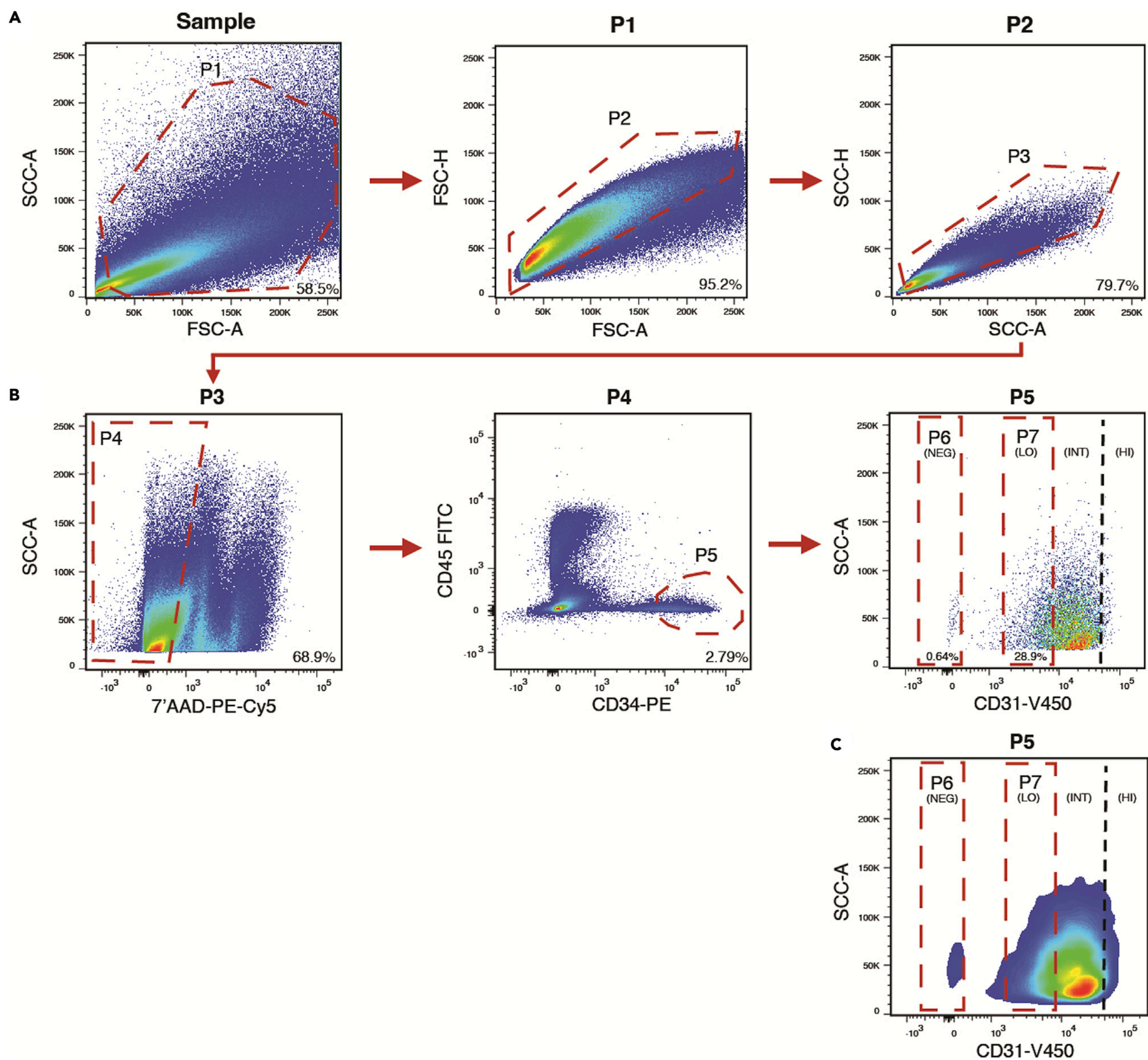


Figure 2. FACS gating strategy for placental ECFC and MSC populations

(A) Representative flow dot plots detailing selection for the whole cell population (P1) and doublet exclusion (P2 and P3). Population frequencies shown as percent (%) parent gate.

(B) Representative flow dot plots detailing selection for the CD45^{NEG}CD34^{POS} parent population (P5) to both CD31^{NEG} (MSC) (P6) and CD31^{LO} (ECFC) (P7). Gates for CD31-intermediate (INT) and CD31-high (HI) expression have been indicated. Population frequencies shown as percent (%) parent gate.

(C) Representative flow pseudo-color smooth plot emphasizing the respective CD31^{NEG} (MSC) (P6) and CD31^{LO} (ECFC) (P7) populations.

Primary cell culture and colony establishment

Timing: 7–28+ days

Here we describe the optimal process for encouraging MSC and ECFC colony formation and subsequent expansion. It is essential that sterile conditions are maintained throughout this process as these stem cells may remain in culture beyond 28 days. Please note that MSCs will often undergo clonogenesis and expansion 2× faster than ECFCs.

47. Following collection, place isolated populations on ice and once back in a BSC top up with 3 mL ice cold DPBS.
48. Pellet cells via centrifugation 500 × *g* (break speed 5) for 5 min at 4°C.
49. Resuspend cells in warmed (37°C) Complete Culture Medium and seed onto a type I collagen-coated plate at approximately 1 × 10^4 cells/cm^2. For example, a 96-well plate is often used for MSCs, while a 24-well plate is used for ECFCs.
50. Culture cells at 37°C in a sterile primary incubator under standard mammalian conditions.
51. Allow 48 h for cells to adequately attach to their plate. Any cells left in suspension following this are not likely to adhere.
52. Following 48 h, refresh the Complete Culture Medium by carefully pipetting out the old media without touching the bottom of the well. To do this, rest the tip of the pipette tip against the wall of the well, close to the bottom. Leave 10% of the old media in the well to prevent cells drying out. Pipette new media in slowly, placing the pipette tip against the wall, approximately 1 cm from the base.
53. Refresh media on a stringent 24-h basis for 5 days following attachment.
54. Refresh Complete Culture Medium on a stringent 48-h basis thereafter until strong High Proliferative Potential ECFCs (HPP-ECFCs) and MSC colonies have formed, taking from 21–28 days for ECFCs and 7–14 days for MSCs (Figures 3D and 3E). Troubleshooting 5.
55. Once adequate HPP-ECFCs and MSC colonies have been established, but before reaching confluency, passage cells (P0). Please note, MSCs may form colonies approximately 2× faster than HPP-ECFCs.
56. To passage, wash the cells by removing the Complete Culture Medium by resting the tip of the pipette tip against the wall of the well, close to the bottom. Leave 10% of the old media in the well to prevent cells drying out.
57. Place enough DPBS to cover the well by slow pipetting, placing the pipette tip against the wall, approximately 1 cm from the base.
58. Remove the DPBS by the same method and repeat the wash 1 time.
59. Complete the final DPBS wash by removing all DPBS from the well. Add a volume of RT TrypLE to the culture plate. For example, add 100 μL to a single well of a 24-well plate.
60. Incubate the cells in the TrypLE in a sterile incubator under standard conditions at 37°C for 3–5 min.
61. Using a microscope, check ECFCs or MSCs have detached, gently tapping the side of the vessel if necessary, and add a volume (e.g., 900 μL) of warm (37°C) Complete Culture Medium to inhibit the TrypLE and create a cell suspension.
62. Ensure all cells have detached by gently washing over the bottom of the well with the Complete Culture Medium cell suspension.
63. Transfer the cell suspension to a sterile 15 mL Falcon tube with an additional 1 mL Complete Culture Medium.
64. To wash cells, pellet via centrifugation at 500 × *g* for 5 min at room temperature and discard the supernatant.
65. Resuspend cells in 1 mL warm Complete Culture Medium.
66. Seed ECFCs into a type I collagen-coated plate of the same size previously used and incubate at 37°C in a sterile incubator under standard conditions. This will encourage HPP-ECFC and Low Proliferative Potential-ECFC (LPP-ECFC) homogeneity and expansion without reducing cell density and causing unnecessary cell stress. For MSCs, graduate to a 12-well plate.
67. Allow 48-h for cells to adequately adhere to their plate.
68. Replenish media every 24-h for the first 5 days following adherence, and every 48-h thereon.
69. Repeat passaging to a minimum of P3 before cryopreservation, seeding cells at a density of 1 × 10^5/cm^2 from P1 onwards. Collagen coating is not required following P0. It will be possible to expand into multiple T175's by P3, therefore allowing biobanking of approximately 2–3 million ECFCs and 5 million MSCs per flask.
70. To cryopreserve both ECFCs and MSCs, lift cells as described in steps 56–64.
71. Rapidly, resuspend cells at a concentration of 1 × 10^6 cells/ mL in cryopreservation solution to make a cryopreservation cell suspension.

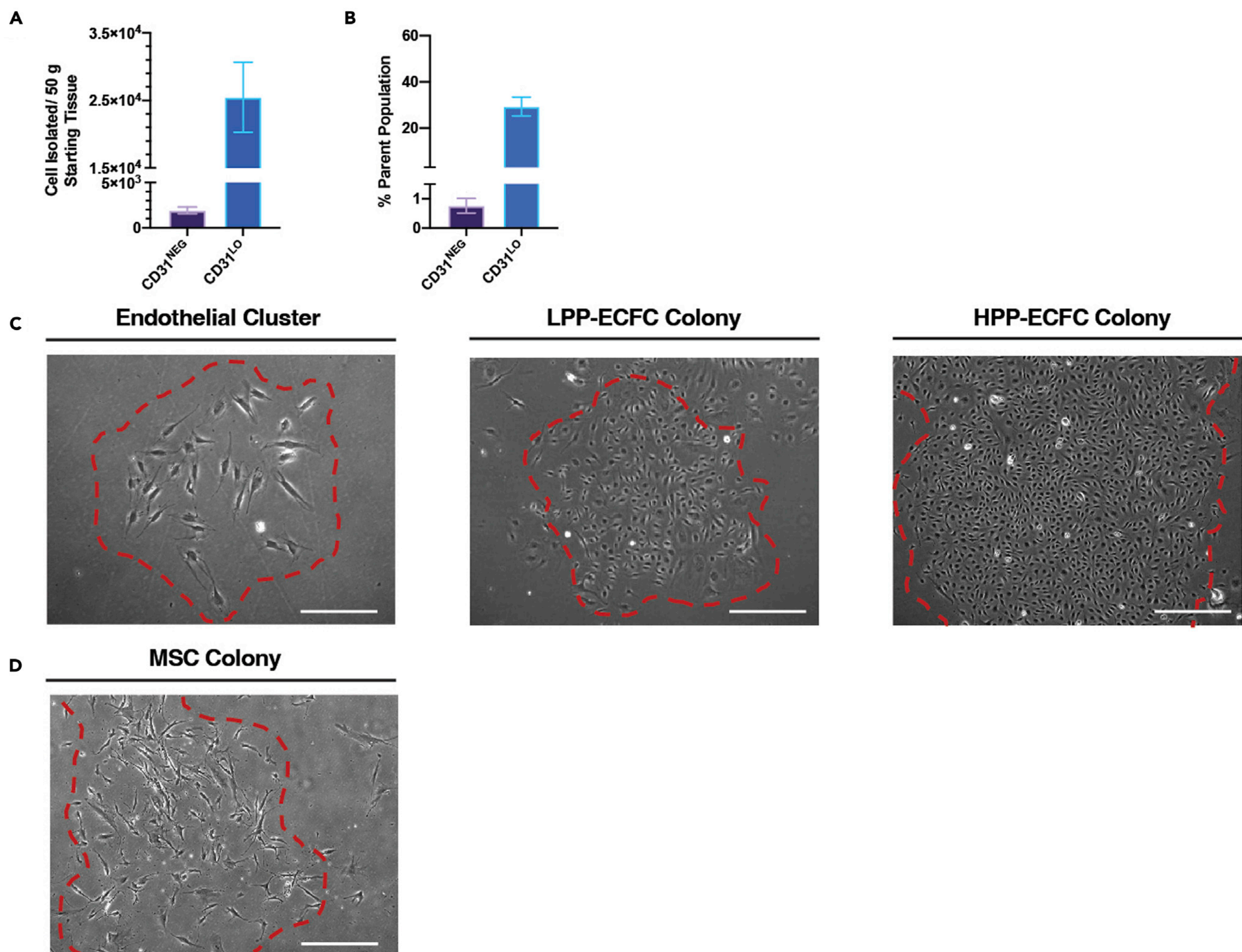


Figure 3. ECFC and MSC colony formation

(A) $CD31^{NEG}$ (MSC) and $CD31^{LO}$ (ECFC) cell frequency per 50 g starting placental tissue via flow cytometry (data presented as mean ± SD, n = 3).
(B) $CD31^{NEG}$ (MSC) and $CD31^{LO}$ (ECFC) cell frequency as a percent (%) of the parent $CD45^{NEG}CD34^{POS}$ population (data presented as mean ± SD, n = 3).
(C) Representative confocal images detailing endothelial cluster (scale bar indicates 10 μm), LPP-ECFC (scale bar indicates 100 μm), and HPP-ECFC (scale bar indicates 100 μm) colony formation. Note that when colonies are small, ECFCs may appear more elongated, adopting a cobble-stone-like morphology with increasing cell-cell contact. Red indicates colony border.
(D) Representative confocal images showing MSC colony formation (scale bar indicates 100 μm). Red indicates colony border.

72. Aliquot 1 mL cryopreservation cell suspension per cryovial and place into a Cool Cell™ Freezing Container.
73. Rapidly, transfer the Cool Cell™ Freezing Container to a −80°C freezer or liquid nitrogen. Cells can be stored in this condition as per standard cell cryopreservation guidelines. Refer to step 15b for thawing procedure for use in downstream assays. Refresh Complete Media every 2–3 days as per standard cell culture guidelines.

EXPECTED OUTCOMES

In terms of pre-depletion and enrichment via MACS, the $CD45^{NEG}CD34^{POS}$ population is rare, and will represent approximately 3.0% ± 1.2% of total placental cells (data not shown here) (Shafiee et al., 2018). Within this, MSC and ECFC identification is even more infrequent. It is expected that from 50 g of starting placental tissue that an average of 2×10^3 MSCs will be isolated, representing

0.76 ± 0.43% of the parent $CD45^{NEG}CD34^{POS}$ FACS population, while 2.5×10^4 ECFCs will be collected, representing 29.30 ± 7.11% (Figures 3A and 3B). Once plated, ECFC colonies will typically appear from 10–28 days post sort, but is dependent on variables such as donor history, cell viability, seeding density, and culture quality. MSCs will typically establish at twice the speed of ECFCs, however, this is also donor dependent.

It is important to maintain a sterile environment throughout the culture process due to prolonged colony formation timelines increasing risk of contamination. From 50 g of tissue a yield of 123 ± 15 HPP-ECFC colonies per 100,000 $CD45^{NEG}CD34^{POS}CD31^{LO}$ cells is expected (data not shown here) (Patel et al., 2013). HPP-ECFC colonies can be identified as large colonies with >1,000 cells which can form secondary and tertiary colonies, while LPP-ECFCs contain >50 cells, and endothelial clusters will be <50 cells and have limited secondary colony formation capability (Figure 3C). MSCs will more often form large colonies which undergo consistent expansion (Figure 3D). It will be important to identify correct ECFC and MSC morphology, as ECFCs that appear inconsistent with the indicated cuboidal cobble-stone-like shape may require confirmative ECFC antigen profiling through FACS (e.g., $CD34^{POS}$, $CD31^{POS}$) in addition to standard endothelial markers, such as VE-Cadherin (CD144), VEGFR2, CD105, CD146, and HLA-ABC, and mesenchymal marker negativity such as CD90 (Patel et al., 2013, 2016). These identifiers can also be confirmed via RT-qPCR or western blot; however, these techniques will not allow for downstream culturing of the same population. Functional analysis may also be required through acetylated low-density lipoprotein uptake or single-cell colony formation assay (Patel et al., 2013, 2016). Conversely, MSCs can be identified in culture via their spindle-like morphology, with their functional capacity confirmed through both osteogenic and adipogenic differentiation assays (Patel et al., 2014). Similarly to ECFCs, MSC status can also be confirmed through RT-qPCR or flow cytometry expression of mesenchymal markers such as CD29, CD44, CD73, CD90, CD105 (Patel et al., 2014).

The characteristics of ECFCs isolated via this procedure reflect that of those obtained through classical umbilical cord blood isolation and are consistent with ECFC morphological standards. Specifically, the placental ECFC fraction contains both high ($CD34^{HI}$) and low ($CD34^{LO}$) proliferative colony forming populations (Patel et al., 2013, 2016). Placental ECFCs also bare genetic and functional equivalence to classical umbilical cord blood ECFCs, with microarray analysis indicating a significant ($p < 0.05$) difference in expression of only 33 genes, such as *IGFBP2*, fibronectin, and elastin (Patel et al., 2013). Gene ontology pathway analysis of these differentially expressed genes ($p < 0.2$) demonstrated variances primarily associated with cell adhesion and migration, a possible nod to circulating versus vessel resident behavior (Patel et al., 2013; Shafiee et al., 2018). Moreover, equivalent vascular integrative and reparative capability has been demonstrated between umbilical cord blood and placental ECFCs via *in vivo* hind limb ischemic rescue (Patel et al., 2013).

In terms of application, this protocol has been used routinely for the isolation of MSCs and ECFCs from human placenta, enabling morphological, functional (e.g., clonogenesis and tubulogenesis), proteomic, and genetic assessment *in vitro* (Patel et al., 2013, 2014, 2016; Shafiee et al., 2017b, 2017c, 2018; Sim et al., 2019; Zhao et al., 2021). Under culture conditions these MSCs and ECFCs can be used to study models of disease, such as wound healing, via isolation from diseased individuals or via pharmacological induction in culture, and can also serve directly as a therapeutic tool in combination, such as in the treatment of intrauterine fetal growth restriction in piglets and other ischemic conditions (Patel et al., 2016; Shafiee et al., 2017a, 2017b, 2017c; Chand et al., 2021; Zhao et al., 2021). In addition, this protocol has been adapted to isolate endovascular progenitors (EVPs), ECFC-like cells, and MSCs cells from *in vivo* murine tissues such as melanoma, lung, and skin for functional and gene ontology analysis (Patel et al., 2017; Donovan et al., 2019; Zhao et al., 2021).

LIMITATIONS

It should be noted that while this protocol outlines the highest yield and purity ECFC isolation method currently available, there are certain limitations that should be considered. This includes

that the number of HPP-ECFC colonies isolated will vary from tissue sample to tissue sample and is limited by the available vascular density found in the starting material, due to the vessel resident nature of placental ECFCs (Shafiee et al., 2018). Moreover, there is no isolation step included here that definitively differentiates HPP-ECFC from LPP-ECFC colonies, with additional flow cytometry with FACS required for separation of cultured ECFCs via CD34 high ($CD34^{HI}$) and CD34 low ($CD34^{LO}$) expression, within the $CD45^{NEG}CD34^{POS}CD31^{LO}$ population (Patel et al., 2016).

In terms of ECFC antigen identification and isolation, a caveat to consider with any marker is the specificity of the antigen. For example, CD31 is often used to identify vascular resident endothelial cells but can also be expressed by some immune cells such as macrophages. Therefore, careful study of ECFC and MSC morphology in culture is essential. However, the nature of this specific crossover is resolved by both MACS and FACS exclusion of CD45 expressing cells prior to adherent cell culture selection, as opposed to after. It should also be noted that specialized core facilities are required to complete this protocol with regards to use of machinery.

In sum, this protocol addresses ethical, purity, and yield hurdles regarding both MSC and ECFC isolation, both of which can be purified without compromise to the opposing stem cell yield. This creates greater clinical implementation capacity for both MSCs and ECFCs in terms of ischemic rescue, particularly in combination therapies.

TROUBLESHOOTING

Problem 1

Step 8: Substantial tissue remains undigested after mechanical and enzymatic digestion. The reasons for this may include that the digestion time was not sufficient or that the enzymes used to make the digestion buffer were out of date.

Potential solution

Increase the incubation and shaking time by 10 min and check the storage and used by date of enzymes, ensuring they are kept dry.

Problem 2

Step 12: The RBS lysis solution appears pink and there is a low cell count. This is due to excessive cell lysis.

Potential solution

Ensure that RBC lysis is exactly 10 min. If this continues to occur, decrease incubation time by increments of 1 min.

Problem 3

Step 43: There is a high percentage of dead cells present on the 7′AAD PE-Cy5A versus SSC-A plot. This may be due to several issues such as initial tissue digestion or RBC lysis being too long, or simply that sample preparation time from step 1–31 was too long.

Potential solution

Shorten digestion or RBS lysis time and ensure each step is completed within the suggested time frame while keeping the samples on ice at all times.

Problem 4

Step 46: Low or no MSC or ECFC yield. There are multiple reasons this may have occurred, ranging from collecting of the wrong CD45 or CD34 fraction, digestion and RBC lysis time was too long, and that sample preparation from step 1–31 took too long. Moreover, donor variance will influence yield.

Potential solution

Read the protocol carefully and clearly label the collection tubes. Shorten digestion or RBS lysis time and ensure each step is completed within the suggested time frame while keeping the samples on ice at all times.

Problem 5

Step 54: No stem cell colonies have formed in culture after 28 days. This can be due to the seeding density being too low, complete culture medium was not used or changed regularly enough, or inter-donor variation has resulted in unviable stem cell populations.

Potential solution

Ensure seeding density is 1×10^4 cells/cm^2 and that complete culture medium is used and refreshed at least every 48 h. This can be increased to 24 h. Please note that colony establishment is donor dependent.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead and technical contact, Dr. Jatin Patel (j7.patel@qut.edu.au).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The ECFC findings of this protocol were originally published by Patel et al. (2013), while placental MSC isolation was originally published by Patel et al. (2014). DOI's are listed in the key resources table. Any additional data is available through the lead and technical contact upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.P.; Methodology and Investigation J.P. and A.S.; Writing – Original Draft, R.N. and S.L.S.; Writing – Review & Editing, R.N, S.L.S., A.S., J.P, and K.K.; Funding Acquisition, J.P. and K.K.; Supervision J.P. and K.K.

DECLARATION OF INTERESTS

J.P. and K.K. are co-inventors in a patent filed using the technology described in this protocol (Patel and Khosrotehrani, 2014).

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