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Isolation of Stem Cells, Endothelial Cells and Pericytes from Human Infantile Hemangioma

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[Abstract] Infantile hemangioma (IH) is a vascular tumor noted for its excessive blood vessel formation during infancy, glucose-transporter-1 (GLUT1)-positive staining of the blood vessels, and its slow spontaneous involution over several years in early childhood. For most children, IH poses no serious threat because it will eventually involute, but a subset can destroy facial structures and impair vision, breathing and feeding. To unravel the molecular mechanism(s) driving IH-specific vascular overgrowth, which to date remains elusive, investigators have studied IH histopathology, the cellular constituents and mRNA expression. Hemangioma endothelial cells (HemEC) were first isolated from surgically removed IH specimens in 1982 by Mulliken and colleagues (Mulliken *et al.*, 1982). Hemangioma stem cells (HemSC) were isolated in 2008, hemangioma pericytes in 2013 and GLUT1-positive HemEC in 2015. Indeed, as we describe here, it is possible to isolate HemSC, GLUT1-positive HemEC, GLUT1-negative HemEC and HemPericytes from a single proliferating IH tissue specimen. This is accomplished by sequential selection using antibodies against specific cell surface markers: anti-CD133 to select HemSC, anti-GLUT1 and anti-CD31 to select HemECs and anti-PDGFRβ to select HemPericytes. IH-derived cells proliferate well in culture and can be used for *in vitro* and *in vivo* vasculogenesis and angiogenesis assays.

Keywords: Hemangioma, Endothelial cells, Pericytes, Vascular tumor, Angiogenesis

[Background] IH occurs in 4-5% of infants; it follows a unique life-cycle of rapid vascular growth, called the proliferating phase, followed by a slow spontaneous involuting phase (Leaute-Labreze *et al.*, 2017). The proliferating phase contains immature vascular endothelial growth factor receptor-2 (VEGFR2)+ cells that appear to be in the process of vasculogenesis–the assembly of new vessels from stem/progenitor cells (Yu *et al.*, 2004; Boscolo and Bischoff, 2009). The involuting phase begins after 12 months of age; well-defined vascular channels become evident yet little is known about mechanisms of involution except that endothelial apoptosis increases in the involuting phase (Mancini and Smoller 1996; Iwata *et al.*, 1996; Razon *et al.*, 1998). The residuum in the involuted phase is characterized by sparse vessels, adipocytes and connective tissue. This natural life-cycle of endothelial maturation and involution distinguishes IH from other vascular tumors and malformations, which do not regress and can grow at any time in a patient's life. In 2008, we isolated a primitive mesenchymal cell from proliferating phase IH that can differentiate into endothelial cells, pericytes and adipocytes and form hemangioma-like (GLUT1-positive) vessels within 7 days after being implanted sub-cutaneously into immune-deficient mice (Khan *et al.*, 2008). We designated these hemangioma stem cells (HemSC); subsequent studies

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validated HemSCs as the IH-initiating cell (Greenberger *et al.*, 2010; Itinteang *et al.*, 2011; Xu *et al.*, 2011; Mai *et al.*, 2013; Harbi *et al.*, 2016; Edwards *et al.*, 2017). Here we describe in detail how to isolate HemSC from proliferating phase IH, and at the same time isolate GLUT1-positive HemEC, GLUT1-negative HemEC and HemPericytes; this allows detailed studies on patient-derived cells that represent the vascular cellular constituents of IH. In principal, the strategy could be broadly applied to other types of vascular tumors and vascular malformations, in particular isolation of endothelial cells from venous malformations (Goines *et al.*, 2018), capillary malformations (Huang *et al.*, 2017), arteriovenous malformations (Couto *et al.*, 2017) and lymphatic malformations (Boscolo *et al.*, 2015).

Materials and Reagents

1. Disposable scalpel
2. 15 ml and 50 ml sterile Falcon® conical tubes
3. 1.7 ml sterile microcentrifuge tube
4. Tissue culture-treated 24-well and 6-well plates
5. Falcon® 100 µm Cell Strainer (Corning, catalog number: 352360)
6. Pre-Separation Filters (70 µm) (Miltenyi Biotec catalog number: 130-095-823)
7. Nalgene Rapid-Flow disposable Filter Units with PES (polyethersulfone) membrane, 0.2 µm (Thermo Scientific, catalog number: 564-0020)
8. MS Columns (Miltenyi Biotec, catalog number: 130-042-201)
9. Dynabeads™ CD31 Endothelial Cell, human (Thermo Fisher, catalog number: 11155D)
10. Liberase™ (Roche, catalog number: 5401119001)
11. Dispase (Corning, catalog number: 354235)
12. Fetal Bovine Serum (HyClone, catalog number: SH30396.03)
13. EGM™-2 Endothelial Cell Growth Medium-2 BulletKit™ (Lonza, catalog number: CC-3162)
14. DMEM, high glucose, GlutaMAX™ Supplement (Thermo Fisher, catalog number: 10566-016)
15. Fibronectin (FN) (Chemicon, catalog number: FC010; stock= 1 µg/µl)
16. Phosphate buffered saline (PBS) 10x, without calcium and magnesium (Lonza, catalog number: 17-517Q)
17. 100x GPS (L-Glutamine-Penicillin-Streptomycin, Corning, catalog number: 30-009-CI)
18. 0.05% Trypsin 0.53 mM EDTA, 1x (Corning, catalog number: 25-052-CI)
19. Red blood cell lysis buffer (Roche, catalog number: 11814389001)
20. FcR Blocking Reagent, human (Miltenyi Biotec, catalog number: 130-059-901)
21. CD133 MicroBead Kit (Miltenyi Biotec, catalog number: 130-050-801)
22. Dynabeads™ Pan Mouse IgG (Thermo Fisher, catalog number: 11041)
23. Dynabeads® M-270 Streptavidin (Thermo Fisher, catalog number: 65305)
24. Human GLUT1 Antibody (R&D Systems, catalog number: MAB1418)
25. Human PDGFRβ Biotinylated Antibody (R&D Systems, catalog number: BAF385)
26. $CaCl_2 \cdot 2H_2O$

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27. $MgSO_4 \cdot 7H_2O$
28. Glucose
29. Sodium citrate
30. Citric acid
31. BSA
32. Na_2CO_3
33. DMSO
34. Heat inactivated FBS (hiFBS) (see Recipes)
35. Liberase™ stock (0.5 mg/ml) (see Recipes)
36. Dispase stock (50 U/ml, 100 ml) (see Recipes)
37. 10x Calcium and Magnesium (Ca^{2+}/Mg^{2+}) solution (see Recipes)
38. Collection Medium (see Recipes)
39. Digestion Buffer (see Recipes)
40. 6% Citrate Dextrose Solution, Solution A (ACD-A) (see Recipes)
41. Buffer A (see Recipes)
42. Buffer B (see Recipes)
43. EGM-2 medium (see Recipes)
44. Fibronectin (FN)-coating buffer (see Recipes)
45. Coating culture plates with fibronectin (FN) (see Recipes)
46. Quenching/Thawing medium (see Recipes)
47. Freezing medium (see Recipes)

Equipment

1. Forcep
2. Pipettes
3. Pestle (Thomas Scientific, catalog number: 3431D94; the smooth pestle is 44 mm x 22 mm)
4. Refrigerator
5. Centrifuge (Eppendorf, model: 5804)
6. Water Bath (Fisher Scientific, model: 2223)
7. MiniMACS Separator (Miltenyi Biotec, catalog number: 130-042-102)
8. DynaMag™-2 Magnet (Thermo Fisher, catalog number: 12321D)
9. -80 °C freezer
10. -20 °C non-defrost freezer

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Procedure

IH specimens should be obtained under an institutional review board (IRB)-approved human subject protocol. The clinical diagnosis should be confirmed by histopathology. Biosafety Level 2 procedures for working with human tissue should be followed. Sterile technique and sterile solutions should be used at each step of tissue homogenization, antibody-mediated selection and cell culture.

A. Tissue collection and digestion
   1. Transfer IH tissue into a sterile container filled with 5-10 ml Collection Medium as soon as possible after resection. Place the tissue on ice and bring it to the lab.
   2. Rinse the tissue twice with sterile PBS to remove surface blood.
   3. If necessary, use a sterile scalpel and forcep to separate the inner soft IH tissue from the outer rough epidermis.
   4. Mince the IH tissue into ~2 mm^3 pieces with a sterile, disposable scalpel.
   5. Transfer minced IH tissue into a 50 ml conical tube and mix with freshly-made Digestion Buffer using gentle pipetting. Use 5 volumes of Digestion Buffer per gram of tumor tissue (*i.e.*, 5 ml Digestion Buffer/1 gram of minced IH).
   6. Incubate mixture at 37 °C. Mix every 5-10 min by tapping the tube. The tissue should appear soft and the solution should become turbid after 40-50 min. Do not over-digest (*i.e.*, longer than 1 h) because the sample will become viscous due to cell lysis and DNA release.
   7. Gently homogenize the IH tissue digest with a Teflon pestle. Move pestle up and down and twist while squeezing the 50 ml tube. Let tumor pieces settle to the bottom of tube and transfer the supernatant to a new tube on ice.
   8. Add 5 ml Collection Medium to the remaining tissue in the original tube and repeat the homogenization; repeat once more. Combine the three supernatants.
   9. Filter the combined supernatants through a 100 µm sterile cell strainer.
   10. Wash cell strainer with 5-10 ml Collection Medium to flush cells through the strainer.
   11. Centrifuge cell suspension at 282 *x g*, room temperature (RT) for 5 min.
   12. Carefully aspirate supernatant and re-suspend the cell pellet with 5-10 ml Buffer A.
   13. Centrifuge cell suspension at 240 *x g,* RT for 5min. Aspirate the supernatant carefully.
   14. Optional: Lysis of red blood cells (RBCs) for the very red (*i.e.*, blood filled) specimen
       a. Resuspend cells in 1 ml PBS and mix well with 7 ml ice-cold RBC lysis buffer.
       b. Incubate at 4 °C for 10 min with gentle shaking.
       c. Centrifuge at 240 *x g,* RT for 5 min. Aspirate the supernatant.
   15. Resuspend the cell pellet in 1 ml Buffer A. Determine cell number. Expect 1 x 10^6 -2 x 10^6 cells from 0.5 cm^3 IH specimen. Use half of the cells for HemSC Purification (Procedure B) and the other for HemEC and HemPericyte Purification (Procedures C-E). See Figure 1 for a schematic of the cell purification strategy.

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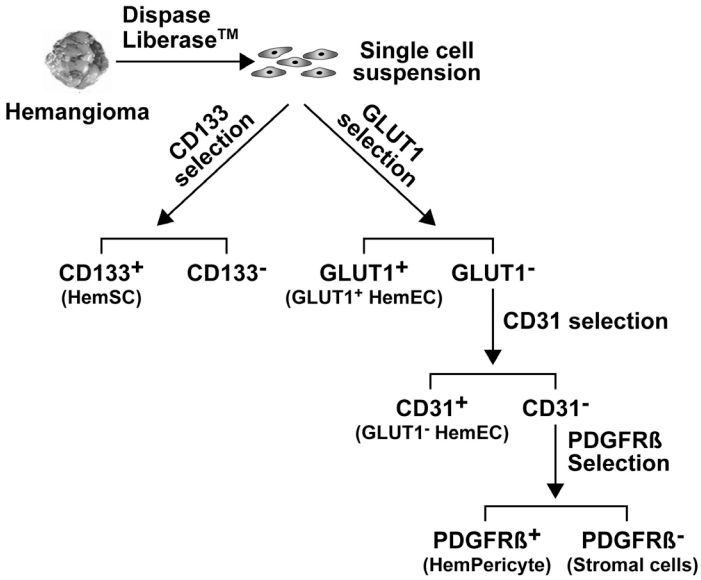

Figure 1. Cell isolation using sequential antibody-coated magnetic beads. Schematic shows work flow for isolating four cell types from IH tissue.

B. HemSC purification (see Miltenyi Biotec protocol for the additional information)

1. Centrifuge cell suspension at 240 *x g,* RT for 5 min. Aspirate supernatant.
2. Resuspend the cells in 300 µl ice-cold Buffer A.
3. Add 100 µl human FcR blocking reagent, mix well by pipetting up and down 2-3 times.
4. Add 100 µl CD133 Microbeads, mix well.
5. Incubate for 30 min in the refrigerator (2-8 °C), gently tap tube every 5-10 min.
6. Wash cells by adding 9.5 ml ice-cold Buffer A.
7. Centrifuge cells at 240 *x g*, RT for 5 min. Aspirate supernatant.
8. Resuspend cells in 500 µl ice-cold Buffer A.
9. Pre-wash MS column by placing the MS column in the magnetic field and rinsing with 500 µl Buffer A. Wait until the column reservoir is empty.
10. Place a 70 µm cell strainer on the top of column reservoir. Load cell suspension and let it run through cell strainer and enter MS column held in the magnet.
11. Collect the effluent.
12. Wash the MS column with 4x 500 µl Buffer A.
13. Collect all effluent fractions and combine together; save as CD133-negative cells.
14. Remove the column from the magnetic separator and place it on a 1.7 ml sterile microcentrifuge tube.
15. Pipette 1 ml Buffer A onto the top of column and collect CD133-positive cells by firmly pushing the plunger (supplied) into the column.
16. Optional: To increase the purity of CD133-positive cells, load the released cell fraction over a second MS column and repeat CD133 selection.

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17. Determine the number of cells in both CD133-positive and CD133-negative cell fractions. Centrifuge cell suspensions at 240 *x g*, RT for 5min. Remove supernatant. The number of CD133+ cells varies from 0.2% to 2% of total digested tumor cells, as reported by Yu *et al.*, 2004. The yield depends on the size of the specimen, the enzyme digestion and batch of anti-CD133-coated beads.
18. Resuspend CD133-positive cells in 1 ml EGM-2 media. Plate them in one-well of 24-well plate pre-coated with 1 μg/cm^2 FN. The CD133-positive HemSC will start to grow rapidly in culture after 7-10 days and will exhibit a mesenchymal morphology (Figure 3) (Khan *et al.*, 2008).
19. Plate CD133-negative cells in one well of 6-well plate pre-coated with 1 μg/cm^2 FN. After primary culture, cryopreserve cells for future use (Procedure H), for example the CD133-negative fraction can be used for selection of CD31$^+$ and/or PDGFRβ$^+$ cells.

C. GLUT1-positive endothelial cell purification (see Pan mouse IgG Dynabeads protocol for the additional information)
   1. Centrifuge the other half of cell suspension at 240 *x g*, RT for 5 min.
   2. Resuspend cells (up to 10^6) in 80 μl ice-cold Buffer A.
   3. Add 20 μl human FcR blocking reagent.
   4. Incubate for 10 min in the refrigerator (2-8 °C).
   5. Add 1 μg of mouse anti-human GLUT1 antibody to 100 μl cell suspension–1 μg antibody/10^6 cells/100 μl buffer.
   6. Incubate for 30 min in the refrigerator (2-8 °C). Mix by gently tapping the tube every 5-10 min.
   7. Add 10 ml ice-cold Buffer A, mix well.
   8. Centrifuge at 240 *x g*, RT for 5min. Resuspend the cell pellet in 100 μl ice-cold Buffer B.
   9. Pre-wash Dynabeads: Transfer 5 μl of the Pan mouse IgG Dynabeads to a 1.7 ml sterile microcentrifuge tube. Add 1 ml ice-cold Buffer B and mix. Place the tube in a magnet for 1 min and aspirate supernatant.
   10. Remove the tube from the magnet and resuspend the washed Dynabeads with 5 μl ice-cold Buffer B.
   11. Add washed beads to anti-GLUT1 treated cells at 1.5 μl beads/10^6 cells/100 μl buffer. Mix well and incubate for 10 min in the refrigerator (2-8 °C). The bead to cell ratio can be increased to 2.5 μl beads/10^6 cells/100 μl buffer but avoid increasing the incubation time because this may increase non-specific binding.
   12. Add 1 ml ice-cold Buffer B. Place the tube in the magnet for 1 min. Anti-GLUT1-bound cells will move towards the magnet leaving the GLUT1-negative cells free in suspension. Gently collect the GLUT1-negative cell fraction into a new 15 ml conical tube.
   13. Remove the tube from the magnet. Add 1 ml ice-cold buffer B and mix well. Place the tube back to the magnet, collect and combine the un-bound cells in the same 15 ml tube.
   14. Repeat this wash step twice more.

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15. After the final wash, remove the tube from the magnet to release the bead-bound GLUT1-positive cells by adding 1 ml EGM-2 media to resuspend cells. Determine the cell number. Plate cells in one well of 24-well plate precoated with 1 μg/cm^2 FN.
16. The GLUT1-negative cells will be further purified using anti-CD31 magnetic beads selection as described in the next section.

    Optional: To increase the yield of GLUT1-positive cells, a second round of anti-mouse IgG beads selection can be applied in GLUT1-negative cell fraction. Repeat magnetic separation and plate cells in a new well of 24-well plate. Do not combine cells from the 1st selection. The GLUT1-positive endothelial cells will start to proliferate after 10-12 days in culture (Huang *et al.*, 2015).

    Note: Almost all of GLUT1-positive cells in proliferating IH are endothelial cells (see Huang et al., 2015 Figure 1C), that is, they express endothelial markers CD31, VE-Cadherin and VEGRR2. After 2-3 weeks in vitro culture and expansion, the cells transition to a mesenchymal phenotype (Figure 3) (Huang et al., 2015).

D. GLUT1-negative endothelial cells purification (see anti-human CD31 Dynabeads protocol for the additional information)
    1. Determine the number of cells in the GLUT1-negative fraction. Centrifuge cell suspension at 240 *x g*, RT for 5 min. Aspirate the supernatant.
    2. Resuspend the cells in 200 μl ice-cold buffer B.
    3. Pre-wash Dynabeads: Transfer 5 μl anti-human CD31 Dynabeads to a 1.7 ml sterile microcentrifuge tube. Add 1 ml ice-cold Buffer B and mix. Place the tube in a magnet for 1 min and aspirate supernatant.
    4. Remove the tube from the magnet and resuspend washed beads with 5 μl ice-cold Buffer B Add 2 μl washed anti-human CD31 Dynabeads to the cells and incubate for 10 min in the refrigerator (2-8 °C).

       Note: 5-8 beads per cell is optimal for selection (Figure 2). If too many beads bind, it will reduce endothelial cell attachment to the FN-coated dish. Do not incubate for longer than 10 min as this might result in non-specific binding and decreased purity.

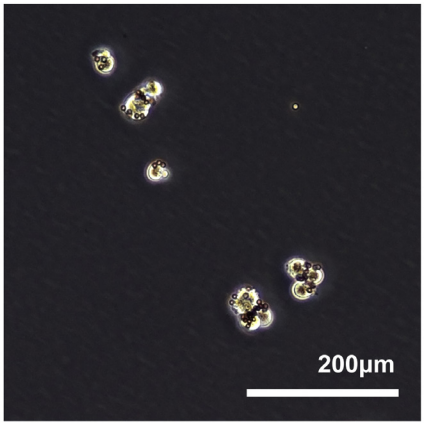

Figure 2. Anti-CD31-bead bound cells

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5. Add 1 ml ice-cold Buffer B. Place the tube in the magnet and hold for 1 min. Anti-CD31 Dynabead bound cells will move towards the magnet (accumulating on the wall of the tube) leaving the unbound cells, the CD31-negative cell fraction, free in suspension. Gently collect the CD31-negative cell fraction into a new 15 ml conical tube.
6. Remove the tube from the magnet. Add 1 ml ice-cold buffer B and mix well. Repeat magnetic separation. Collect and combined bead un-bound cells into the same 15 ml tube.
7. Repeat wash step two more times.
8. After the final wash, remove the tube from the magnet and release the bead-bound CD31-positive cells in 1 ml EGM-2 media (Figure 2). Determine the cell number and plate cells in one well of 24-well plate precoated with 1 μg/cm^2 FN.
9. GLUT1-negative HemECs will require 7-10 days to begin rapid proliferation. If needed, repeat the anti-CD31 selection to increase the endothelial cell purity. GLUT1-negative HemEC show typical endothelial morphology (Figure 3).

   Optional: To increase the yield of CD31-positive cells, a second round of anti-CD31 beads selection can be applied in the CD31-negative cell fraction. Repeat magnetic separation and plate cells in a new well of 24-well plate. Do not combine cells from the first selection.

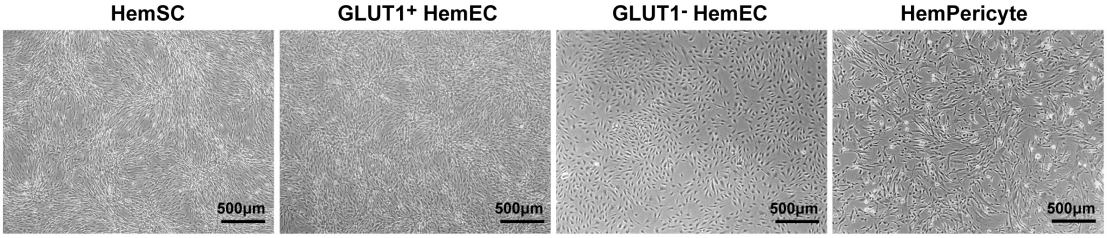

Figure 3. IH cells in the primary culture

E. PDGFRβ-positive cell purification (see Dynabeads® M-270 Streptavidin protocol for the additional information)
   1. Determine the number of cells in the CD31-negative cell fraction. Centrifuge the cell suspension at 240 *x g*, RT for 5 min. Aspirate the supernatant.
   2. Resuspend the cells in 80 μl ice-cold buffer A.
   3. Add 20 μl human FcR blocking reagent.
   4. Incubate for 10 min in the refrigerator (2-8 °C).
   5. Add 1 μg of biotinylated anti-PDGFRβ antibody to the 100 μl cell suspension.
   6. Incubate for 30 min in the refrigerator (2-8 °C). Mix by gently tapping tube every 5-10 min.
   7. Add 10 ml ice-cold buffer A, mix well.
   8. Centrifuge cell suspension at 240 *x g*, RT for 5min. Aspirate supernatant and resuspend cells in 100 μl ice-cold Buffer B.

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9. Pre-wash Dynabeads: Transfer 5 µl Streptavidin-coupled Dynabeads to a 1.7 ml microcentrifuge tube. Add 1 ml ice-cold Buffer B and mix. Place the tube in a magnet for 1 min and aspirate supernatant. Remove the tube from the magnet and resuspend the washed beads with 5 µl ice-cold Buffer B.
10. Add 1.5 µl washed Streptavidin-coupled Dynabeads to the cells, mix well and incubate for 10 min in the refrigerator (2-8 °C).
11. Add 1ml ice-cold Buffer B. Place the tube in the magnet for 1 min. PDGFRβ-positive cells will move towards the magnet leaving the PDGFRβ-negative cell fraction free in suspension. Gently collect the PDGFRβ-negative cell fraction into a new 15 ml conical tube.
12. Remove the tube from the magnetic field. Add 1 ml ice-cold buffer B and mix gently by pipetting. Place the tube back to the magnet, collect and combine the un-bound cells in the same 15 ml tube.
13. Repeat wash step two more times, collect and combine PDGFRβ-negative cells.
14. After the final wash, collect PDGFRβ-positive cells in 1 ml EGM-2 media, determine cell number and plate into one well of 24-well plate precoated with 1 µg/cm^2 FN.
    Note: PDGFRβ-positive cells–HemPericytes–will start to expand after 5-7 days (Figure 3).
15. Determine the number of PDGFRβ-negative cells. Plate cells into one well of 6-well plate precoated with 1 µg/cm^2 FN and filled with 2ml EGM-2 media. These are stromal cells.
16. Alternatively, the Hem Pericytes can be plated on a non-coated dish in 10% FBS-DMEM as described in Boscolo *et al*., 2013.

F. Expanding bead-selected IH cells
1. Forty-eight hours after plating, carefully remove media and non-adherent cells with a pipette. Add fresh EGM-2 media into each well. You should see attached single cells and/or cell clusters.
2. Change the media every 2-3 days.

G. Passaging IH-derived cells
1. To passage cells, first prepare FN-coated plates at concentration 0.1 µg/cm^2.
   Note: This is 10 fold lower than the amount of FN coated on plates used for the initial plating.
2. Wash plates with sterile PBS twice before use. The PBS from the 2nd wash should be removed right before the cells are ready to be plated.
3. Wash cell monolayer with PBS twice.
4. Aspirate PBS and add pre-warmed trypsin-EDTA (0.8 ml/p60, 1.5 ml/p100, 5 ml/p150). Gently rock the plates to evenly distribute the trypsin-EDTA.
5. Incubate in the 37 °C incubator for 1-2 min. Gently tap the plates to detach cells.
6. Inactivate the trypsin with quenching/thawing medium (5 ml/p60, 8 ml/p100; 15 ml/p150), transfer the trypsinized cells to a sterile tube.

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7. Recover remaining cells from the plate by adding quenching/thawing medium to rinse the plate. Collect the rinse and combine the cells into the same tube.
8. Determine cell number, centrifuge the cell suspension at 240 *x g*, RT for 5min, and aspirate the supernatant.
9. Resuspend the cells in EGM-2 media and plate cells on a FN-coated, tissue culture-treated dish at a cell density 5,000 cells/cm^2 (sparse) or 10,000 cells/cm^2.
    a. HemSC, GLUT1-positive HemEC and HemPericyte are usually plated at 5,000 cells/cm^2. A 90% confluent p100 plate has 5 x 10^6-6 x 10^6 cells.
    b. GLUT1-negative HemEC are usually plated at 10,000 cells/cm^2. A 90% confluent p100 plate has 3 x 10^6-4 x 10^6 cells.

H. Freezing IH-derived cells
1. When cells are confluent, wash the monolayer twice with PBS.
2. Trypsinize the cells, collect the cell suspension and determine cell number.
3. Centrifuge cell suspension at 240 *x g*, RT for 5 min.
4. Label a cryovial with the passage number (+1), cell number, and date.
5. Suspend the cell pellet at 1 x 10^6-5 x 10^6 cells in 1 ml freezing medium and aliquot 0.5-1 ml per cryovial.
6. Put cryovials into the “Mr. Frosty” and place at -80 °C overnight.
7. The next day, move cryovials to the liquid nitrogen tank for long-term storage.

I. Thawing IH-derived cells
1. Prepare FN-coated, tissue culture-treated plates (0.1 µg/cm^2), pre-warmed EGM2 medium and quenching/thawing medium.
2. Prepare 15 ml Falcon tubes. Add 5 ml quenching/thawing medium to each tube.
3. Wash FN-coated plates with PBS twice.
4. Quickly thaw frozen cells directly after removing from liquid nitrogen by placing in a 37 °C water bath for 1-2 min. Gently swirl the tube to facilitate thawing.
5. Transfer cells into the 15 ml tube pre-filled with quenching/thawing medium (avoid vigorous pipetting). Determine cell number.
6. Centrifuge cell suspension at 240 *x g*, RT for 5 min.
7. Aspirate quenching/thawing medium. Resuspend cells in EGM-2 media and plate on FN-coated, tissue culture-treated dish (3 ml/p60; 10 ml/p100; 25 ml/p150).
8. Label the coated plates with cell type, passage number, date, and initials.
9. Cells should attach within 4-6 h after plating. Add fresh media every 2-3 days.

Data analysis

Analyze cellular phenotype and purity by flow cytometry, immunofluorescence and qPCR at passage

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2-3. HemSC are positive for CD90 and VEGFR1 and negative for CD31 (Khan *et al*., 2008). GLUT1$^+$ HemECs are initially positive for CD31, VE-cadherin and VEGFR2 by qPCR but with time in culture, these endothelial markers are no longer detected but instead the cells express the mesenchymal marker CD90 (see Huang *et al*., 2015). GLUT1-negative/CD31$^+$ HemEC express CD31, VE-cadherin and VEGFR2 and do not express CD90 (Huang *et al*., 2015), HemPericytes express PDGFRβ, NG2, calponin,α-smooth muscle actin, NOTCH3 but not CD31 (Boscolo *et al*., 2013). Cells are re-analyzed as needed, for example before an *in vivo* experiment, to verify the phenotype. We typically use the cells between passages 4 and 10.

Notes

1. The percentage of CD133$^+$ cells varies among different IH specimens (Yu *et al*., 2004).
2. The percentage of GLUT1$^+$ endothelial cells is reduced in IH specimens from patients over one year of age (Huang *et al*., 2015).
3. 1% gelatin in PBS can be substituted for FN for coating the culture plates.
4. Always use freshly prepared EGM2-media to insure full and consistent activity of the growth factors in the Single Quots (VEGF-A, basic FGF, EGF and IGF1). Once the media is prepared it can be stored in working aliquots at -20 °C until use. Media stored at 2-8 °C should be used within 3-4 days of preparation or thawing.

Recipes

1. Heat inactivated FBS (hiFBS)
   a. Thaw a 500 ml bottle of HyClone FBS in warm water (for several hours) or at 4 °C overnight.
   b. Put the thawed FBS into the 56 °C pre-heated water bath for 30 min (make sure the water covers all of FBS in the bottle. Mix the FBS in the bottle by shaking/inverting every 10 min to achieve an even temperature throughout the 500 ml bottle.
   c. Allow FBS to cool to room temperature.
   d. Aliquot 45 ml into sterile 50 ml tubes and store in a -20 °C non-defrost freezer.
   e. Thaw a 45 ml frozen aliquot of heat-inactivated (hi)-FBS at 37 °C for about 20 min or at 4 °C overnight before use.
2. Liberase^{TM} Stock (0.5mg/ml)
   5 mg lyophilized Liberase^{TM}
   a. Dissolve in 10 ml sterile ddH_2O
   b. Make 0.5 ml aliquots and store at -20 °C
3. Dispase stock (50 U/ml, 100 ml)
   Make 5 ml aliquots and store at -20 °C
4. 10x Ca^{2+}/Mg^{2+} solution, 500 ml
   927 mg $CaCl_2 \cdot 2H_2O$ (final concentration: 1.26 mM)

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1.0 g $MgSO_4 \cdot 7H_2O$ (final concentration: 0.8 mM)

a. Dissolve in sterile ddH_2O to a final volume of 500 ml

b. Filter (0.2 µm) and store at room temperature

5. Collection medium, 100 ml

87 ml DMEM (high glucose, GlutaMAX™ Supplement)

10 ml 10x Ca^{2+}/Mg^{2+} Solution

2 ml hi-FBS

1 ml 100x GPS

a. Mix and filter (0.2 µm)

b. Aliquot to 5 or 10 ml/tube and store at -20 °C for up to 3 months

6. Digestion buffer, 6 ml (make fresh)

a. Thaw 5 ml Collection Medium

b. Add 0.5 ml Liberase™ (0.5 mg/ml) (1:10; working concentration: 50 µg/ml)

c. Add 0.5 ml Dispase (50 U/ml) (1:10; working concentration: 5 U/ml)

7. 6% ACD-A solution, 1 L

22.3 g Glucose

22.0 g Sodium citrate

8.0 g Citric acid

a. Dissolve in ddH_2O to a final volume of 1 L

b. Filter (0.2 µm) and store at 4 °C

8. Buffer A (PBS/0.6% ACD-A/0.5% BSA), 500 ml

2.5 g BSA

50 ml 6% ACD-A solution

450 ml PBS

Dissolve, filter (0.2 µm) and store at 4 °C

9. Buffer B (PBS/0.6% ACD-A/0.1% BSA), 500 ml

0.5 g BSA

50 ml 6% ACD-A solution

450 ml PBS

Dissolve, filter (0.2 µm) and store at 4 °C

10. EGM-2 Medium, 500 ml

445 ml EBM-2

50 ml hiFBS

5 ml 100x GPS

EGM-2 Single Quot supplements (all except hydrocortisone)

Filter (0.2 µm), aliquot (45 ml/tube) and store at -20 °C until needed or store at 4 °C and use within 3-4 days

11. Fibronectin (FN)-coating buffer (0.1 M Na_2CO_3), 500 ml

5.3 g of Na_2CO_3

a. Dissolve in ddH_2O to a final volume of 500 ml
b. Adjust pH to 9.4 with 12 N HCl
c. Filter (0.2 µm) and store at room temperature

12. Coating culture plates with fibronectin (FN)
    a. Calculate the amount of FN needed; the stock is 1 µg/µl
    b. Use 1 µg/cm^2 for freshly-isolated cells and 0.1 µg/cm^2 for expansion of primary culture cells
    c. Dilute FN into just enough FN-coating buffer to cover the plate(s)
    d. Mix well and add to plates with a sterile pipette
    e. Incubate in the 37 °C incubator for 30 min or up to overnight (add more coating buffer for overnight incubation in case of evaporation)
13. Quenching/Thawing medium (500 ml)
    445 ml DMEM (high glucose, GlutaMAX™ Supplement)
    50 ml hiFBS (final 10%)
    5 ml 100x GPS
    Filter (0.2 µm) and store at 4 °C
    EGM-2 media can also be used to quench but it is more expensive than DMEM
14. Freezing medium (make fresh)
    95% hiFBS
    5% DMSO
    Filter (0.2 µm) and store at 4 °C

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Competing interests

The authors have no financial and non-financial competing interests to disclose.

Ethics

IH tissue used for these isolation procedures was obtained under an IRB-approved protocol from the Committee on Clinical Investigation at Boston Children's Hospital (04-12-175R; 11/11/2010 – 6/6/2020). Informed consent was obtained from parents or guardians of the subjects.

References

1. Boscolo, E. and Bischoff, J. (2009). Vasculogenesis in infantile hemangioma. *Angiogenesis* 12(2): 197-207.
2. Boscolo, E., Mulliken, J. B. and Bischoff, J. (2013). Pericytes from infantile hemangioma display proangiogenic properties and dysregulated angiopoietin-1. *Arterioscler Thromb Vasc Biol* 33: 501-509.
3. Boscolo, E., Coma, S., Luks, V. L., Greene, A. K., Klagsbrun, M., Warman, M. L. and Bischoff, J. (2015). AKT hyper-phosphorylation associated with PI3K mutations in lymphatic endothelial cells from a patient with lymphatic malformation. *Angiogenesis* 18 (2): 151-162.
4. Couto, J. A., Huang, A. Y., Konczyk, D. J., Goss, J. A., Fishman, S. J., Mulliken, J. B., Warman, M. L. and Greene, A. K. (2017). Somatic MAP2K1 mutations are associated with extracranial arteriovenous malformation. *Am J Hum Genet* 100(3): 546-554.
5. Edwards, A. K., Glithero, K., Grzesik, P., Kitajewski, A. A., Munabi, N. C., Hardy, K., Tan, Q. K., Schonning, M., Kangsamaksin, T., Kitajewski, J. K., Shawber, C. J. and Wu, J. K. (2017). NOTCH3 regulates stem-to-mural cell differentiation in infantile hemangioma. *JCI Insight* 2(21).
6. Goines, J., Li, X., Cai, Y., Mobberley-Schuman, P., Metcalf, M., Fishman, S. J., Adams, D. M., Hammill, A. M. and Boscolo, E. (2018). A xenograft model for venous malformation. *Angiogenesis* 21(4): 725-735.
7. Greenberger, S., Boscolo, E., Adini, I., Mulliken, J. B. and Bischoff, J. (2010). Corticosteroid suppression of VEGF-A in infantile hemangioma-derived stem cells. *N Engl J Med* 362(11): 1005-1013.
8. Harbi, S., Wang, R., Gregory, M., Hanson, N., Kobylarz, K., Ryan, K., Deng, Y., Lopez, P., Chiriboga, L. and Mignatti, P. (2016). Infantile hemangioma originates from a dysregulated but not fully transformed multipotent stem cell. *Sci Rep* 6: 35811.
9. Huang, L., Couto, J. A., Pinto, A., Alexandrescu, S., Madsen, J. R., Greene, A. K., Sahin, M. and Bischoff, J. (2017). Somatic GNAQ mutation is enriched in brain endothelial cells in sturge-weber syndrome. *Pediatr Neurol* 67: 59-63.
10. Huang, L., Nakayama, H., Klagsbrun, M., Mulliken, J. B. and Bischoff, J. (2015). Glucose transporter 1-positive endothelial cells in infantile hemangioma exhibit features of facultative stem cells. *Stem Cells* 33(1): 133-145.
11. Itinteang, T., Vishvanath, A., Day, D. J. and Tan, S. T. (2011). Mesenchymal stem cells in infantile haemangioma. *J Clin Pathol* 64(3): 232-236.
12. Iwata, J., Sonobe, H., Furihata, M., Ido, E. and Ohtsuki, Y. (1996). High frequency of apoptosis in infantile capillary haemangioma. *J Pathol* 179(4): 403-408.
13. Khan, Z. A., Boscolo, E., Picard, A., Psutka, S., Melero-Martin, J. M., Bartch, T. C., Mulliken, J. B. and Bischoff, J. (2008). Multipotential stem cells recapitulate human infantile hemangioma in immunodeficient mice. *J Clin Invest* 118(7): 2592-2599.
14. Leaute-Labreze, C., Harper, J. I. and Hoeger, P. H. (2017). Infantile haemangioma. *Lancet*

390(10089): 85-94.

15. Mai, H. M., Zheng, J. W., Wang, Y. A., Yang, X. J., Zhou, Q., Qin, Z. P. and Li, K. L. (2013). CD133 selected stem cells from proliferating infantile hemangioma and establishment of an *in vivo* mice model of hemangioma. *Chin Med J (Engl)* 126(1): 88-94.
16. Mancini, A. J. and Smoller, B. R. (1996). Proliferation and apoptosis within juvenile capillary hemangiomas. *Am J Dermatopathol* 18(5): 505-514.
17. Mulliken, J. B., Zetter, B. R. and Folkman, J. (1982). *In vitro* characteristics of endothelium from hemangiomas and vascular malformations. *Surgery* 92(2): 348-353.
18. Razon, M. J., Kraling, B. M., Mulliken, J. B. and Bischoff, J. (1998). Increased apoptosis coincides with onset of involution in infantile hemangioma. *Microcirculation* 5(2-3): 189-195.
19. Xu, D., O, T. M., Shartava, A., Fowles, T. C., Yang, J., Fink, L. M., Ward, D. C., Mihm, M. C., Waner, M. and Ma, Y. (2011). Isolation, characterization, and *in vitro* propagation of infantile hemangioma stem cells and an *in vivo* mouse model. *J Hematol Oncol* 4: 54.
20. Yu, Y., Flint, A. F., Mulliken, J. B., Wu, J. K. and Bischoff, J. (2004). Endothelial progenitor cells in infantile hemangioma. *Blood* 103(4): 1373-1375.