Editorial summary: Rat or human lung samples are enzymatically digested and CD31 expressing cells positively selected using magnetic-activated cell sorting prior to plating in endothelial-specific growth conditions.

The isolation and culture of endothelial colony forming cells from human and rat lungs

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Abstract

Blood vessels are crucial for the normal development, lifelong repair and homeostasis of tissues. Recently, vascular progenitor cell-driven “postnatal vasculogenesis” has been suggested as an important mechanism contributing to new blood vessel formation and organ repair. Among several described progenitor cell types contributing to blood vessel formation, endothelial colony forming cells (ECFCs) have received widespread attention as lineage-specific 'true' vascular progenitors. Herein, we describe a protocol for the isolation of pulmonary microvascular ECFCs from human and rat lung tissue. Our technique takes advantage of an earlier protocol for isolating circulating ECFCs from the mononuclear cellular fraction of peripheral blood. We adapted the earlier protocol to isolate resident ECFCs from the distal lung tissue. Following enzymatic dispersion of rat or human lung samples into a cellular suspension, CD31 expressing cells are positively selected using magnetic-activated cell sorting (MACS) and plated in endothelial-specific growth conditions. The colonies arising after 1-2 weeks in culture are carefully separated and expanded to yield pure ECFC cultures after a further 2-3 weeks. The resulting cells demonstrate the defining characteristics of ECFCs such as: (i) 'cobblestone' morphology of cultured cell monolayers; (ii) acetylated low-density lipoprotein uptake and *Ulex* lectin binding; (iii) tube-like network formation in Matrigel; (iv) expression of endothelial cell-specific surface markers and the absence of hematopoietic/myeloid surface antigens; (v) self-renewal potential displayed by the most proliferative cells; and (vi) contribution to *de novo* vessel formation in an *in vivo* murine implant model. Assuming typical initial cell adhesion and proliferation rates, the entire procedure can be completed within 4 weeks. Isolation and culture of lung vascular ECFCs will allow assessment of the functional state of these cells in experimental and human lung diseases, providing newer insights into their pathophysiological mechanisms.

INTRODUCTION

The role of vasculature in the development, lifelong repair and maintenance of the lung is unquestionably paramount. Chronic lung disease is predicted to become the third most common cause of death by 2020[1]. It has long been recognised that many lung ailments across lifespan, such as chronic lung disease of prematurity or emphysema in adults, display vascular abnormalities[2-5]. A better understanding of how the lung vasculature contributes to normal lung development and is impaired during disease is crucial to develop new therapeutic strategies for debilitating lung diseases[5].

Since the description of circulating endothelial progenitor cells (EPCs) by Asahara *et al*[6], postnatal vasculogenesis has been purported as an important mechanism contributing to new blood vessel formation[7,8]. Extensive attempts were made to identify and more precisely characterize EPCs. More specifically, efforts have been made to correlate the number and function of EPCs with outcome in various diseases [9,10], including lung disorders [11,12]. Among the various described cell types with an "EPC phenotype", endothelial colony forming cells (ECFCs), also referred to as late-outgrowth EPCs, have received widespread attention as lineage-specific 'true EPCs' participating in neovasculogenesis [10,13,14]. ECFCs may reside throughout the vascular endothelium contributing to generalized vascular integrity[15] . ECFCs are mobilized into a circulating pool of endothelial progenitors, potentially involved in neovasculogenesis via tissue recruitment and homing[16], however the mechanism for this is not known at present. ECFCs may also contribute to new vessel formation during lung organogenesis as well as support lifelong lung repair and maintenance. These characteristics may mean ECFCs are implicated in the disease processes of several childhood and adult lung diseases. Therefore, studying the functional characteristics of tissue resident ECFCs will widen our understanding of chronic lung diseases and may contribute to the development of more efficient treatments.

Development of the protocol:

A method for the isolation and culture of ECFCs from rat and human lung samples was established and standardized in our laboratory to study the survival capacity and other functional characteristics of pulmonary vascular ECFCs [17]. This technique takes advantage of an earlier

protocol for isolating circulating ECFCs from umbilical cord blood or peripheral blood mononuclear cells [18] and has been extensively revised and optimized for successful isolation of resident ECFCs from a solid organ such as the lung. A comprehensive overview of this method is provided in **Figure 1.** Following enzymatic dispersion of rat or human lung samples (**Figure 2**), CD31 expressing cells are positively selected using magnetic-activated cell sorting (MACS) and plated in endothelial-specific growth conditions (**Figure 3**). The ECFC colonies that arise after 1-2 weeks in culture are carefully isolated and expanded to yield pure ECFC cultures. The phenotype of ECFCs is confirmed by (i) classical 'cobblestone' morphology of cultured cell monolayers, (ii) assessment of endothelial cell characteristics via acetylated low-density lipoprotein uptake and Ulex lectin binding, (iii) tube-like network formation in Matrigel, (iv) endothelial specific surface antigen expression, (v) self-renewal potential displayed by the most proliferative cells, and (vi) assessment of their ability to contribute to de novo vessel formation in an *in vivo* murine implant model [13].

Comparison with existing methods:

Recently, three papers have demonstrated the existence of endothelial progenitor-like cells with a high potential for proliferation in the peripheral microvasculature of rat [19,20] and mouse [21] lungs. The Stevens group isolated ECFC-like cells from peripheral rat lung tissue following collagenase digestion, plating and selection of late-outgrowth endothelial colonies[19]. The cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics and observed to demonstrate ECFC-like phenotype, on the basis of both expression of surface antigens and functional behaviours such as uptake of low-density lipoprotein (LDL), maintenance of high barrier resistance, *in vitro* and *in vivo* vasculogenesis in Matrigel [20]. Weich and colleagues[21,22], on the other hand, selected out CD31+ cells from mouse peripheral lung microvasculature by successively using magnetic and fluorescence-activated cell sorting (MACS and FACS). They expanded these cells in DMEM enriched with 20% FBS and demonstrate their expression of panendothelial cell surface markers[22] and ability to bind *Griffonia simplicifolia* lectin. Their cells were also negative for CD45, for CD117 (c-kit or SCF receptor) and CD41, which are markers for leukocytes and hematopoietic stem and progenitor cells. Using limiting dilution assays at single-cell seeding density, they show that at least 15% of those cells were capable of very high

proliferation rate. These cells also generated blood vessels of donor origin in Matrigel plugs implanted subcutaneously in recipient mice.

In a recent study, we combined CD31-positive selection and clonal ring isolation of endothelial colonies and expanded the derived cells to establish primary endothelial cell lines from rat and human peripheral lung vasculature[17]. These cells were assessed for surface marker expression and a panel of functional assays that defined them as ECFCs [13,18]. In this article, in addition to providing a detailed step-by-step description of this technique, we also provide a troubleshooting guide for the routine issues that might arise during the isolation process. The focus of this protocol is to resident lung ECFCs and we have not compared lung and other organ ECFCs. Rafii et al[23] used lectin staining to isolate human bone marrow microvascular cells and showed they displayed the same antigens as human umbilical vein endothelial cells (CD31, CD34, von Willebrand factor, and uptake of AcLDL).

Applications of the method

We have tested this protocol in adult human and rat lung samples[17]. It also allows isolation of ECFCs from developing rat and human lungs, i.e. lungs from newborn rats as early as 14-days of age or aborted human fetuses at 18-20 weeks gestation[17]. Isolation and culture of lung resident ECFCs from these samples allows functional assessment of these cells in experimental and clinical lung diseases. ECFCs isolated and expanded by this technique can also be used in therapeutic studies. In addition, the described protocol enables the isolation and culture of a properly defined subset of endothelial cells with demonstrable progenitor-like characteristics (i.e a population of 'lineage-specific' endothelial progenitor cells); this will enable an improved understanding of endothelial cell biology.

Limitations

The sensitivity of the current protocol to successfully isolate lung ECFCs is limited by the vascular density of the starting tissue that is being processed. The existing method probably requires capturing a threshold number of CD31+ cells for successful expansion and establishment of pure ECFC cultures. This was evident from our inability to successfully isolate

and establish ECFC cultures from lungs of term rat fetuses. Secondly, it was more difficult to establish pure ECFC cultures from rat lungs compared to human. Greater numbers of contaminating cells, due to non-specific binding of rat anti-CD31, could possibly explain this difference. Finally, using this technique, we are able to obtain pure cultures of lung ECFCs by passage 4 or above. Differences in functional properties of ECFCs due to experimental or clinical disease processes may be lost is cells are passaged many times and this will need to be checked for each new batch of ECFCs produced.

MATERIALS

Reagents

- DMEM, high glucose 1x (GIBCO-Invitrogen, cat. no. 11965)
- Antibiotic-antimycotic: Penicillin (10 000 U/mL)/streptomycin (10 000 μg/mL)/amphotericin (25 μg/mL) (PSA [100x]) (Invitrogen, cat. no. 15240)
- Human lung tissue sample (e.g., we typically use human embryonic lung samples of 15-18 week gestational age, stored in DMEM with 2% antibiotic-antimycotic) or freshly isolated rat lung tissue. Caution: Experiments involving human tissue require informed consent to have been obtained. Experiments involving human or rats must conform to National and Institutional regulations. All procedures described here were approved by the Animal Health Care Committee of the University of Alberta. Human lung tissue collection was reviewed and approved by the Institutional Review Board at the University of New Mexico.
- Dulbecco's phosphate buffered saline (PBS), without calcium and magnesium, pH 7.2 (Sigma, cat. no. D8537)
- Trypan blue solution 0.4% (GIBCO-Invitrogen, cat. no. 15250-061)
- Endothelial basal medium (EBM)-2 (Lonza, cat. no. cc-3156)
- EGM-2 growth factor supplement singlequots (Lonza, cat. no. cc-4176)
- Trypsin-EDTA (0.25%) (Invitrogen, cat. no. 25200)
- Collagen Type I solution (BD Biosciences, cat. no. 354236)
- Fetal bovine serum (FBS; Sigma Life Science, cat. no. F1051-100 mL)

- Collagenase/dispase (Roche Diagnostics, cat. no. 11097 113001)
- Streptavidin tagged M-280 dynabeads (Dynal, cat. no. 112.05D)
- Biotinylatyed anti-CD31 antibody (human- Abcam, cat. no. ab7385; rat- BD Pharmingen, cat. no. 555026)
- Bovine serum albumin (Sigma, cat. no. A7906)
- Glacial acetic acid (17.4 N) (Fisher Scientific, cat. no. 505216)
- 70% ethanol
- Vacuum grease, sterilized (Dow Corning, cat. no. 1658832)
- Dimethyl sulphoxide (DMSO), sterile filtered (Sigma, cat. no. D2650)

Equipment:

- 6-, 24- and 96-well plates (BD Biosciences Falcon)
- 25 and 75 cm^2 vented tissue culture flasks (BD Biosciences Falcon)
- 15 mL and 50 mL conical centrifuge tubes (BD Biosciences Falcon)
- 10 cm petri dish, sterile (Coning, cat. no. 430167)
- 1 mL, 10 mL syringes, sterile (BD Biosciences, cat. nos. 309602 & 301604)
- Blades, sterile & disposable
- Pasteur pipettes (VWR, 14673-043), sterilized
- Round bottomed polystyrene culture tubes (12 mm x 75 mm H) (BD Biosciences Falcon, cat. no. T405-3)
- Cryovials (Nunc, cat. no. 375418)
- Anatomical scissors, tweezers
- Mcllwain tissue chopper (Brinkmann, Westbury, NY, USA)
- Cell strainers, nylon (Fisher Scientific; cat. no. 22363547 [40 μm], 22363548 [70 μm])
- 0.22 μm vacuum filtration system (Millipore, cat. no. SCGPU05RE)
- Neubauer chamber (Reichert, Buffalo, N.Y.)
- Magnet (Dynal MPC-STM/MPC-LTM or DynaMagTM -2/-15)
- Assorted pipettes (2-20 μL, 20-200 μL, 100-1000 μL) with sterile, disposable tips
- Centrifuge
- 37^oC cell culture incubator

- 37^{o}C water bath
- Inverted microscope
- Fine tipped marker
- Aluminium foil
- Glass dish
- Cloning cylinders, sterile (Fisher Scientific, cat. no. 07-907-10)

Reagent Setup

MACS buffer

Dissolve Dulbecco's PBS with 0.1% (w/v) bovine serum albumin. Can be stored up to 2 weeks at 4^{o}C

Serum-free 'basal' DMEM (basal-DMEM)

DMEM + 1% (v/v) PSA. Can be stored in 4^{o}C for up to 2 months.

10% FBS 'complete' DMEM (complete-DMEM)

DMEM + 10% (v/v) fetal bovine serum + 1% (v/v) PSA. Can be stored in 4^{o}C for up to 2 months.

Complete EGM-2 (cEGM-2)

Supplement EBM-2 with all components of EGM-2 growth factor bullet-kit except human epidermal growth factor (hEGF), 10% (v/v) fetal bovine serum and 1% (v/v) PSA. Store up to 1 month at 4^{o}C.

Collagenase/dispase digestive solution

Make up 5X stock solution by dissolving 5 mg of lyophilized collagenase/dispase (0.1 U collagenase & 0.8 U dipase/mg lyophilizate) per mL double distilled (dd)-water. Store at -20^{o}C up to one month. On the day of experiment, dilute 5X stock 5 times with Dulbecco's PBS. *Critical: Prepare working dilution on the day of the experiment.*

Collagen type I solution

Dilute 0.575 mL of glacial acetic acid (17.4 N) in 495 mL dd-water (final concentration, 0.02 N) and sterile filtere with 0.22 μm vacuum filtration system. Dilute Collagen type I stock with 0.02 N acetic acid to yield 50 μg/mL working dilution. The volume of collagen to use will vary depending on the supplied collagen stock concentration. Store up to 1 week at 4°C.

Sterile vacuum grease

Spread a small amount of vacuum grease into a thin layer in an autoclavable glass dish. After setting the lid, wrap the glass dish in aluminium foil, autoclave and cool completely.

Coating of cell culture surfaces with collagen type I. Add the working dilution (50 μg/mL) of collagen type I solution to the surface to be coated. Use 1ml for each well of a six well plate, 300 μL/well for 24 well plates, 2-3 mL/25-cm^2 flask and 5-6 mL/75-cm^2 flask. Incubate 90 min to overnight at 37°C. CRITICAL Plates must be used by 24h after coating.

Procedure

Critical: Work in strictly aseptic conditions (preferably under the cell culture hood) at all times

Preparation of reagents

1. Prepare two to three 15 or 50 mL centrifuge tubes with fresh DMEM + 1% (v/v) antibiotic/anti-fungal solution and place over ice (4°C), ready to use.
2. Preheat the incubator to 37°C.

Preparation of anti-CD31 tagged magnetic beads

3. Take 1 mg (100 μL of supplied suspension) of streptavidin tagged M-280 dynabeads in a 1.8 mL cryovial.
4. Add 500 μL MACS buffer and mix well.
5. Place the cryovial in the magnet for 1 min and pipette off supernatant completely.

6. Remove the cryovial from the magnet and resuspend the washed dynabeads with 500 µL MACS buffer.
7. Repeat steps 5 and 6 two more times
8. At the end of the third wash, resuspend the dynabeads in MACS buffer and add 10 µg of biotinylated anti-CD31 antibody while mixing, to reach an overall volume of 100 µL

<u>Critical Step:</u> *The volume of antibody solution containing 10 µg of antibody varies depending on the supplied concentration. Add appropriate volume of antibody solution first and then make it up to 100 µL by adding MACS buffer.*

9. Incubate for 30 min at room temperature (RT; 18-25^{o}C) with gentle tilting and rotation.
10. Add 500 µL MACS buffer and pipette up and down several times.
11. Wash dynabeads three times as described in steps 5 and 6.
12. Resuspend dynabeads in 100 µL MACS buffer. Leave on ice (4^{o}C) until use.

<u>PAUSEPOINT:</u> *Anti-CD31 tagged magnetic beads can be prepared as described above, 1 day before the experiment and stored at 4^{o}C until use. This reduces effort on the day of the experiment, especially if performing this protocol for the first time.*

Preparation of lung samples

13. If isolating human cells, follow option A. If isolating rat cells, follow option B.

    A) Human cells.

    i) Collect fetal or adult biopsied/autopsied lung samples into 15 or 50 mL centrifuge tubes with basal DMEM (prepared in step 1) and immediately store on ice

<u>PAUSEPOINT</u>*: Biopsies can be stored on ice for up to 24 hrs.*

The lung samples should ideally be processed for ECFC isolation, the same day. In some circumstances, immediate processing may not be possible, if for example, lungs are shipped from another centre. In this case, carefully store lungs at 4^{o}C in basal DMEM for not more than 24 hrs. In the authors' experience, ECFC colonies can be isolated following this holding period; however, the number of colonies will decrease as the time between sample collection and processing increases. *Samples should not be allowed to freeze at any time during shipment.*

B) Rat cells.

i) Sacrifice the rat using sodium phenobarbitol (65 mg/kg, i.p.).

ii) Wet the chest of the rat with 70% ethanol and open the chest of the rat with anatomical scissors.

iii) Remove whole lungs of the rat with anatomical scissors and tweezers.

iv) Transfer the lungs into 15 or 50 mL centrifuge tubes with basal DMEM (prepared in step 1) and immediately store on ice.

Isolation of Total Lung Cells

14. Transfer lungs from the centrifuge tubes into a Petri dish.
15. Wash whole lungs with serum-free basal DMEM
16. If using rat tissue, slice the peripheries of lung lobes, discarding the contents of the hilum and the proximal alveolar tissue. Remove thin strips of alveolar tissue subjacent to the pleural surface. Alternatively, for the human lung, trace the pleural surface of the supplied lung piece and remove strips of tissue immediately underlying the pleural surface.
17. Add a few drops of basal-DMEM and chop the lung tissue using sterile, disposable blades to very small pieces (approximately 1-2 mm^2) that could be drawn into a 5 mL pipette.
18. Transfer the pieces into a 15 mL centrifuge tube containing 1x working dilution of collagenase/dispase digestive solution (7-10 mL per 0.5 g wet tissue).
19. Digest the lung at 37^{o}C for 45 min under continuous horizontal shaking (300 r.p.m.).
20. Quench the lung-digestive solution mixture with equal volume of complete-DMEM
21. Draw the mixture up and down several times using a 5 mL pipette.
22. Place 70 μm cell strainer on a 50 mL tube
23. Under sterile conditions, strain the lung digest through the 70 mm cell strainers by releasing the lung digest on the cell strainer using a 5 mL pipette.

Caution: Slowly release the lung digest on the strainer to avoid overflowing of digested suspension. More than one 70 mm cell strainers may be needed at this step.

24. Wash the cell strainer with 2-3 mL of complete-DMEM
25. Re-strain the cell suspension through a 40 μm cell strainer into a 50 mL centrifuge tube
26. Centrifuge the 50 mL tube at 300 g at 4ºC for 10 min.
27. Decant the supernatant and resuspend the cell pellet in 10 mL of complete-DMEM.
28. Centrifuge the 50 mL tube at 300 g at 4ºC for 10 min.
29. Decant the supernatant and resuspend the cell pellet in 5 mL of MACS buffer.

TROUBLESHOOTING:

Magnetic selection of CD31+ cells

30. Determine cell number of each sample of total lung cells using a modified Neubauer chamber. Prepare 1:10 and 1:100 dilutions of each sample in addition to the undiluted suspension. Mix 10 μL of these dilutions in a 1:1 ratio with trypan blue and count cells.
31. Centrifuge the unstained cell suspension at 300 g for 10 min.
32. Decant the supernatant and resuspend the cell pellet in 1 mL of MACS buffer per 10^7 total cells. Strain the cell suspension through a 40 μm cell strainer to remove any cell aggregates.
33. Add 25 μL dynabeads per 10^7 total cells.

Caution: The volumes used above for magnetic selection are for up to 10^7 total cells. When working with fewer than 10^7 cells, use the same volumes as indicated. If a sample contains more than 10^7 cells, scale up all reagent and total volumes accordingly.

34. Incubate for 30 min at 2-8ºC with gentle tilting and rotation.

Critical Step: Use a mixer that provides tilting and rotation of the tubes to ensure dynabeads do not settle at the bottom of the tube. Strictly adhere to the incubation time and temperature to reduce phagocytic activity and other metabolic processes and to avoid lower purity or viability of the isolated cells.

35. Double the cell suspension volume with MACS buffer to limit trapping of unbound cells.
36. Place the tube in a magnet in aseptic conditions for 2 min.
37. Discard the supernatant and gently wash the bead-bound cells with 1 mL MACS buffer.
38. Place the tube in the magnet for 1 min and discard the supernatant.

39. Repeat steps 37 and 38 four times. At the end, add enough MACS buffer to thoroughly resuspend all cells that would have adhered to the walls of the tube. Centrifuge the cell suspension at 300 g for 10 min. Aspirate the supernatant.
40. Resuspend cells in cEGM-2 to obtain a suspension of $7.5x10^4$ to $1.0x10^5$ cells/mL.

TROUBLESHOOTING:

Plating of CD31+ cells in culture

41. Remove the rat tail collagen type I solution from a collagen-coated 6-well tissue culture plate and wash the surface twice with PBS (using 2 mL PBS per well). Critical step : This step must be done immediately prior to plating the well plate with cells.

42. Pipette 2 mL (approx. 4000-5000 cells) into each well of the 6 well plate precoated with rat tail collagen type I and place in a 37^{o}C, 5% CO_2 humidified incubator.
43. After 24 hr (day 1), slowly remove the medium from the well with a pipette.

Critical step: Aspirate medium slowly at a rate of 1 mL per 4-5 sec. A small quantity of medium should be left behind in the well to prevent drying of the plate surface.

44. Slowly add 2 mL of cEGM-2 to the well. Return the plates to the incubator.
45. Refresh the medium daily (as described in steps 43 and 44) until day 7 and every other day thereafter. ECFC colonies should appear between day 4 and day 14 of culture as well circumscribed areas of cobblestone-appearing cells (Figure 3 and 4A, white arrows). Individual colonies should be isolated and expanded as described in the following steps between days 7 to day 14. Mesenchymal stem cells (MSC)-like contaminating cells appear as dendritic/fibroblast-like cells (Figure 3 and 4A, white arrowhead and 4B and C) that can overgrow ECFC.

Critical Step: ECFC colony isolation should be done very soon after contaminating cells are seen, to ensure a pure subculture of ECFC.

ECFC colony isolation

46. Visualize the ECFC colonies using an inverted microscope and outline the colony boundaries with a fine tipped marker on the underside of the well.
47. Using sterile forceps pick a cloning cylinder from its packing and dip the bottom surface into autoclaved vacuum grease to acquire a uniform coating.

Critical Step: *Always apply the minimum amount of vacuum grease necessary to uniformly coat the bottom surface of the cloning cylinder and form a good seal with the culture plate. Too much grease will interfere with cell collection. Prepare cloning cylinders just prior to use.*

48. Aspirate the culture medium and wash the well with 2-3 mL PBS.
49. After aspirating the PBS, set a clonal ring around each colony and press firmly against the plate with forceps.

Caution: *Carefully apply vertical pressure to seal the cloning cylinder against the culture plate. Avoid any inadvertent sliding of the cylinder as this can disrupt the ECFC colony.*

50. Using a 20-200 µL pipette, add 50-150 µL of warm trypsin/EDTA into each cloning cylinder.

Critical Step: *Make sure that sufficient quantity of trypsin/EDTA is added to cover the entire area within the cloning cylinder.*

51. Incubate plates up to 5 min until the cells have completely detached.
52. Draw 200 µL of cEGM-2 into a 20-200 µL pipette.
53. When all cells within the cloning cylinder have detached, place the tip of the pipette into the center of the cylinder and pipette up and down several times.
54. Collect the entire volume into a sterile microcentrifuge tube containing an equal volume of cEGM-2 and mix well.
55. Wash the area within the cloning cylinder 1-2 times with 200 µL cEGM-2 and transfer washes into the sterile microcentrifuge tube until all detached cells are collected into the microcentrifuge tube.
56. Remove the rat tail collagen type I solution from a collagen-coated 24-well tissue culture plate and wash the surface twice with PBS (using 1 mL PBS per well). Critical step : This step must be done immediately prior to plating the well plate with cells.
57. Seed the cells from each ECFC colony into one well of the 24-well cell culture plate precoated with rat tail type-I collagen in a total volume of 1 mL of cEGM-2 and culture in a 37ºC, 5% CO_2 humidified incubator for expansion

Critical Step: *If the ECFC colony is small (fewer than 100 cells), cells from 2-3 colonies can be pooled and plated into one well of a 24-well cell culture plate.*

TROUBLESHOOTING:

Further Purification and Expansion of ECFC

58. When cells in the 24-well plate approach confluence, remove cell culture medium and wash the culture surface with PBS.
59. After removal of PBS, add 100 μL of trypsin/EDTA per well of the 24-well plate and incubate plates up to 5 min until the cells have completely detached.
60. Pipette up and down thoroughly with 500 μL of cEGM-2 to collect the detached cells into a 15 mL centrifuge tube.
61. Wash the well 1-2 times with 500 μL cEGM-2 until all detached cells are collected into the centrifuge tube.

Critical Step: Cells from up to 2-3 confluent wells can be pooled into one centrifuge tube.

62. Make the volume of the cell suspension up to 12-14 mL with cEGM-2 and mix well by pipetting up and down gently.
63. Remove the rat tail collagen type I solution from a collagen-coated 75 cm^2 vented tissue culture flask and wash the surface twice with PBS (using 10 mL PBS per well). Critical step : This step must be done immediately prior to plating the well plate with cells.
64. Plate the cells in the 75 cm^2 vented tissue culture flask precoated with rat tail type-I collagen and culture in a 37ºC, 5% CO_2 humidified incubator for expansion.
65. Replace culture medium with fresh cEGM-2 every other day and culture until cells approach 80-90% confluence.

Critical Step: Cultures should reach 80-90% confluence in 4-7 days.

TROUBLESHOOTING:

66. When the tissue culture flasks have approached 80-90% confluence, carefully examine the cultured cells using inverted microscope to spot areas of MSC-like contaminating cell colonies as opposed to uniform cobblestone-appearing ECFC monolayer.

Critical Step: It is not uncommon to find MSC-like contaminating cell colonies interspersed with the ECFCs in early passages of the culture. The contaminating cells appear as described under step 47. The ECFCs can be purified further by performing approximately 3-5 cycles of CD31-positive magnetic selection and subculturing, as described in steps 67-75.

67. Remove culture medium from the cell culture flasks and wash the culture surface liberally with PBS.

Critical Step: *For a 75 cm^2 tissue culture flask, use 8-10 mL of PBS for the washes.*

68. Aspirate PBS and add 3 mL trypsin/EDTA per 75 cm^2 culture flask.

Critical Step: *Warm trypsin/EDTA to 37^oC prior to use. .*

69. Incubate the flask at 37^{o}C until the cells detach completely.

TROUBLESHOOTING:

70. Add 8-10 mL of cEGM-2 and collect the cells completely into a 15 mL centrifuge tube.
71. Centrifuge the 15 mL tube at 300 g at 4^{o}C for 5 min.
72. Decant the supernatant and resuspend the cell pellet in 1 mL of MACS buffer.
73. Take up 10 µL of the ECFC suspension and mix with 10 µL trypan blue.
74. Apply 10 µL of this mixture into a modified Neubauer chamber and count the cells

Critical Step: *Usually a confluent 75 cm^2 tissue culture flask contains 2-4 million ECFCs. Up to 10^7 cells can be suspended in 1 mL of MACS buffer.*

75. Perform a MACS selection of CD31 positive cells following the procedure described in steps 31-40.

Critical Step: *This step is to remove the MSC-like contaminating cells* ***(Figure 4)*** *and to obtain a pure culture of ECFCs. If contaminating cells are still present upon further passage, repeat MACS selection of CD31 positive cells at each passage until a pure ECFC culture is obtained. Fluorescence activated cell sorting (FACS)-based selection for CD31+ cells may also be considered at this step. Usually pure ECFC cultures are obtained after 2-4 cycles of CD31-positive selection performed over 2-4 passages.*

Troubleshooting

See Table 1 for Troubleshooting guidance.

Timing

Isolation of CD31+ cells from lung samples and their initial plating in culture (steps 1-40) requires ~4.5 hrs. The approximate timeline for the different steps this involves are as follows:

- Preparation of reagents: 2 hrs (Steps 1-12)
- Remove lungs from rat: 5 min (Step 13)
- Isolation of total lung cells: 1 hr 45 min (Steps14-29)
- Magnetic selection: 30 min (Steps31-40)

Late-outgrowth ECFC colonies usually arise in culture of CD31+ lung cells between days 4 and 14. These colonies can be isolated and expanded in culture in two to three 75-cm^2 flasks in ~2 weeks for human lung samples and ~3 weeks for rat lungs (Steps 41-57). The expanded cells should be processed through 2-4 cycles of CD31+ selection done over 2-4 passages to obtain pure cultures. This may take an additional 2-3 weeks (Steps 58-75). There is variability in the number of primary ECFC colonies obtained and their population doubling times between donors for human lungs. For instance, we obtained approximately 20 ± 5 colonies/5000 CD31+ cells plated (mean ± SD from 4 independent trials). For rats, since routinely lung tissue is pooled from several animals, not much variability is observed. Here, approximately 40 ± 5 ECFC colonies were obtained by plating 5000 CD31+ cells (data from 5 independent trials).

Anticipated Results

Initiation and propagation of ECFCs

ECFC colonies appear between 7 and 14 days of culture. Routinely, embryonic human lung samples can be expanded to 10^7 cells in 5-6 weeks. Rat lungs, on the other hand, may require a longer time (6-8 weeks) to yield the same number of ECFCs. Both rat and human lung vascular ECFCs have the potential to be cultured for up to 15 passages before they start expressing morphological indications of senescence such as slow multiplication and appearance of cytoplasmic vacuoles. These observations are consistent with previous studies on human adult EPCs[15,18].

Phenotypic characterization of lung vascular ECFCs

Lung vascular ECFCs grow as a monolayer with the characteristic "cobblestone" morphology. They ingest AcLDL, bind Ulex lectin and form capillary tube-like networks when plated on Matrigel **(Figure 5A).** ECFCs uniformly express typical endothelial cell surface antigens CD31, CD146, CD105 and CD144, but do not express hematopoietic cell specific surface antigen CD45 or monocyte/macrophage marker CD14 (see online supplement of reference[17] for detailed methods) **(Figure 5B).** In addition, human lung ECFC are capable of giving rise to clusters (up to 50 cells) or colonies (50-500 cells or more than 500 cells) in 96-well plates where ECFCs are plated at a seeding density of 1 cell per well. In three independent trials by the authors, 6.0 ± 1.2 % and 7.5 ± 2.9 % (mean ± SD) of single plated human lung ECFCs formed colonies with 50-500 cells and more than 500 cells, respectively **(Figure 5C).** This characteristic of "single-cell colony forming potential" is important in distinguishing ECFCs from mature endothelial cells[13].

The most stringent means to verify the functionality of ECFCs is to assess their ability to contribute to de novo vasculogenesis. In order to assess this property, ECFCs are loaded on collagen-fibronectin matrices and implanted subcutaneously in NOD/SCID mice. Cellularized collagen-fibronectin implants are excised after 14 days post-implantation and examined for vascularization by immunohistochemical staining. RBC perfused anti-human CD31+ vessels are identified in implants loaded with human lung ECFCs **(Figure 6)**.

Rat lung ECFCs isolated by this protocol also demonstrate all the defining characteristics of ECFCs, as shown in Alphonse *et al*[17]. Rat ECFCs express endothelial surface markers VCAM-1 and ICAM-2[15,24]. They also express CD34 and c-Kit, cell-surface antigens previously identified on endothelial and hematopoietic progenitor cells[15,25] **(Figure 7)**. In addition to binding *Ulex europaeus* lectin, rat lung ECFCs also bind the lectin from *Griffonia simplicifolia*, indicating its likely origin from lung microvasculature[26].

CFI:

The authors declare that they have no competing financial interests.

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Author Contributions Statement:

R.S.A. developed the protocol design, troubleshot/perfected steps of the procedure, performed experiments, analysed data and wrote the manuscript. A.V. and S.Z. performed experiments, helped with experimental design and contributed to manuscript preparation. S.M. and R.O. helped with human tissue acquisition and experimental design. M.C.Y. contributed to protocol design, guided troubleshooting and edited the manuscript. B.T. supervised the project, contributed to experimental design, guided protocol development and edited the manuscript.

Table 1 Troubleshooting Guide

Step	Problem	Possible Reason	Solution
29	Too few viable cells after enzymatic disintegration	Poor storage conditions or prolonged storage of lung samples	For rat lung, it is best to process them immediately after excision. For human lung, if immediate processing is not feasible or if being shipped from another centre, carefully store at 4^{o}C in basal DMEM for not more than 24 hrs. Samples should never be allowed to freeze anywhere during this storage period.
		Too long or too vigorous shaking during enzymatic digestion step	Do not leave the chopped lung sample for more than 45 min in pre-warmed collagenase/dispase. Do not shake at speeds greater than 300 r.p.m.
40	Too few viable cells after CD31+ MACS selection	Miscalculation during anti-CD31 tagging of dynabeads	Recheck the amount of anti-CD31 antibody added per unit volume of dynabeads
		Miscalculation during coating of cells with anti-CD31 tagged dynabeads	Recheck the amount of anti-CD31 tagged dynabeads added per 10^7 cells
		Incubation temperature too high or too low	Strictly adhere to the incubation time and temperature specified in the protocol
57	No ECFC colonies appear	Harsh treatment of plated CD31+ cells	Remove and replace medium very slowly and gently during the first week of culture. Do not use vacuum suction to remove medium.
		Very low CD31+ cell seeding density	Plate at least 3-5 x 10^3 CD31+ cells/well at step 42.
		Insufficient enzyme digestion	Make sure the tissue is chopped adequately (to obtain 1-2 mm^3 pieces). Also make sure there is sufficient enzyme added to the chopped tissue (~ 10 mL of enzyme solution [working dilution] per 0.5 g tissue)
		Insufficient washing of cells (with beads attached)	In steps 37 and 38, make sure the cells (with attached beads) are washed sufficiently and properly suspended in MACS buffer to minimize CD31+ cell loss
57	Too many contaminating cells around ECFC colonies **(Figure 4)**	Antibody problem	Antibodies may not be very specific. Use antibodies from standard manufacturers, preferably the same ones used in this protocol. Do not subject antibodies to repeated freeze-thaw cycles and leave on ice all the

Step	Problem	Possible Reason	Solution
			time before use.
		Ineffective coating of cells with anti-CD31 tagged dynabeads	Strictly adhere to the incubation time and temperature specified in the protocol. High phagocytic activity will result in non-CD31+ cells being selected out in large numbers.
		Improper timing for ECFC colony selection	ECFC colonies generally appear anytime between 4 and 14 days of culture. If colonies are not selected promptly after appearance of ECFC colonies, MSC-like contaminating cells will quickly overgrow the cultures.
65	Insufficient purity of yields	Overgrowth of contaminating cells over time	Positive selection for CD31+ cells via MACS or FACS over 2-4 passages will yield clean ECFC cultures.
		Contaminating cells picked up along with ECFC colonies	After circling ECFC colonies with a marker pen, scratch out any contaminating cells around the colonies with a pipette tip. Select the size of the cloning cylinders to be just enough to cover the colonies.
65	ECFCs do not attach and grow	Ineffective type I collagen coating of culture surfaces	If plated on uncoated culture surfaces, ECFCs never attach and grow. Use fresh and properly constituted type I collagen solution for coating. If expired collagen stock or improperly constituted working dilutions are used, ECFCs may fail to attach and grow.
65	ECFCs are not dividing	Cells are senescent	ECFC-derived mature endothelial cells eventually undergo senescence. For best results in functional assays, use ECFCs between passages 5 and 10.
69	ECFCs do not detach	Serum may block trypsin activity	Wash 2-3 times with PBS to remove leftover serum components, prior to adding trypsin.
		Trypsin activity is low	Use fresh, warm trypsin. Freeze trypsin in smaller aliquots so that once thawed, the entire aliquot is used and not exposed to repeated warming and cooling.

Figure Legends

Figure 1. Flow diagram of the steps involved in lung ECFC isolation.

Figure 2. Schematic representation of lung dissection and lung cell suspension preparation (protocol steps 14 to 29). After thoracotomy and careful excision of the rat lung, the peripheral rims of all lung lobes are dissected. The lung tissue is chopped into approximately 1-2 mm^2 pieces and dispersed into a single cell suspension by incubation in collagenase/dispase digestion solution.

Figure 3. Schematic representation of CD31+ cell selection, plating and ECFC colony isolation (protocol steps 30 to 57). CD31+ cells are selected from the total lung cell suspension using magnetic-activated cell sorting (MACS). CD31+ lung cells are plate and cultured in type I collagen-coated 6 well plates containing complete-Endothelial Growth Medium-2 (cEGM-2). Late-outgrowth 'cobblestone-like' ECFC colonies appear between 4-14 days. The individual ECFC colonies are carefully marked and isolated using cloning cylinders. ECFCs are expanded in cEGM-2 culture and purified with 2-4 cycles of CD31+ selection and passaging. Scale bars in the microscopy images of ECFC colonies represent 100 μm.

Figure 4. Representative photomicrographs of contaminating cells in ECFC culture. (A) Contaminating cells with mesenchymal stromal cell (MSC)-like or fibroblast-like phenotype (white arrowheads) are seen around a cobblestone-like rat lung ECFC colony (white arrow) using phase contrast microscopy (B) Contaminating cell cluster with fibroblast-like phenotype observed in passage 2 rat lung ECFC culture. (C) Contaminating cell colony observed in passage 2 human lung ECFC culture. The scale bar in the images represents 100 μm. All procedures were approved by the Animal Health Care Committee of the University of Alberta.

Figure 5. Representative phenotypic analysis of human lung vascular ECFCs. (A) Human lung ECFCs form cobblestone-like colonies when cultured as a monolayer. Cells are observed by phase contrast microscopy. Cultured ECFCs demonstrate Dil-acLDL uptake and Ulex europaeus-lectin binding. The cells ingest Dil-AcLDL (red) following 4 hrs of incubation and bind Ulex europaeus-lectin (green) following 1 hr of incubation after fixation. Counterstaining with Heochst 33258 (blue) illustrates that all adherent cells are positive for LDL-uptake and Ulex-lectin binding. It was observed that a modest number of stray MSC-like contaminating cells did not take up LDL. But these cells were totally lost following 2-4 cycles of CD31-positive selection and passaging. ECFCs formed tube-like structures when suspended in matrigel and observed by light microscopy. **(B)** Human lung ECFCs were positive for endothelial-specific cell surface antigens CD31, M-CAM (CD146), VE-cadherin (CD144), endoglin (CD105) and negative for hematopoietic cell specific CD45 and monocyte/macrophage specific CD14. Filled grey histograms represent antigen staining with negative isotype controls overlaid in white. **(C)** Human lung ECFC were capable of giving rise to clusters (2 to 50 cells) or colonies (51-500 cells or 501 and above cells/colony) in 96-well plates, when ECFCs were plated at a seeding density of 1 cell per well. Results represent the average ± standard error of mean of 3 independent experiments. The y axis represents percentage of plated single cells. Human lung tissue collection was reviewed and approved by the Institutional Review Board at the University of New Mexico. Specific details about the methods used for phenotypic analysis are available in

our earlier report[17] and information about the antibodies used for cell labeling are provided in the supplementary information.

Figure 6. Human lung ECFCs contribute to de novo vasculogenesis. Human lung ECFCs form blood vessels de novo when seeded on fibronectin-collagen plugs (10^6 ECFCs per implant) and implanted subcutaneously into the flanks of NOD/SCID mice. 7 days post implantation, the cellularized implants were excised, paraffin embedded and stained with hematoxylin and eosin (H&E) and anti-human CD31 (brown). Black arrow heads indicate red blood cell (RBC)-perfused, anti-human CD31+ vessels within the gel implant. Scale bars represent 65 μm. Human lung tissue collection was reviewed and approved by the Institutional Review Board at the University of New Mexico.

Figure 7. Surface antigen expression and lectin binding characteristics of rat lung vascular ECFCs. Rat lung ECFCs express the cell surface antigens CD34, ICAM-2, VCAM-1 (CD19) and c-Kit. Rat ECFCs bind lectins from Ulex europaeus and Griffonia simplicifolia following 1 hr of incubation after fixation. Dark coloured histograms represent antigen staining with negative isotype controls overlaid in pale grey. Specific details about the antibodies used for cell staining have been provided in the supplementary information. The specificity of these antibodies is suboptimal when working with rat ECFCs.

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Supplementary Information

The isolation and culture of endothelial colony forming cells from human and rat lungs

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Human ECFC Surface Marker Characterization

Surface Marker	Antibody	Supplier Information
CD31	Anti-human CD31 antibody, FITC	BD Pharmingen, Cat. No. 555445
CD45	Anti-human CD45 antibody, FITC	BD Pharmingen, Cat. No. 555482
CD14	Anti-human CD14 antibody, FITC	BD Pharmingen, Cat. No. 555397
CD144 (VE-Cadherin)	Anti-human CD144 antibody, PE	eBioscience, Cat. No. 12-1449-80
CD146 (M-CAM)	Anti-human CD146 antibody, PE	BD Pharmingen, Cat. No. 550315
CD105 (Endoglin)	Anti-human CD105 antibody, PE	Invitrogen, Cat. No. MHCD10504

Rat ECFC Surface Marker Characterization

Surface Marker	Primary Antibody	Supplier Information	Secondary Antibody	Supplier Information
CD34	Anti-CD34 antibody [EP373Y]	Abcam, Cat. No. AB81289-□2	Alexa Fluor 488 goat anti-rabbit IgG (H+L)	Life Technologies, Cat. No. A-11008
ICAM-2	ICAM-2 Antibody (H-159)	Santa Cruz, Cat. No. sc-7933	Alexa Fluor 488 goat anti-rabbit IgG (H+L)	Life Technologies, Cat. No. A-11008
VCAM-1	VCAM-1 Antibody (C-19)-R	Santa cruz, Cat. No. sc-1504-R	Alexa Fluor 488 goat anti-rabbit IgG (H+L)	Life Technologies, Cat. No. A-11008
c-Kit	c-kit (2B8, biotin), rat monoclonal antibody	Abcam, Cat. No. ab25022	Streptavidin-Cy3	Sigma, Cat. No. s6402
Ulex europaeus lectin, FITC conjugate		Sigma, □ Cat. No. L9006		
Griffonia simplicifolia lectin, TRITC conjugate		Sigma, Cat. No. L5264		