Video Article

Isolation and Culture Expansion of Tumor-specific Endothelial Cells

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Abstract

Freshly isolated tumor-specific endothelial cells (TEC) can be used to explore molecular mechanisms of tumor angiogenesis and serve as an *in vitro* model for developing new angiogenesis inhibitors for cancer. However, long-term *in vitro* expansion of murine endothelial cells (EC) is challenging due to phenotypic drift in culture (endothelial-to-mesenchymal transition) and contamination with non-EC. This is especially true for TEC which are readily outcompeted by co-purified fibroblasts or tumor cells in culture. Here, a high fidelity isolation method that takes advantage of immunomagnetic enrichment coupled with colony selection and *in vitro* expansion is described. This approach generates pure EC fractions that are entirely free of contaminating stromal or tumor cells. It is also shown that lineage-traced Cdh5^{cre}:ZsGreen$^{l/s/l}$ reporter mice, used with the protocol described herein, are a valuable tool to verify cell purity as the isolated EC colonies from these mice show durable and brilliant ZsGreen fluorescence in culture.

Video Link

The video component of this article can be found at http://www.jove.com/video/53072/

Introduction

Endothelial cells (EC) are essential during the development of solid tumors. From initiation of the angiogenic switch in dormant tumors to dissemination and seeding of metastases at distant sites, EC form the conduits that provide blood, oxygen, and nutrients to sustain tumor growth [1]. As recently suggested, EC also have perfusion-independent functions and form a niche that supports the growth of cancer stem cells and other tumor stromal cells [2-5]. Thus, highly purified tumor-specific EC (TEC) for *in vitro* culture allows for routine functional studies that will shed light on novel molecular mechanisms mediating tumor angiogenesis and cross talk with tumor cells.

EC are highly specialized depending on the tissue of origin [6]. Due to the heterogeneous nature of different tumor types and the tumor microenvironment, TEC may also display unique features that reflect a tumor-specific specialization of the vasculature. For example, there is striking variability in the gene expression signatures in TEC isolated from different types or grades of tumors [7,8]. However, frequent co-purification of non-EC, especially tumor-associated fibroblasts and tumor cells, with TEC can confound genome-wide expression analyses. These unwanted cell types are especially problematic in studies that rely on long-term *in vitro* expansion of TEC cultures.

Described here is a high-fidelity method that consistently produces pure EC cultures from tumors and other tissues. Following immunomagnetic column enrichment of EC fractions and removal of co-purified non-EC, an additional cloning-ring step to capture pure EC colonies is used [9]. Each colony can be expanded in culture for multiple passages without the emergence of contaminating non-EC. This method also yields multiple EC clones from a single isolation procedure, which is ideal for the study of endothelial heterogeneity. In addition, it is shown that Cdh5^{cre}:ZsGreen$^{l/s/l}$ reporter mice are a valuable tool for generating “fate-mapped” and indelibly-marked EC which maintain ZsGreen fluorescence in culture [10]. With minor adjustments to the protocol, this method should be adaptable to different tumor types or normal tissues.

Protocol

The following protocol is carried out according to guidelines established by the Department of Laboratory Animal Medicine at the University of North Carolina at Chapel Hill.

1. Prepare the Following Material and Reagents Before Starting

1. Prepare EC media by supplementing 400 ml low glucose (1 g/L D-glucose or LG) Dulbecco's Modified Eagle's medium (DMEM) with 50 ml heat-inactivated fetal bovine serum, 50 ml Nu-Serum IV, 5 ml antibiotic-antimycotic, and the hFGF, VEGF, hEGF, R3-IGF-1, and heparin components from the commercial kit.
2. Prepare 500 ml FACS buffer (0.5% BSA and 2 mM EDTA in PBS); filter through a sterile 0.22 µm filter cup.
3. Sterilize or disinfect dissecting board.
4. Sterilize dissection pins, surgical scissors, and dissectors.

2. EC Isolation (Day 1, ~5 hr)

1. Euthanize mouse with carbon dioxide or other methods compliant with the Institutional Animal Care and Use Committee (IACUC) policies.
   Note: Multiple mammary tumors varying in size (5 - 15 mm in diameter) may develop in a single genetically modified mouse such as C3-TAg, be sure to harvest all of them for TEC isolation. If using tumors that are orthotopically engrafted in mice, pool two to three 1 cm^3 tumors for a single EC isolation. Here we use mammary tumors as a demonstration, but the protocol can be modified for other types of tumors.
2. Disinfect mouse by spraying or wiping the mouse ventral side with ample amount of 75% v/v ethanol.
3. Resect tumors with one pair of scissors and dissectors using aseptic techniques in a sterile hood or at a clean bench.
   1. Stretch out and pin the limbs of the mice on a dissecting board. Make a midline ventral incision with the scissors without opening the peritoneum. Dissect laterally between the skin and the peritoneum towards the mammary glands where tumors are located. Do not open the peritoneum.
   2. Excise only tumor tissue from the mammary glands, leaving out normal mammary margins. Carefully trim off non-tumor tissues such as skin and muscles, and place dissected tumors in a conical tube containing 30 ml of LG-DMEM on ice.
4. Bring tumor samples to tissue culture hood; wash tissues with sterile LG-DMEM 1 - 2 times.
5. Transfer tumors from the conical tube to a sterile tissue-culture petri dish, add 2 ml of LG-DMEM in the dish, and mince with a pair of sterile scissors into pieces <5 mm.
6. Add 5 ml of collagenase (stock = 2 mg/ml in Hank's Balanced Salt Solution, hereafter HBSS), 1 ml of dispase (stock = 2.5 U/ml in HBSS) and 75 µl of deoxyribonuclease (stock = 1 mg/ml in PBS) into the petri dish. Total volume is now ~10 ml.
7. Transfer the collagenase/tissue mix from the petri dish to a tissue-dissociator tube and run on a tissue dissociator for 60 sec twice (pre-set dissociation program on the dissociator: 1,270 total rounds per run). Incubate with light shaking on a shaker for 75 min at 37 °C.
8. Filter digested tissue through a 100 µm cell strainer over a 50 ml conical tube. Rinse filter with 5 ml of FACS buffer to wash any remaining cells. Spin at 280 x g for 5 min and carefully aspirate the supernatant without disturbing the cell pellet.
9. Dilute 1 ml of stock RBC lysis buffer (10x) in 9 ml of sterile water. Lyse red blood cells with 10 ml of lysis buffer (1x), and immediately spin 5 min at 280 x g. Note: This step can be skipped if little blood is visible.
10. Resuspend in 10 ml of FACS buffer. Mix 10 µl of cell suspension with 10 µl of trypan blue, and count live cells using a hemocytometer .
11. Resuspend cells at ~10^7 cells/100 µl. Add 10 µl of FcR block per 100 µl of cell suspension, and incubate on ice for 10 min.
12. Add rat-anti mouse PE-conjugated CD31 antibody according to **Table 1**. Incubate on ice for 10 - 15 min and flick tube occasionally.
13. Add 10 ml of FACS buffer to the tube and spin at 280 x g for 5 min. Carefully remove the supernatant, and wash the cell pellet again with 5 ml of FACS buffer. Centrifuge to pellet cells. Aspirate supernatant without disturbing cell pellet.
14. Add FACS buffer and anti-PE microbeads according to **Table 2**. Incubate on ice for 10 - 15 min. Flick tube occasionally.
15. Add 10 ml of FACS buffer and spin samples at 280 x g for 5 min; wash once with 5 ml of FACS buffer and centrifuge again. Aspirate supernatant without disturbing pellet.
16. Bring volume to 300 µl in FACS buffer. Spin through 35 µm cell-strainer capped tube 5 min at 280 x g. Use 2 tubes and a larger volume if needed.
17. Set up magnetic multistand and magnetic columns in hood, attach a column to the separator and equilibrate the column with 2 ml of FACS buffer.
18. Aspirate the supernatant and resuspend the cell pellet in 0.5 - 1 ml of FACS buffer.
19. Pass the cell suspension through the equilibrated magnetic column.
20. Wash the column three times with 2 ml of FACS buffer, and collect flow-through (FT) in a 15 ml tube (FT fraction).
21. Take the column off the separator and elute with 2 ml of FACS buffer into another 15 ml tube (eluate fraction). Use plunger to ensure all cells are off the column. Repeat the elution two more times with 2 ml of FACS buffer each time.
22. Spin the eluate at 280 x g for 5 min.
23. Remove the supernatant and resuspend the pellet in 10 ml of EC media.
24. Equally divide the eluate fraction (~ 6 ml) into 10 cm gelatin-coated dishes. For three 1 cm^3 tumors, plate eluted cells in at least four plates. Alternatively, plate eluted cells at different concentrations in multiple plates (*e.g.*, seed 0.5 ml, 1 ml, 1.5 ml, and 3 ml of the eluate into four plates) to ensure that at least one plate is sparsely seeded with eluted cells. Check that the confluency of the attached cells is at ~1% the next day (*i.e.,* approximately 1.0 - 2.0 X 10^5 attached cells).
    Note: The cells need to be plated sparse so that EC colonies can form without being contaminated by other cell types.
25. Plate FT fraction in one 10 cm dish to let cells recover O/N. Check that the plate is 80 - 100% confluent the next day. Freeze down the cells (-80 °C) in 250 µl cell-freezing media in a cryotube and store them in liquid nitrogen the next day. Note: FT fraction can be used for tumor cell isolation at a later stage and/or as a negative control for EC gene expression analysis of the isolated EC clones.
26. Change media every 2 - 3 days. Colonies start to form after 7 - 10 days. Small EC colonies can be identified as early as day 3. Mark the colonies with a fine-tip marker on the bottom of the dish.
27. Scrape off non-specific cells surrounding the identified colonies with a sterile 200 µl pippet tip.

3. Colony Selection Using Cloning Rings

1. Change media every 2 - 3 days. EC purity can be checked by LDL (DiI-Ac-LDL) addition at about 5 - 7 days. Add 50 µl of LDL per 10 ml EC medium and incubate for 3 - 4 hr before checking the cells under fluorescence microscope.
   Note: Multiple LDL$^+$ EC clones could be observed at ~day 7.
2. Start harvesting EC colonies when they reach diameters of 3 - 5 mm in size. (Choose big colonies that are packed with small LDL$^+$ cells for best results.)
3. Before harvesting EC with cloning rings, pre-coat a few 6-well plates with 0.5% gelatin. Aspirate gelatin, add 2 ml of EC media to each well, and keep the plates in an incubator until needed.
4. Scrape off non-EC on the edges of the colonies to make sure no other cell types will be trapped within the cloning ring.
5. Using a phase-contrast microscope (4X or 10X objective), outline with a fine-tip marker on the bottom of the culture dish the areas containing EC colonies.
6. Wash the plate with 10 ml of PBS and leave a very thin layer of PBS in the plate when aspirating. (Important: a small amount (~0.5 ml) of PBS will keep cells alive during the cloning-ring procedure; also, tissue adhesive needs water to bond.)
7. Choose a cloning ring of appropriate size. Use a pair of dissecting forceps to pick up a ring, and with a 10 µl pipet tip evenly apply a small amount of tissue adhesive onto the cloning ring.
   Note: use only minimal amount (~0.2 µl for a small ring) of tissue adhesive on cloning rings, and make sure tissue adhesive is spread out evenly around the bottom surface to ensure good sealing. Excessive tissue adhesive produces heat and forms films that may kill cells.
8. Place the cloning ring over the EC colony. Gently press down the cloning ring to glue the ring onto the plate. Ensure that the colonies are not dried out before gluing the ring.
9. Immediately pipet 25 µl of enzymatic cell detachment solution into the cloning ring and incubate ~1 min or until the cells are loosely attached.
10. Pipet cells in the cloning ring drop-wise into one well of a 6-well plate containing pre-warmed EC media. (Important: Do not shake the plate to disperse cells; EC prefer to grow in tight clusters.) Wash the cloning ring with 50 - 100 µl EC media to collect as many cells as possible, and transfer all washes into the same 6-well.
11. If some colonies in the 10 cm dish are too small to harvest, add 10 ml fresh media, let the colonies grow for a few more days, and repeat the cloning ring procedure again.
12. Grow harvested colonies in 6-well plates until 80 - 100% confluent, and transfer cells to 3 wells of a 6-well plate, before expanding them in a 10 cm tissue culture dish. Scrape off contaminant cells. Repeat another round of cloning ring procedure (Steps 3.5 - 3.10) if necessary. Keep cells relatively confluent (~60 - 70%) when expanding. EC may stop growing if plated too sparse.
13. Characterize EC by FACS, staining, PCR, etc. Note that DiI-Ac-LDL is fluorescent and may interfere with PE or other fluorescent antibodies for FACS.

Representative Results

EC represent only minor a fraction of the total cell population in most adult tissues [11]. It is therefore important to fully digest the harvested tissue into a single-cell suspension that ensures the maximal release of EC from extracellular matrix (ECM) and connective tissues. In our experience, CD31-mediated immunomagnetic selection only provides enriched but not pure EC fractions; therefore, another crucial step is the physical removal of co-purified non-EC and selection/expansion of EC colonies using cloning rings (**Figure 1**). For example, when CD31-enriched EC were plated without further colony selection, non-EC rapidly proliferated and intermingled with EC, producing impure EC cultures as revealed by DiI-Ac-LDL$^+$ and DiI-AC-LDL$^-$ populations (**Figure 2A**). However, when column eluates were plated at low density, the EC colonies grew with relatively few surrounding non-EC, which were removed by scraping with a pipet tip (**Figure 2B**). The growth and purity of the expanded colonies can be further monitored by addition of DiI-Ac-LDL. Small EC colonies can be observed at ~day seven after CD31-mediated column enrichment (**Figure 2B**, top panels). After selection and expansion of EC colonies, DiI-Ac-LDL$^-$ cells are eliminated, and the cell population is highly pure for DiI-Ac-LDL$^+$ EC as indicated by DiI-Ac-LDL uptake (**Figure 2B**, bottom panels).

To test if the isolation method can produce consistent results, multiple clones derived from either mammary tumors resected from C3-TAg (FVB/N $C3_1$-TAg) mice or normal mammary glands from age-matched wild-type FVB mice were isolated and expanded [12]. The purity of each TEC and normal EC (NEC) population was then carefully characterized to ensure purity. Flow cytometry analyses of three independent samples showed a single, uniformed CD31$^+$ population that was distinct from IgG isotype controls (**Figure 3A**). The mRNA expression of EC markers, including *Cd31*, *Cdh5* (VE-Cadherin), *Cd133*, and *Vegfr2* in all four EC clones tested was ~200 to 7,000 times higher than that of mouse embryonic fibroblasts (MEFs) which was used as a negative control (**Figure 3B**). As expected, all EC clones expressed nearly undetectable levels of the mesenchymal marker genes *Col1a1* and *Tagln* when compared to MEFs. Immunofluorescent staining showed that all cells in a representative TEC clone were uniformly CD31$^+$ (**Figure 3C**). Furthermore, these EC clones were able to form spontaneous vessel-like structures *in vitro*, indicating they retain endothelial functions after culturing (**Figure 3D**).

The isolation method was further validated using Cdh5^{cre}:ZsGreen$^{l/s/l}$ mice where all EC are indelibly labeled with the green fluorescent protein, ZsGreen (**Figure 4A**, a). Lung tissue that contains abundant EC was used as proof-of-principle for the isolation procedure (**Figure 4A, b**). ZsGreen$^+$ cells comprised ~30% of the total cell population in the lung homogenate, and were partially enriched after CD31-mediated immunomagnetic column selection (**Figure 4B**). As Cdh5^{cre}:ZsGreen$^{l/s/l}$ mice carry a constitutive *cre* gene, a proportion of hematopoietic cells are also labeled with ZsGreen [10]. Thus, the actual EC percentage in the lungs may be lower than the observed ~30%. Notably, approximately 20% of cells co-isolated with ZsGreen$^+$/CD31$^+$ EC were ZsGreen$^-$ (**Figure 4B**). Because contamination by these non-EC may persist in culture, the enriched EC population at this stage is not suitable for studies that require further *in vitro* cell culture. However, after applying cloning rings to capture pure EC colonies, a 100% pure ZsGreen$^+$ population was obtained that could be further expanded in culture (**Figures 4C and 4D**).

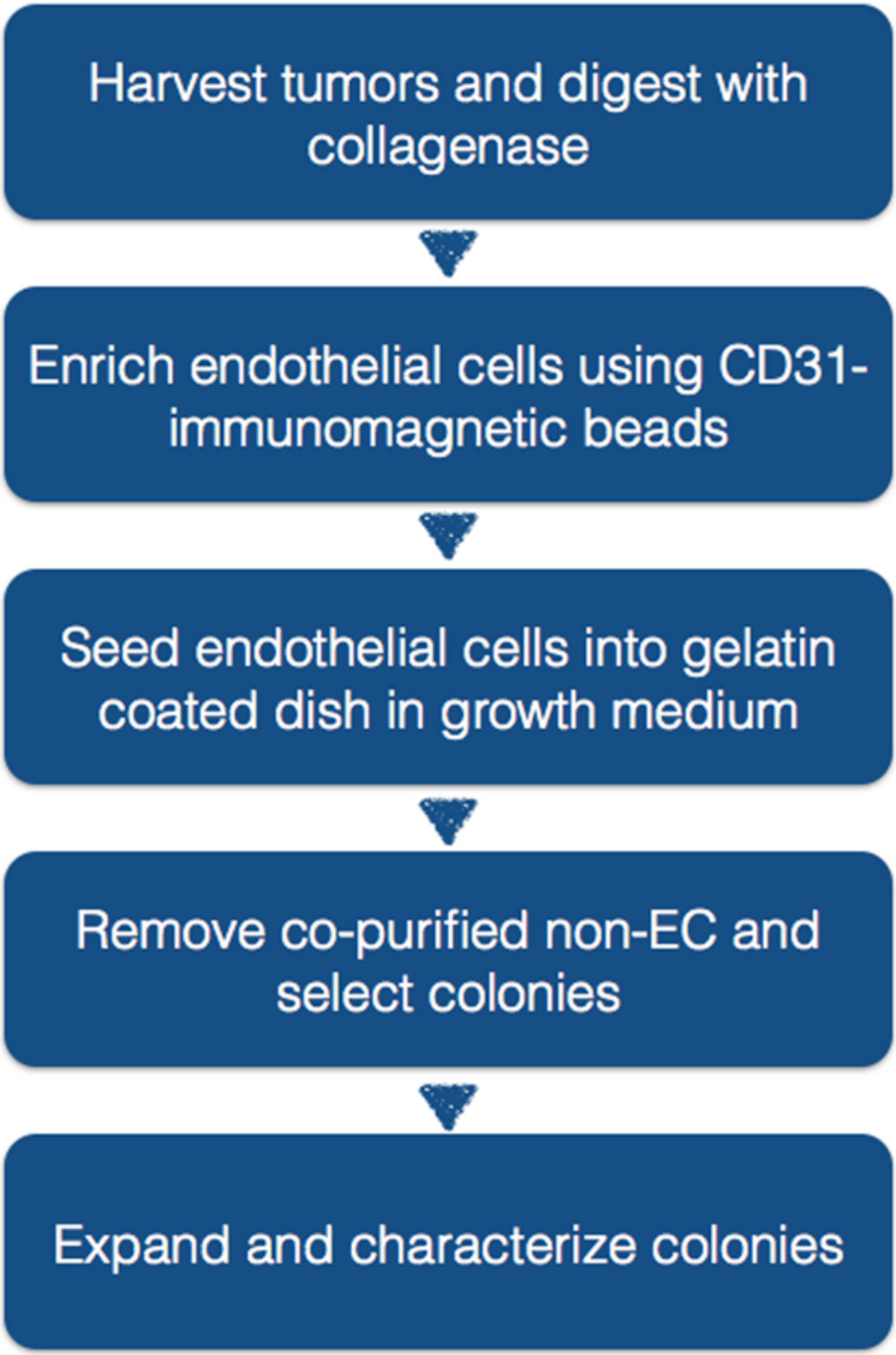

Figure 1. Flow Chart and Overview of the EC Isolation Procedure.

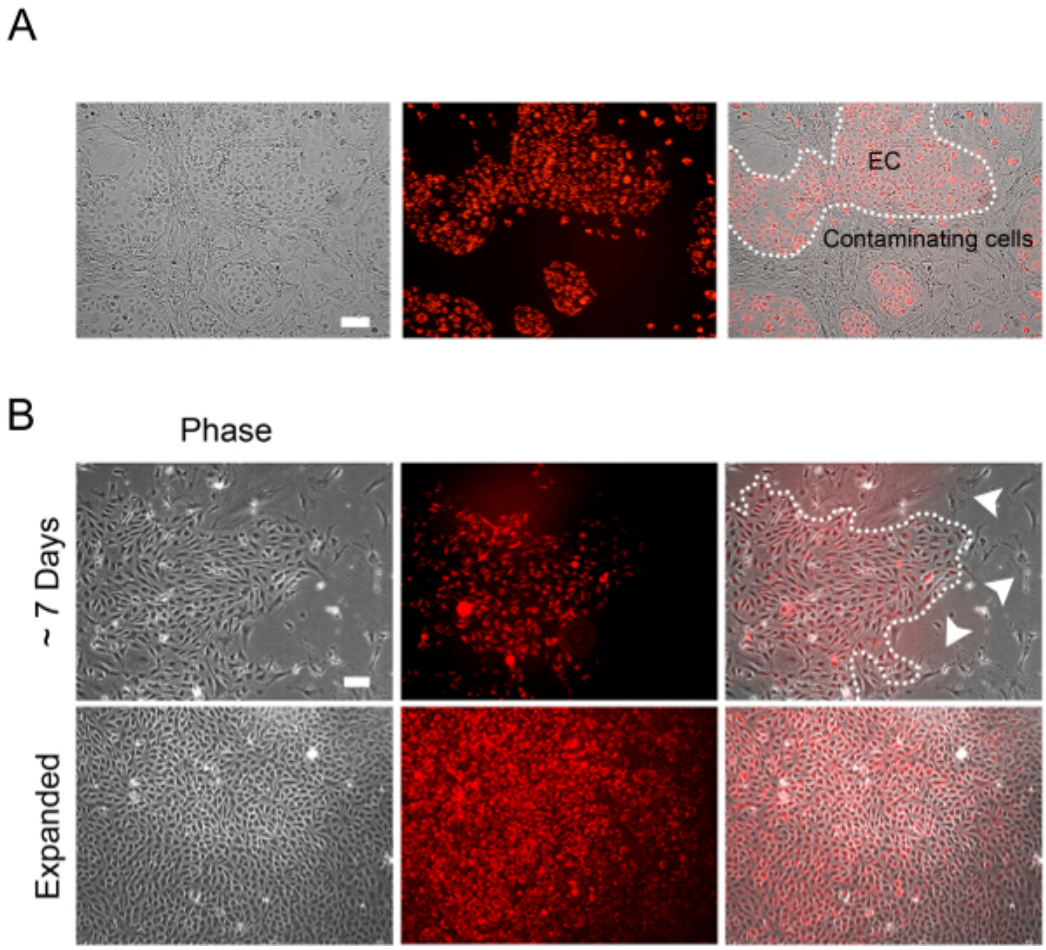

Figure 2. Dil-Ac-LDL Distinguishes EC from Contaminating Non-EC in Live Cultures. (**A**) Phase contrast and fluorescent images showing EC and co-isolated non-EC without cloning-ring selection of EC colonies. (**B**) Phase contrast and fluorescent images of EC colonies at different stages of isolation. Dotted lines mark the boundaries of Dil-Ac-LDL^{+} EC and surrounding contaminating cells. White arrowheads indicate non-EC outside an EC colony that should be removed with a pipette tip. Scale bars represent 100 µm. Please click here to view a larger version of this figure.

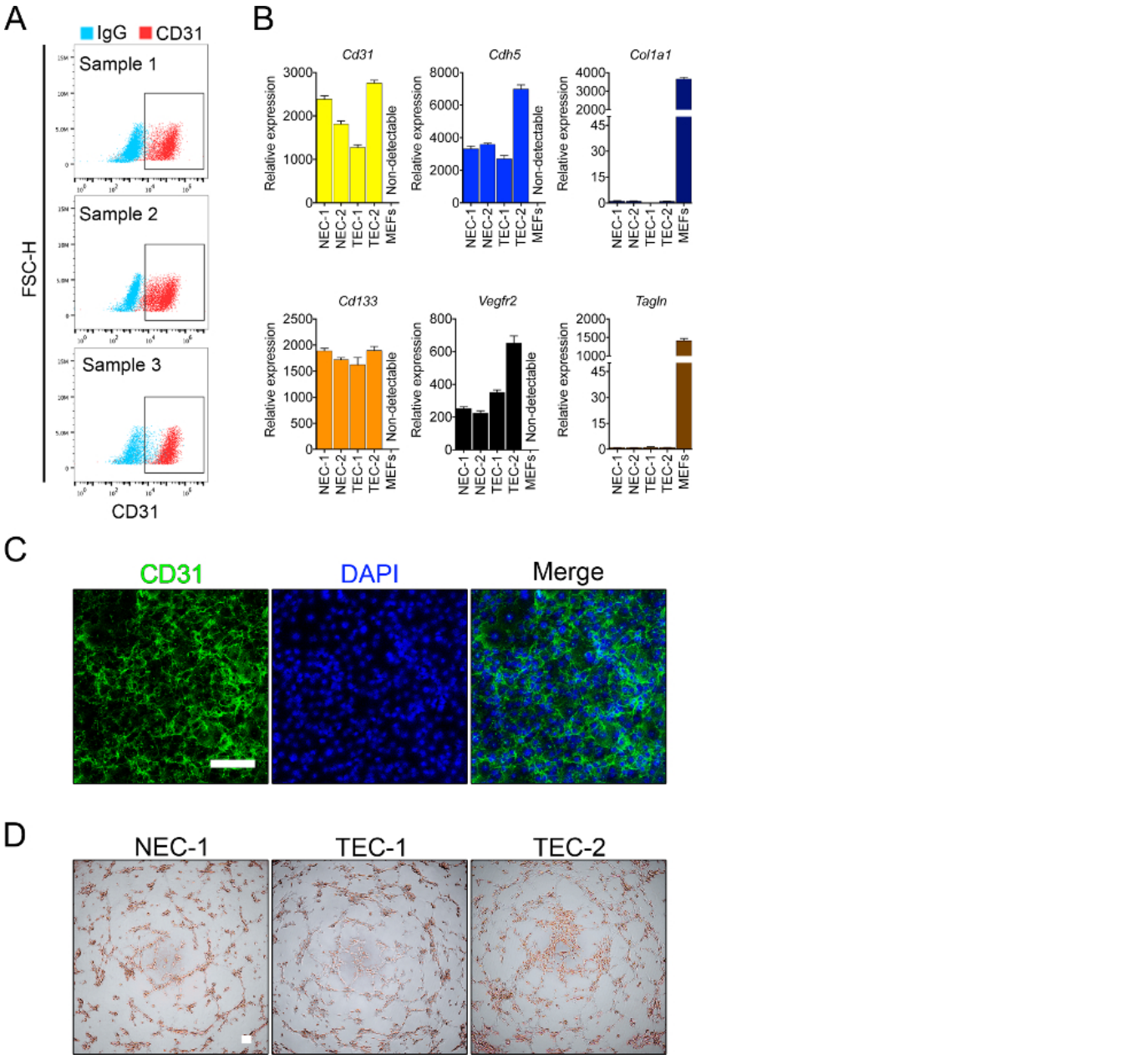

Figure 3. Cloning Rings and Physical Removal of Non-EC Produce Pure and Functional Long-term EC Cultures. (**A**) Representative FACS dot plots of CD31 staining of different EC clones. Three representative samples are shown. The open black rectangle in each plot outlines the $CD31^{+}$ population. An IgG isotype is used as a negative control. (**B**) Measurement of endothelial gene expression in selected NEC and TEC clones. The mRNA levels of endothelial genes *Cd31*, *Cd133*, *Cdh5*, and *Vegfr2* are expressed relative to those of mouse embryonic fibroblasts (MEFs), and the mRNA levels of mesenchymal genes *Col1a1* and *Tagln* are expressed relative to those of NEC-1. (**C**) Representative immunofluorescent staining of an expanded TEC clone. CD31 is shown in green and nuclei are stained blue with 4'6'-diamidino-2-phenylindole (DAPI). (**D**) Representative images of tube formation by different EC clones plated on Matrigel. Scale bars represent 100 µm. Please click here to view a larger version of this figure.

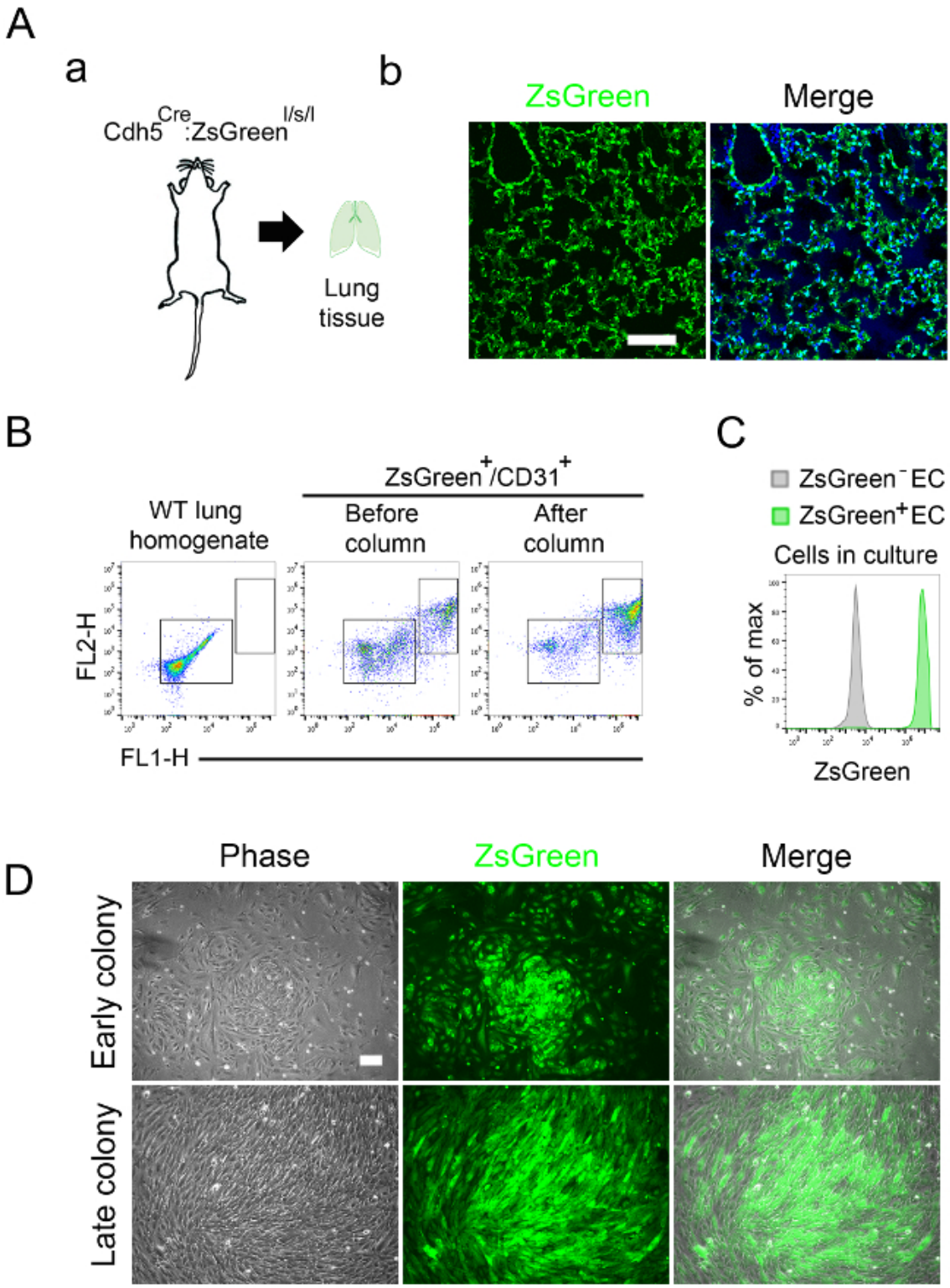

Figure 4. EC Isolated from Cdh5^{cre}:ZsGreen$^{l/s/l}$ Mice Maintain Brilliant ZsGreen Fluorescence in Culture. (**A**) The endothelial-lineage tracing mouse Cdh5^{cre}:ZsGreen$^{l/s/l}$ carries a floxed ZsGreen transgene that is induced by Cdh5^{cre} expressed by EC. (a) Lung tissue from the Cdh5^{cre}:ZsGreen$^{l/s/l}$ mice were harvested and digested for EC isolation. (b) Representative images of Cdh5^{cre}:ZsGreen$^{l/s/l}$ mouse lung tissue. ZsGreen (green) labels the endothelium and nuclei are stained with DAPI (blue). (**B**) FACS dot blots showing the percentages of ZsGreen^{+} cells before (middle panel) and after (right panel) the CD31-mediated column enrichment from the homogenized lung tissue of a Cdh5^{cre}:ZsGreen$^{l/s/l}$ mouse. Unstained wild-type (WT) lung homogenate (left panel) was used as a negative control for gating. The large open rectangles indicate ZsGreen^{-} cells and the small open rectangles indicate cells double positive for ZsGreen (FL1-H channel) and CD31 (FL2-H channel). (**C**) FACS histogram plot showing that ZsGreen^{+} EC (green peak) remain 100% pure after *in vitro* culturing. A ZsGreen^{-} EC population was used as a negative control (gray peak). (**D**) Representative phase contrast and fluorescent images of an early-stage ZsGreen^{+} EC colony and an expanded ZsGreen^{+} EC colony. Scale bar represents 100 µm. Please click here to view a larger version of this figure.

Cell number	FACS buffer (µL)	CD31-PE antibody (µl)
1 x 10^7	100	5
2 x 10^7	200	6
3 x 10^7	300	7
4 x 10^7	400	8
5 x 10^7	500	9
6 x 10^7	600	10

Table 1. PE-conjugated Anti-CD31 Antibody Volumes Required for Different Cell Numbers.

Cell number	FACS buffer (µl)	Anti-PE magnetic microbeads (µl)
1 x 10^7	80	20
2 x 10^7	160	40
3 x 10^7	240	60
4 x 10^7	320	80
5 x 10^7	400	100
6 x 10^7	480	120

Table 2. Anti-PE Microbead Volumes Required for Different Cell Numbers.

Discussion

Due to the difficulties in obtaining pure primary TEC cultures, many *in vitro* studies substitute TEC with commercially available EC lines or primary EC such as human umbilical vein EC (HUVEC) [13]. However, these EC populations from normal tissues may only serve as a proxy for TEC which differ markedly from their normal counterparts. For example, TEC are phenotypically and functionally abnormal *in vivo* and some of these abnormalities may be transmittable *in vitro* [14-18]. TEC have aberrant growth, migratory, and differentiation abilities and may coalesce with $CD31^+$ tumor cells to form vessel-like structures [16,19,20]. Studies using either cultured TEC or laser capture micro-dissected tumor vessels have demonstrated that TEC deviate from normal EC in gene expression, cytogenetic, and epigenetic profiles [12,18,21-23]. Despite deepening our understanding of tumor angiogenesis over the past few decades, very few functional and mechanistic studies using cultured primary TEC are available.

EC were isolated from human umbilical veins in the early 1970s [24]. Subsequent isolation and culturing of microvascular EC from other human and mouse tissues has provided a powerful tool for investigating endothelial functions in both health and disease [25-28]. Most EC isolation protocols involve three major steps: obtaining a single-cell suspension after mechanical dissociation or enzymatic digestion, labeling EC with an endothelial-specific antibody that is conjugated to either a fluorophore or magnetic microbeads, and enriching the EC population using cell sorting or magnetic columns. However, isolation of mouse EC, especially from tumors, has proven difficult due to the low yield of viable EC and frequent contamination of other cell types including tumor cells and fibroblasts. In addition, *in vitro* phenotypic drift of EC into mesenchymal-like cells (endothelial-to-mesenchymal transition, EndMT) poses an additional challenge for long-term culturing of EC from normal tissues, tumors, and reprogrammed precursors.

The major challenge during TEC isolation is contamination by tumor cells, which can easily outcompete the slower-growing EC colonies that typically appear after ~7 - 10 days in culture. In addition, a commonly used selection marker for isolating EC is CD31 which is abundantly expressed in the endothelium but also marks hematopoietic cells and in some cases tumor cell subpopulations [20,30]. Furthermore, while enriching for EC, CD31-mediated immunomagnetic column selection cannot remove every single contaminating cell that can eventually take over EC cultures after a few passages. By adding a cloning-ring step, one is able to select and expand pure clonally-derived EC populations that are free of these contaminating cell types. A second challenge is the long-term maintenance of pure EC without promotion of EndMT. EndMT occurs during development, may be recapitulated during vascular dysfunction, and is common in cultured EC (especially in the presence of TGFβ) [31-33]. To minimize EndMT, remove contaminating cells that may act as a source TGFβ, keep cultures at high densities, and maintain high concentrations of bFGF in the media at all times. As we have shown recently, bFGF is essential to preserve EC specification as it antagonizes TGFβ-driven expression of smooth muscle actin (an EndMT marker) [34].

Using this methodology, isolation of EC from a “fate-mapped” reporter mice was also shown. These mice are particularly useful in studies where intravital imaging is needed [35]. Although some labeling of hematopoietic cells may occur in the $Cdh5^{cre}$:$ZsGreen^{l/s/l}$ mice used here, this model provides a relatively rapid and easy method for generating fluorescent EC *in vivo*. An alternative EC lineage-labeling model is a tamoxifen-inducible mouse ($Cdh5^{cre/ERT2}$) crossed with a floxed reporter mouse strain ($ZsGreen^{l/s/l}$) that will have EC faithfully and irreversibly marked [36,37]. Fluorescent EC from the inducible *cre* mice may also be purified using FACS or using the methodology we describe herein.

EC are heterogeneous across different vascular beds and “intra-vessel” heterogeneity may also exist among EC within the same vessel [38,39]. In addition, a hierarchy of EC with different proliferative potential has been observed in EC populations isolated from veins, and a small fraction of clonal EC can be passaged beyond 40 population doublings [40]. Therefore, limitations of the described method here include: 1) possible selection

of these highly proliferative EC residing in the vessel wall; and 2) enrichment of only one specific vascular subtype derived from arteries, veins, or capillaries, which may produce clones that do not completely represent the total EC population *in vivo*. Nevertheless, as multiple clonal subpopulations can be obtained from one isolation procedure, this method can be useful for functional and genetic analyses of EC heterogeneity that exists within tumors and other tissues [12]. Taken together, described here is an EC isolation method that produces pure EC colonies devoid of contaminating non-EC. The isolated cells should be useful for *in vitro* functional studies, genome-wide expression profiling, and molecular characterization of new pathways that control tumor angiogenesis.

Disclosures

The authors have nothing to disclose.

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