Journal of Visualized Experiments

Directed Differentiation of Hemogenic Endothelial Cells from Human Pluripotent Stem Cells

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE62391R1
Full Title:	Directed Differentiation of Hemogenic Endothelial Cells from Human Pluripotent Stem Cells
Corresponding Author:	Karen Hirschi, Ph.D. University of Virginia Charlottesville, Virginia UNITED STATES
Corresponding Author's Institution:	University of Virginia
Corresponding Author E-Mail:	kkh4yy@virginia.edu
Order of Authors:	Elizabeth Nelson
	Jingyao Qiu
	Nicholas Chavkin
	Karen Hirschi, Ph.D.
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please specify the section of the submitted manuscript.	Developmental Biology
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Charlottesville, VA USA
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	
Please indicate whether this article will be Standard Access or Open Access.	Open Access ($3900)

TITLE:

Directed Differentiation of Hemogenic Endothelial Cells from Human Pluripotent Stem Cells

AUTHORS AND AFFILIATIONS:

Elizabeth A. Nelson[1–2], Jingyao Qiu[3–4], Nicholas E. Chavkin[1–2], Karen K. Hirschi[1–5]

[1]Department of Cell Biology, University of Virginia, Charlottesville, VA, USA
[2]Cardiovascular Research Center, University of Virginia, Charlottesville, VA, USA
[3]Department of Medicine, Yale University School of Medicine, New Haven, CT, USA
[4]Department of Genetics, Yale University School of Medicine, New Haven, CT, USA
[5]Yale Cardiovascular Research Center, Yale University School of Medicine, New Haven, CT, USA

Email addresses of co-authors:

Elizabeth A. Nelson	(en2b@virginia.edu)
Jingyao Qiu	(jingyao.qiu@yale.edu)
Nicholas W. Chavkin	(nwc6qu@virginia.edu)
Karen Hirschi	(kkh4yy@virginia.edu)

Corresponding author:

Karen Hirschi	(kkh4yy@virginia.edu)

KEYWORDS:

pluripotent human stem cells, human endothelial cells, hemogenic endothelial cells, arterial endothelial cells, cell culture protocol, directed differentiation

SUMMARY:

Presented here is a simple protocol for the directed differentiation of hemogenic endothelial cells from human pluripotent stem cells in approximately 1 week.

ABSTRACT:

Blood vessels are ubiquitously distributed within all tissues of the body and perform diverse functions. Thus, derivation of mature vascular endothelial cells, which line blood vessel lumens, from human pluripotent stem cells is crucial for a multitude of tissue engineering and regeneration applications. In vivo, primordial endothelial cells are derived from the mesodermal lineage and are specified toward specific subtypes, including arterial, venous, capillary, hemogenic, and lymphatic. Hemogenic endothelial cells are of particular interest because, during development, they give rise to hematopoietic stem and progenitor cells, which then generate all blood lineages throughout life. Thus, creating a system to generate hemogenic endothelial cells in vitro would provide an opportunity to study endothelial-to-hematopoietic transition, and may lead to ex vivo production of human blood products and reduced reliance on human donors. While several protocols exist for the derivation of progenitor and primordial endothelial cells, generation of well-characterized hemogenic endothelial cells from human stem cells has not been described. Here, a method for the derivation of hemogenic endothelial cells from human embryonic stem cells in approximately 1 week is presented: a differentiation protocol with

primitive streak cells formed in response to GSK3β inhibitor (CHIR99021), then mesoderm lineage induction mediated by bFGF, followed by primordial endothelial cell development promoted by BMP4 and VEGF-A, and finally hemogenic endothelial cell specification induced by retinoic acid. This protocol yields a well-defined population of hemogenic endothelial cells that can be used to further understand their molecular regulation and endothelial-to-hematopoietic transition, which has the potential to be applied to downstream therapeutic applications.

INTRODUCTION:

Endothelial cells (ECs) are a heterogeneous population of cells that perform multiple functions throughout the human body and in engineered tissues. In addition to supporting and regulating other cell types (i.e., cardiomyocytes[1], osteoblastic cells[2]), these functions include forming a selective barrier between blood and tissues and assisting in tissue formation[3]. Differentiation of mature ECs during normal development requires diverse signaling pathways. Primordial ECs are derived from mesoderm progenitors, and are then specified toward mature arterial, venous, capillary and lymphatic phenotypes[4]. Additionally, a small subset of ECs in the extraembryonic yolk sac and embryonic Aorta-Gonad-Mesonephros (AGM) region are also specified to become hemogenic ECs, which give rise to hematopoietic stem and progenitor cells (HSPCs) that migrate to the fetal liver and fetal bone marrow, where they remain postnatally and generate all blood cell types throughout life[4]. The diverse range of EC phenotypes is essential for all tissue development and maintenance.

Thus, ECs and their derivatives are critical components of studies aimed at modeling, and elucidating mechanisms of, human development and/or disease, as well as regenerative medicine and tissue engineering applications[5–8]. However, the main limitation for these types of studies is the lack of availability of primary human ECs in the quantity required. It has been estimated that a minimum of 3 x 10^8 ECs would be required for the majority of therapeutic applications[6]. To solve this problem, the use of human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) has been proposed due to their diverse lineage potential and their ability to generate large numbers of progeny[6,9].

Indeed, the usefulness of cells derived from hESCs or hiPSCs has been demonstrated in multiple studies focused on disease modeling and drug screening[10–12]. Organ-on-a-Chip (OOC) technology has been used to more faithfully recapitulate the physiology of the human body by integrating cells and tissues into three-dimensional scaffolds. Furthermore, connection of multiple individual OOCs (a so-called body- or human-on-a-chip, BOC/HOC) can be accomplished via microfluidics to allow for crosstalk between the organs of interest[13–15]. Supporting tissues, such as the vasculature, are critical components of OOCs and BOC/HOCs; incorporating vasculature allows for the transport of nutrients, oxygen, and paracrine factors throughout the tissues, thereby promoting the requisite tissue-specific microenvironment[3,12]. Thus, methods for deriving mature human ECs, such as arterial, venous, lymphatic, and hemogenic ECs, are crucial to advancing these tissue engineering approaches.

Multiple protocols have been published detailing steps for the derivation of human primordial or progenitor ECs from hESCs or hiPSCs[5,16–26]. Many of these protocols rely on embryoid body (EB)

formation or co-culture of ESCs/iPSCs with a murine feeder layer of stromal cells. These strategies tend to be difficult and time consuming, with low EC yields and/or contamination of human ECs with murine cells. Protocols that rely strictly on 2D culture without the use of stromal cells often require long inductions, utilize complex combinations of growth factors and/or inhibitors for induction, have extended expansion periods following cell separation, or a combination of these factors. Advancing knowledge of signaling pathways and factors involved in the derivation of mature EC types in vivo provides the groundwork for a straightforward and robust in vitro differentiation protocol.

Previously, key roles for Notch and Retinoic Acid (RA) signaling pathways in the specification of murine arterial and hemogenic ECs, respectively, during development were identified. The Notch signaling pathway plays multiple roles in the specification and maintenance of the arterial EC phenotype. Work using the murine retinal vascularization model identified a pathway in which fluid shear stress induces a Notch-Cx37-p27 signaling axis, promoting G1 cell cycle arrest, which enables arterial EC specification[27]. Cell cycle states have been hypothesized to play a role in cell fate decisions by providing distinct windows of opportunity in which cells are receptive to certain signals that can induce gene expression and phenotypic changes[28]. This Notch-mediated G1 arrest enabled the expression of genes enriched in arterial ECs, including ephrinB2, Cx40, DLL4, Notch1, and Notch 4 (reviewed in[29,30]). It has also been shown that hemogenic EC specification is promoted in vivo via RA signaling[31,32]. Additional studies identified that, downstream of RA signaling, expression of c-Kit and Notch upregulate p27, which enables hemogenic specification in the murine yolk sac and AGM[33]. Murine hemogenic ECs can be minimally identified by expression of both endothelial (i.e., CD31, KDR) and hematopoietic (i.e., c-Kit, CD34) markers[4]. Finally, hemogenic ECs undergo an endothelial-to-hematopoietic transition (EHT) to form HSPCs, which can give rise to all blood cell types[4,34,35].

Recent work tested whether this same signaling hierarchy can promote human hemogenic EC specification. To do so, a serum- and feeder-free 2D culture protocol to derive hemogenic ECs from hESCs was developed, and these hemogenic ECs were characterized on a single cell level as $CD31^+$ KDR^+ $c\text{-}Kit^+$ $CD34^+$ $VE\text{-}Cadherin^-$ $CD45^-$. This study also took advantage of the Fluorescent Ubiquitination Cell Cycle Indicator (FUCCI) reporter, which identifies different cell cycle states, using H9-hESCs that express the FUCCI reporter construct (H9-FUCCI-hESC)[36]. In studies with these cells, it was demonstrated that RA promotes early G1 cell cycle arrest in ECs, and early G1 state enables hemogenic specification in vitro[37]. Herein, a detailed protocol for the differentiation of these human hemogenic endothelial cells and assays confirming their identity are provided. This straightforward method provides a useful means of generating this specialized subset of ECs for future studies of mechanisms of human blood cell development.

PROTOCOL:

1. Reagents and reagent preparation

NOTE: A list of reagents is provided in **Table of Materials**.

1.1. Obtain the human pluripotent stem cell lines: H1-hESC, H9-Fucci-hESC.

NOTE: The generation of hemogenic ECs may be more efficient in the H1 cell line.

1.2. Prepare matrix protein stocks: Aliquot the matrix protein into pre-chilled 1.5 mL tubes (on ice) so that each tube contains 1 mg of matrix protein. 1 mg of matrix protein is enough to coat all wells of two 6-well plates (12 wells total). Store the aliquots at -20 °C until use.

NOTE: Perform all the steps involving matrix protein on ice or at 4 °C. Thaw the frozen stock vial of matrix protein on ice at 4 °C overnight. Once thawed, swirl the vial to ensure the contents are mixed. Prechill 1.5 mL microcentrifuge tubes for at least 1 h at -20 °C and transfer to ice immediately prior to aliquoting.

1.3. Prepare matrix protein-coated plates: Thaw matrix protein aliquots on ice at 4 °C. Add 12.5 mL of ice-cold DMEM:F12 to a prechilled conical tube on ice. Utilizing pre-chilled pipette tips, transfer one aliquot (1 mg) of matrix protein to the conical tube and mix well by pipetting up and down. Aliquot 1 mL of the diluted matrix protein to each well of pre-chilled 6-well plates (on ice) using a pre-chilled serologic pipette. Swirl and rock the plates so that the entire well is coated evenly. Incubate the plates for a minimum of 30 min at room temperature to allow the matrix protein to solidify. Wrap the plates with parafilm and store at 4 °C until use. Use matrix protein-coated plates within 2 weeks of preparation.

NOTE: Use the matrix protein-coated 6-well plates for routine passaging of cells and differentiation to primordial and hemogenic endothelial cells. Perform all the steps on ice. Pre-chill 6-well plates, pipette tips, serologic pipettes, and conical tubes prior to use for at least 1 h at -20 °C. Transfer these items to ice when ready to use.

1.4. Prepare the base differentiation medium by adding 5 mL of PFHM to 100 mL of pluripotent stem cell differentiation medium. Store at 4 °C.

1.5. Prepare 0.1% BSA-PBS by dissolving 0.1 g BSA in 100 mL PBS. Filter sterilize BSA-PBS by passing through a 0.22 μm filter and store at 4 °C.

1.6. Prepare 5 mM HCl by diluting stock HCl (12 M) 1:2400 with water. Adjust the pH to 3.0 utilizing NaOH. Filter sterilize the solution by passing through a 0.22 μm filter and store at 4 °C.

1.7. Prepare bFGF, BMP4, VEGF-A, Retinoic Acid (RA), and DLL4 stocks.

1.7.1. bFGF: Reconstitute the lyophilized powder to 100 μg/mL in 0.1% BSA-PBS. Aliquot and store at -20 °C. Use bFGF stocks within 3 months of preparation. Thaw the aliquots immediately prior to use.

1.7.2. BMP4: Reconstitute the lyophilized powder to 1 mg/mL in 5mM HCl, pH 3.0. Further dilute to 50 μg/mL in 0.1% BSA-PBS. Aliquot and store at -20 °C. Use the BMP4 stocks within 3 months of preparation. Thaw the aliquots immediately prior to use.

1.7.3. VEGF-A: Reconstitute the lyophilized powder to 1 mg/mL in dH_2O. Further dilute to 100 μg/mL in 0.1% BSA-PBS. Aliquot and store at -20 °C. Use the VEGF-A stocks within 3 months of preparation. Thaw the aliquots immediately prior to use.

1.7.4. RA: Reconstitute the lyophilized powder to 100 mM in DMSO. Aliquot and store at -80 °C. Use the RA stocks within 1 month of preparation. Thaw the aliquots immediately prior to use.

NOTE: Protect RA stocks from light.

1.7.5. DLL4: Reconstitute the lyophilized powder to 1 mg/mL in PBS. Aliquot and store at -20 °C. Use the DLL4 stocks within 12 months of preparation. Thaw the aliquots immediately prior to use.

1.8. Prepare the endothelial cell differentiation medium immediately prior to use by diluting VEGF-A and BMP4 in base differentiation medium (step 1.4) so that the final concentrations are 25 ng/mL and 50 ng/mL, respectively.

1.9. Prepare the working RA immediately prior to use by diluting 100 mM stock 1:1,000 in DMSO to 100 μM.

NOTE: Protect working RA from light.

1.10. Prepare the hemogenic endothelial cell differentiation medium immediately prior to use by diluting VEGF-A, BMP4, and working RA in base differentiation medium (step 1.4) so that the final concentrations are 25 ng/mL, 50 ng/mL, and 0.5 μM, respectively.

1.11. Prepare the antibody staining buffer by making HBSS containing 10% FBS and supplement with 1:500 diluted antimicrobial reagent. Sterile filter the buffer and use immediately.

1.12. Prepare the cell sorting buffer by making HBSS containing 1% FBS and supplement with 1:500 diluted antimicrobial reagent. Sterile filter the buffer and use immediately.

1.13. Prepare 1 mg/mL fibronectin stocks by adding 5 mL of sterile water to 5 mg lyophilized fibronectin. Aliquot and store at -20 °C. Use the fibronectin stocks prior to the expiry date on the lyophilized product label. Thaw the aliquots immediately prior to use.

1.14. Prepare the fibronectin-coated 35 mm dishes. Dilute the fibronectin stocks (1 mg/mL) to 4 μg/mL in sterile water. Add 1 mL of this fibronectin coating solution to each dish and incubate at 37 °C for 30 min to 1 h. Use the dishes immediately following coating.

1.15. Prepare 3 or 4 mL aliquots of methylcellulose-based medium following the manufacturer's instructions. Store the aliquots at -20 °C until use. Use the methylcellulose-based medium aliquots prior to the expiry date indicated on the stock product label. Thaw the aliquots immediately prior to use.

1.16. Prepare the DLL4 coating solution by diluting recombinant human DLL4 stock to a final concentration of 10 μg/mL in PBS.

1.17. Prepare and store the endothelial cell growth medium according to the manufacturer's instructions.

1.18. Prepare the flow cytometry analysis buffer by making PBS containing 0.1% BSA. Sterile filter the buffer and store at 4 °C until use.

2. Cell culture and passaging of hESCs

2.1. Allow the matrix protein-coated plates, stem cell growth medium, and DMEM:F12 to warm to room temperature.

2.2. Grow the hESC cell lines in stem cell growth medium (2 mL/well) on matrix protein-coated 6-well plates in a 37 °C, 5% CO_2 incubator.

2.3. Check the cells daily and remove the differentiated cells as necessary by gently scraping them off the plate using a p200 pipette tip.

NOTE: Differentiated cells will appear on the periphery of colonies. Refer to the stem cell growth medium product manual for examples of differentiated cells in culture.

2.4. Passage the cells once they reach 70%–80% confluency. To passage cells, perform the following steps.

NOTE: Cells should be split prior to reaching 70%–80% confluency if increased differentiation occurs.

2.4.1. Remove the medium above the cells and gently wash with 1 mL DMEM:F12 per well.

2.4.2. Add 1 mL of DMEM:F12 per well.

2.4.3. Add 160 μL/mL of Dispase per well and incubate the cells at 37 °C in a 5% CO_2 incubator for 45 min.

2.4.4. Following Dispase incubation, add an additional 1 mL of DMEM:F12 per well and gently pipette to lift the cells.

NOTE: Avoid dissociating the cells into a single-cell suspension. Passage the cells as small clumps.

2.4.5. Transfer the cells to a conical tube containing 12 mL of DMEM:F12 and allow the cells to settle by gravity (~5–10 min).

2.4.6. Remove the supernatant and gently resuspend the pellet in 0.5 mL of stem cell growth medium per well of lifted cells to obtain small clumps of cells.

NOTE: Avoid dissociating the cells into a single-cell suspension. Passage the cells as small clumps.

2.4.7. Aspirate the matrix protein coating solution from the wells of the prepared matrix protein-coated plate and add 1.5 mL of the stem cell growth medium per well.

2.4.8. Add the desired volume of resuspended cells (step 2.4.6) to each well of the prepared matrix protein-coated plate.

2.4.9. Incubate the plate in a 37 °C, 5% CO_2 incubator. Change the medium every 24 h to fresh stem cell growth medium.

3. Differentiation of hESCs to primordial endothelial cells

3.1. Day -1: Culture and passage the cells as described above in section 2. Seed the cells (step 2.4.6) in small clumps (~50 μm) at a density of approximately 2 clumps per square centimeter[38].

NOTE: Evaluate the seed density and refine empirically if necessary.

3.2. Day 0: 24 h after seeding the cells, aspirate the medium from each well and gently wash the cells with 1 mL DMEM:F12 per well. Add 1 mL of the base differentiation medium containing 5 μM GSK3i (CHIR99021, added fresh) to each well and incubate for 24 h (37 °C, 5% CO_2).

NOTE: For all the wash steps, slowly add the indicated wash media to the plate by pipetting against the wall of the plate well. Gently swirl the plate so that the entire surface of the well is covered by wash media. Slightly tilt the plate so that the wash media pools at the six o-clock position and carefully aspirate the wash media.

3.3. Day 1: Aspirate the medium from each well and gently wash the cells with 1 mL DMEM:F12 per well. Add 1 mL of the base differentiation medium containing 50 ng/mL bFGF (added fresh, 1:2,000 from frozen stock) to each well and incubate 24 h (37 °C, 5% CO_2).

3.4. Day 2: Aspirate the medium from each well and gently wash the cells with 1 mL of DMEM:F12 per well. Add 1 mL of the endothelial cell differentiation medium to each well and incubate 24 h (37 °C, 5% CO_2).

3.5. Day 3: Replace the medium above the cells with 1 mL of freshly prepared endothelial cell differentiation medium per well and incubate 24 h (37 °C, 5% CO_2).

3.6. Day 4: Replace the medium above the cells with 1 mL of freshly prepared endothelial cell differentiation medium per well and incubate 24 h (37 °C, 5% CO_2).

3.7. Day 5: FACS purify the cells to assess EC phenotype (sections 4–5) or keep in culture and differentiate towards hemogenic endothelial cells (section 6).

4. FACS purification of primordial endothelial cells

4.1. Aspirate the medium above the cells and gently wash once with 1 mL of DMEM:F12 per well.

4.2. Add 1 mL of cell detachment solution per well and incubate the cells for 12 min in a 37 °C, 5% CO_2 incubator, or until cells have dissociated.

4.3. Transfer the dissociated cells to a conical tube containing 12 mL of DMEM:F12 and pellet by centrifugation for 5 min, 1,000 x *g*.

4.4. Remove the supernatant and resuspend the pellet in 12 mL of DMEM:F12 to wash.

4.5. Pellet the cells by centrifugation for 5 min, 1,000 x *g*.

4.6. Remove the supernatant and resuspend the cell pellet in ice-cold antibody staining buffer and count the cells. Adjust the concentration using ice-cold antibody staining buffer to 1 x 10^5 cells/mL.

4.7. Divide the cells evenly into microcentrifuge tubes on ice, each containing a minimum of 600 μL cells at 1 x 10^5 cells/mL, for antibody staining.

NOTE: Four tubes of cells are required for staining of primordial ECs: unstained control, CD31 single antibody control, CD45 single antibody control, and sample containing both CD31 and CD45 antibodies. Information about antibodies is provided in **Table of Materials**.

4.8. Add antibodies to the tubes containing cells, as appropriate, and incubate on ice and protected from light for 30 min.

4.8.1. Unstained control: Do not add any antibody.

4.8.2. CD31 single antibody control: Add only CD31 antibody.

4.8.3. CD45 single antibody control: Add only CD45 antibody.

4.8.4. Sample: Add both CD31 and CD45 antibodies.

NOTE: The final antibody concentration in the sample, as well as the fluorescent conjugates, should be optimized based on the specific antibodies and cell sorter used.

4.9. Pellet the cells by centrifugation in a 4 °C tabletop microcentrifuge for 5 min at 1,000 x *g*.

4.10. Remove the supernatant and resuspend the cell pellets in 600 µL of ice-cold sorting buffer.

4.11. Strain the samples through the mesh filter caps of 5 mL FACS tubes and store the cells on ice, protected from light, for immediate FACS.

4.12. Obtain primordial endothelial cells ($CD31^+$ $CD45^-$), by performing the following steps.

4.12.1. Identify the CD45-negative cell population and gate ($CD45^-$).

4.12.2. Within ($CD45^-$), identify the CD31-positive ($CD31^+$) cell population and sort cells into 6 mL ice-cold cell sorting buffer. Use these cells for downstream applications (see section 5).

5. Assay to confirm primordial endothelial cell phenotype

5.1. Coat three wells of a 6-well plate with 1 mL/well DLL4 coating solution for 30 min in a 37 °C, 5% CO_2 incubator. As a control, mock-coat the other three wells of the plate with 1 mL/well PBS.

5.2. Aspirate the DLL4 coating solution and PBS, and plate 25,000 sorted primordial endothelial cells (see step 4.12) in 2 mL of endothelial cell growth medium per well.

5.3. Incubate the cells for 24 h in a 37 °C, 5% CO_2 incubator.

5.4. Aspirate the medium above the cells and wash once with 2 mL/well PBS. Analyze the cells by qPCR or flow cytometry (for cells expressing the FUCCI construct).

5.4.1. To analyze the cells by qPCR, perform the following steps.

5.4.1.1. Aspirate the liquid above the cells and isolate the RNA in the cells using an RNA extraction kit according to the manufacturer's protocol.

5.4.1.2. Perform reverse transcription reactions with a reverse transcription master mix according to the manufacturer's protocol.

5.4.1.3. Perform qPCR reactions with a SYBR Green master mix according to the manufacturer's protocol.

NOTE: Primers utilized (EFNB2, GJA5, GJA4, NR2F2, EPHB4, HEY2) are listed in **Table 1**.

5.4.2. To analyze cells expressing the FUCCI construct by flow cytometry, perform the following steps.

5.4.2.1. Aspirate the liquid above the cells and add 500 µL/well 0.25% trypsin-EDTA.

5.4.2.2. Incubate the cells at 37 °C in a 5% CO_2 incubator until lifted, ~5 min.

5.4.2.3. Transfer the cells to a microcentrifuge tube and pellet the cells in a 4 °C microcentrifuge for 5 min at 1,000 x *g*.

5.4.2.4. Aspirate the supernatant and resuspend the pellet in 500 µL of ice-cold flow cytometry analysis buffer.

5.4.2.5. Strain the samples through the mesh filter cap of a 5 mL FACS tube and store the cells on ice, protected from light, for immediate flow cytometry analysis.

5.4.2.6. Analyze the percentage of cells plated on DLL4 vs. PBS control in early G1 (no color), late G1 ($mCherry^+/mVenus^-$), G1/S ($mCherry^+/mVenus^+$), and S/G2/M ($mCherry^-/mVenus^+$).

6. Differentiation of hESCs to hemogenic endothelial cells

NOTE: Differentiate the cells to day 4 primordial ECs, as described above in sections 3.1–3.6.

6.1. Day 5: Aspirate the medium above the cells and gently wash the cells with 1 mL of DMEM:F12 per well. Add 1 mL of freshly prepared hemogenic endothelial cell differentiation medium per well and incubate 24 h (37 °C, 5% CO_2).

6.2. Day 6: Replace the medium above the cells with 1 mL of freshly prepared hemogenic endothelial cell differentiation medium per well and incubate 24 h (37 °C, 5% CO_2).

6.3. Day 7: Replace the medium above the cells with 1 mL of freshly prepared hemogenic endothelial cell differentiation medium per well and incubate 24 h (37 °C, 5% CO_2).

6.4. Day 8: FACS isolate hemogenic endothelial cells (see section 7).

7. FACS-isolation of hemogenic endothelial cells

7.1. Lift and wash the cells as described above in sections 4.1–4.6.

7.2. Divide the cells evenly into microcentrifuge tubes on ice, each containing a minimum of 600 µL cells at 1 x 10^5 cells/mL, for antibody staining.

NOTE: Eight tubes of cells are required for antibody staining of hemogenic ECs: unstained control, CD31 single antibody control, CD45 single antibody control, KDR single antibody control, c-Kit single antibody control, CD34 single antibody control, VE-cadherin single antibody control, and sample containing all six antibodies.

NOTE: Antibody information is provided in **Table of Materials**.

7.3. Add antibodies to the tubes containing cells, as appropriate, and incubate on ice and protected from light for 30 min.

7.3.1. Unstained control: Do not add antibody.

7.3.2. CD31 single antibody control: Add only CD31 antibody.

7.3.3. CD45 single antibody control: Add only CD45 antibody.

7.3.4. KDR single antibody control: Add only KDR antibody.

7.3.5. c-Kit single antibody control: Add only c-Kit.

7.3.6. CD34 single antibody control: Add only CD34 antibody.

7.3.7. VE-cadherin single antibody control: Add only VE-cadherin antibody.

7.3.8. Sample: Add CD31, CD45, KDR, c-Kit, CD34, and VE-cadherin antibodies.

NOTE: The final antibody concentration in the sample, as well as the fluorescent conjugates, should be optimized based on the specific antibodies and cell sorter used.

7.4. Pellet the cells by centrifugation in a 4 °C microcentrifuge for 5 min at 1,000 x *g*.

7.5. Remove the supernatant and resuspend the pellets in 600 µL of ice-cold sorting buffer.

7.6. Strain the samples through the mesh filter cap of 5 mL FACS tubes and store cells on ice, protected from light, for immediate FACS.

7.7. To obtain hemogenic endothelial cells ($CD31^+$ KDR^+ $c\text{-}Kit^+$ $CD34^+$ $VE\text{-}Cadherin^-$ $CD45^-$), perform the following steps.

7.7.1. Identify the $CD45^-$ cell population and gate ($CD45^-$).

7.7.2. Within ($CD45^-$), identify the $CD31^+$ cell population and gate ($CD31^+$).

7.7.3. Within ($CD31^+$), identify the VE-$Cadherin^-$ cell population and gate ($CDH5^-$).

7.7.4. Within ($CDH5^-$), identify the c-Kit^+ cell population and gate (KIT^+).

7.7.5. Within (KIT^+), identify the $CD34^+$ cell population and gate ($CD34^+$).

7.7.6. Within ($CD34^+$), identify and sort the KDR^+ cells into 6 mL ice-cold cell sorting buffer. Use these cells in downstream applications (see section 8).

8. Colony forming unit assay

8.1. Thaw the aliquots of methylcellulose-based medium following the manufacturer's instructions.

NOTE: One 3 mL aliquot is sufficient for two samples and one 4 mL aliquot is sufficient for three samples.

8.2. Count the sorted hemogenic endothelial cells obtained in step 7.7.

8.3. Following the manufacturer's instructions, calculate the volume of sorted cells to add to each methylcellulose-based medium aliquot so that each sample will contain a minimum of 1,000 hemogenic endothelial cells.

NOTE: The number of cells can be adjusted as necessary.

8.4. Add the calculated volume of cells to the methylcellulose-based medium aliquot and vortex thoroughly. Allow the methylcellulose-based medium cultures to sit at room temperature for 10 min, or until the bubbles dissipate.

8.5. Aspirate the fibronectin coating solution from the prepared 35 mm dishes. Following the manufacturer's instructions, dispense 1 mL of methylcellulose-based medium culture per 35 mm dish. Gently rotate the dish to evenly distribute the culture.

8.6. Place these 35 mm dishes, as well as two additional 35 mm dishes filled with sterile water, within a 15 cm tissue culture dish.

8.7. Incubate the cultures in a 37 °C, 5% CO_2 incubator, and observe the cultures on days 8 and 14.

8.7.1. On day 8, count the CFU-E and BFU-E colonies.

8.7.2. On day 14, count the CFU-GM and CFU-GEMM colonies.

NOTE: Refer to the methylcellulose-based medium manual for morphological identification of colony types.

REPRESENTATIVE RESULTS:

A schematic outlining the specification of primordial ECs and hemogenic ECs from hESCs, and a representative image of cells 24 h after plating are shown in **Figure 1**. Following specification, primordial ECs and hemogenic ECs are FACS purified on days 5 and 8, respectively. Primordial ECs are defined as $CD31^+$ $CD45^-$ and hemogenic ECs are defined as $CD31^+$ KDR^+ c-Kit^+ $CD34^+$ VE-$Cadherin^-$ $CD45^-$. A representative flow cytometric gating strategy for primordial ECs and hemogenic EC purification is shown in **Figure 2**. Cells are initially gated based on the negative expression of CD45 and positive expression of CD31 to obtain purified primordial ECs (**Figure 2A**). To obtain purified hemogenic ECs, cells are initially gated as in **Figure 2A** and are then further purified based on positive or negative expression of (in order) VE-Cadherin (CDH5), c-Kit (KIT), CD34, and KDR (**Figure 2B**).

To assess the potential of H9-Fucci-hES-derived primordial $CD31^+$ $CD45^-$ endothelial cells, isolated via FACS at day 5 of differentiation (protocol section 4), to give rise to endothelial subtypes, the purified cells are seeded onto plates coated with either the Notch ligand DLL4 to induce arterial specification, or PBS (control), and incubated for 24 h in a 37 °C, 5% CO_2 incubator. The cells are then lysed with RNA lysis buffer, the RNA extracted, and reverse transcribed to cDNA, and qPCR is performed to compare gene expression levels in the DLL4-treated vs. control cells. As expected, endothelial cells grown on DLL4 have increased expression of the Notch-responsive gene HEY2, as well as the arterial-associated genes EFNB2, GJA5, and GJA4. Additionally, these cells also have decreased expression of the venous transcription factor NR2F2 (**Figure 3A**). Alternatively, to determine the effect of DLL4 treatment on cell cycle state, the FACS purified $CD31^+$ $CD45^-$ cells are incubated on plates coated with either DLL4 or PBS for 24 h, lifted, and analyzed based on the expression of hCdt1(30/120)-mCherry (late G1) and hGem(1/110)-mVenus (S/G2/M). Consistent with the findings that Notch signaling promotes late G1 cell cycle arrest[27], a greater percentage of primordial ECs are arrested in late G1 after growth in the presence of DLL4, compared to control cells (**Figure 3B,C**).

To verify the hematopoietic potential of hemogenic endothelial cells, the $CD31^+$ KDR^+ c-Kit^+ $CD34^+$ VE-$Cadherin^-$ $CD45^-$ endothelial cells isolated through FACS (methods in section 7) are seeded in a methylcellulose-based medium formulated for growth of hematopoietic progenitor cells in colony-forming unit (CFU) assays and are allowed to grow for 14 days. CFU-E erythroid colonies and blast-forming unit (BFU)-E erythroid colonies are counted on day 8 (**Figure 4A,B**), and CFU-GM granulocyte/macrophage and GFU-GEMM (granulocyte, erythroid, macrophage, and megakaryocyte) multipotent hematopoietic progenitor colonies are counted on day 14 (**Figure 4C and D**). Per 1,000 hemogenic ECs plated, approximately 20 CFU are generated **(Figure 4F)**. Cells with endothelial cell morphology can also be seen in the cultures (**Figure 4E)**; these are the hemogenic endothelial cells that give rise to multi-lineage hematopoietic progenitors on a single cell level[37].

FIGURE AND TABLE LEGENDS:

Figure 1: Protocol for the specification of primordial and hemogenic ECs. (**A**) Schematic diagram of the differentiation protocol. Embryonic stem cells are plated on Day -1 on matrix protein-coated plates and are allowed to attach overnight. The cells are then treated on Days 0 and 1 with GSK3i inhibitor (CHIR99021) and bFGF, respectively, to induce primitive streak and mesoderm specification, respectively. Beginning on Day 2, the cells are treated with a combination of BMP4 and VEGF-A to promote primordial EC development. Primordial ECs (red circle) are FACS purified on Day 5. Alternatively, to generate hemogenic ECs, the medium above the primordial ECs is exchanged on Day 5 to fresh hemogenic differentiation medium containing BMP4, VEGF-A, and RA. This medium is replaced daily until Day 8, when hemogenic ECs (red star) are FACS purified. (**B**) Colonies on Day 0 of differentiation, scale bar= 100 μm. Panel A has been modified from Qiu et al.[37] with permission from Elsevier.

Figure 2: FACS analysis of hemogenic ECs derived from hESCs. Representative flow cytometric gating strategy for the purification of (**A**) primordial ECs and (**B**) hemogenic ECs. Note that since hemogenic ECs are derived from primordial ECs, the flow cytometry gating strategy for CD45 and CD31 is identical for both cell populations. Shown in the top row of each panel (sample) are cells that were differentiated to hemogenic ECs as described in protocol section 6 and stained with antibodies as described in protocol section 7.3.8. Shown in the bottom row of each panel (control) are unstained cells that were differentiated for 8 days without RA treatment.

Figure 3: DLL4-induction of H9-Fucci CD31$^+$ CD45$^-$ primordial endothelial cells results in late G1 arrest and increased arterial gene expression. (**A**) DLL4 treatment of CD31$^+$ CD45$^-$ H9-hESC-derived primordial endothelial cells expressing the Fucci construct purified at day 5 of differentiation results in increased expression of arterial genes (i–iii) and the Notch responsive gene Hey2 (v), which is accompanied by a concomitant decrease in the expression of the venous gene NR2F2 (iv). (**B**) Representative FACS plots showing the cell cycle state distribution of 5,000 H9-Fucci-derived CD31$^+$ CD45$^-$ grown on PBS (control) or DLL4 for 24 h. (**C**) DLL4 induction results in a 15% increase in cells in the late G1 phase compared to control. Data is the average of triplicate samples from the same experiment shown in panel (**B**). Error bars indicate standard deviation.

Figure 4: Analysis of hematopoietic potential of hemogenic ECs derived from hESCs. Representative images showing morphology of H1-hESC derived (**A**) CFU-E erythroid colony (scale bar = 35 μm), (**B**) BFU-E erythroid colony (scale bar = 75 μm), (**C**) CFU-GM granulocyte/macrophage colony, (**D**) CFU-GEMM multipotent hematopoietic progenitor colony, and (**E**) CFU-GM granulocyte/macrophage colony with underlying endothelial cells (ECs) (red arrows). (**F**) number and distribution of CFUs formed per 1,000 plated hemogenic endothelial cells. Scale bar = 100 μm in (**C–E**). Additional images of CFUs differentiated using this protocol can be found in Qiu et al.[37]. Panel F has been modified from Qiu et al.[37] with permission from Elsevier.

Table 1: qPCR primer information

DISCUSSION:

Herein, the steps for producing hemogenic endothelial cells from human embryonic stem cells in approximately 1 week using a murine feeder- and serum-free 2D culture system (**Figure 1**) are outlined. This protocol expands on a method described by Sriram et al. (2015) to obtain primordial ECs[38]. The primordial nature and specification potential of the $CD31^+$ $CD45^-$ ECs is demonstrated by culturing these cells on DLL4-coated plates and observing gene expression changes consistent with arterial specification (**Figure 3**). Additionally, the gain of arterial identity is associated with late G1 cell cycle arrest (**Figure 3**), which is consistent with previous studies[27]. After culturing primordial ECs for an additional 3 days in the presence of 0.5 μM RA, 25 ng/mL BMP4, and 50 ng/mL VEGF-A, it was possible to generate and FACS-isolate hemogenic ECs (**Figure 2**) that are capable of giving rise to CFU-erythroid, BFU-erythroid, CFU-granulocyte/macrophage, and CFU-granulocyte, erythrocyte, macrophage, and megakaryocyte colonies (**Figure 4**). Using this method in a recently published study, gene expression changes over an 8-day time period consistent with loss of pluripotency, primitive streak, and mesoderm induction, acquisition of endothelial cell identity, and finally hematopoietic identity were observed[37]. Furthermore, RA treatment induced early G1 cell cycle arrest to enable hemogenic EC specification[37].

Recently, Ohta et al. (2019) described a protocol for the differentiation of hemogenic ECs from hPSCs[39]. However, the protocol described above offers significant advantages: 1) this method does not require the formation of spheroids; 2) this protocol utilizes a standard 37 °C, 5% CO_2 incubator rather than a hypoxic incubator, eliminating the need for dedicated specialty equipment; and 3) this protocol utilizes only one medium (pluripotent stem cell differentiation medium supplemented with PFHM), a cost-saving advantage, whereas the Ohta protocol requires mediums for induction. Another recently published study by Galat et al. (2017) described a protocol in which CHIR99021 induction was utilized to generate a population of $CD34^+$ hemogenic endothelial cells[40]. These cells also expressed CD31 and were capable of giving rise to endothelial cells when cultured under monolayer conditions or cells expressing myeloid and lymphoid markers after co-culturing with OP9 or OP9-DLL4 cells, respectively, in the presence of additional cytokines. The requirement for additional co-culture could lead to potential contamination of desired cell populations with murine cells. Additionally, although Ohta et al. and Galat et al. utilized a hemogenic induction period that was shorter than the one described here (4 days and 5 days, respectively vs. 8 days), both defined hemogenic ECs as $CD34^+$, whereas this protocol utilized a more stringent definition: $CD31^+$ KDR^+ $c\text{-}Kit^+$ $CD34^+$ $VE\text{-}Cadherin^-$ $CD45^-$. While CD34 is recognized as a marker of hematopoietic cells, it is also expressed by other non-hematopoietic cell types, such as mesenchymal stromal cells and endothelial cells[41]. The definition of hemogenic ECs in this protocol ($CD31^+$ KDR^+ $c\text{-}Kit^+$ $CD34^+$ $VE\text{-}Cadherin^-$ $CD45^-$) is therefore more rigorous and represents a more defined population.

One limitation to the use of hESCs or hiPSCs in therapeutic applications is the large number of cells required, and standard 2D derivation methods are primarily restricted to small-scale differentiations. Utilizing hiPSC lines, Olmer et al. (2018) demonstrated the feasibility of scaling up production of functional $CD31^+$ ECs that expressed both arterial (DLL4) and venous (EPHB4) cell markers utilizing either suspension culture or a stirred-tank bioreactor[6]. Importantly, they

showed that they were able to obtain 1.18 x 10^7 $CD31^+$ ECs that co-express CD34 and KDR from a single flask containing 20 mL suspension culture. In order to obtain the requisite 3 x 10^8 ECs necessary for the majority of therapeutic applications, just over two 500 mL flasks would be required[6]. Future experiments should explore the application of scaling techniques to the protocol presented here for large-scale production of hemogenic ECs.

ACKNOWLEDGMENTS:

This work was partially supported by NIH grants HL128064 and U2EB017103. Further support was provided by CT Innovations 15-RMB-YALE-04 grant.

DISCLOSURES:

The author has nothing to disclose.

REFERENCES:

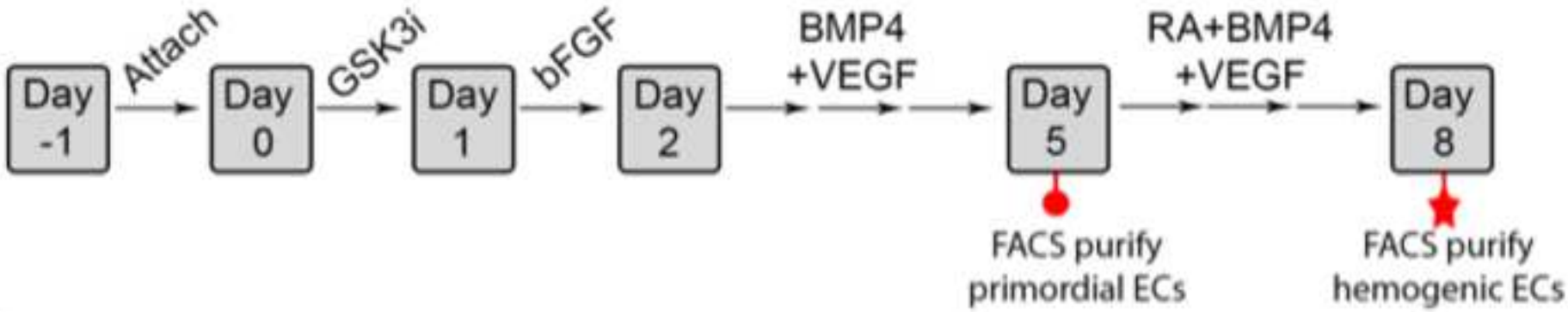
1. Giacomelli, E. et al. Three-dimensional cardiac microtissues composed of cardiomyocytes and endothelial cells co-differentiated from human pluripotent stem cells. *Development*. **144** (6), 1008–1017 (2017).
2. Wu, J., Wu, Z., Xue, Z., Li, H., Liu, J. PHBV/bioglass composite scaffolds with co-cultures of endothelial cells and bone marrow stromal cells improve vascularization and osteogenesis for bone tissue engineering. *RSC Advances*. **7** (36), 22197–22207 (2017).
3. Lee, H., Chung, M., Jeon, N. L. Microvasculature: An essential component for organ-on-chip systems. *MRS Bulletin*. **39** (1), 51–59 (2014).
4. Gritz, E., Hirschi, K. K. Specification and function of hemogenic endothelium during embryogenesis. *Cellular and Molecular Life Sciences*. **73** (8), 1547–1567 (2016).
5. Williams, I. M., Wu, J. C. Generation of endothelial cells from human pluripotent stem cells: Methods, considerations, and applications. *Arteriosclerosis, Thrombosis, and Vascular Biology*. **39** (7), 1317–1329 (2019).
6. Olmer, R. et al. Differentiation of human pluripotent stem cells into functional endothelial cells in scalable suspension culture. *Stem Cell Reports*. **10** (5), 1657–1672 (2018).
7. Cossu, G. et al. Lancet Commission: Stem cells and regenerative medicine. *The Lancet*. **391** (10123), 883–910 (2018).
8. Fox, I. J. et al. Use of differentiated pluripotent stem cells in replacement therapy for treating disease. *Science*. **345** (6199), 1247391 (2014).
9. Mahla, R. S. Stem cells applications in regenerative medicine and disease therapeutics. *International Journal of Cell Biology*. **2016**, 1–24 (2016).
10. Ebert, A. D., Liang, P., Wu, J. C. Induced pluripotent stem cells as a disease modeling and drug screening platform: *Journal of Cardiovascular Pharmacology*. **60** (4), 408–416 (2012).
11. Rowe, R. G., Daley, G. Q. Induced pluripotent stem cells in disease modelling and drug discovery. *Nature Reviews Genetics*. **20** (7), 377–388 (2019).
12. Liu, C., Oikonomopoulos, A., Sayed, N., Wu, J. C. Modeling human diseases with induced pluripotent stem cells: from 2D to 3D and beyond. *Development*. **145** (5), dev156166 (2018).
13. Wnorowski, A., Yang, H., Wu, J. C. Progress, obstacles, and limitations in the use of stem cells in organ-on-a-chip models. *Advanced Drug Delivery Reviews*. **140**, 3–11 (2019).
14. Ramme, A.P. et al. Autologous induced pluripotent stem cell-derived four-organ-chip.

Future Science OA. **5** (8), 1–12 (2019).
15. Ronaldson-Bouchard, K., Vunjak-Novakovic, G. Organs-on-a-Chip: A fast track for engineered human tissues in drug development. *Cell Stem Cell*. **22** (3), 310–324 (2018).
16. Kane, N. M. et al. Derivation of endothelial cells from human embryonic stem cells by directed differentiation: analysis of microrna and angiogenesis in vitro and in vivo. *Arteriosclerosis, Thrombosis, and Vascular Biology*. **30** (7), 1389–1397 (2010).
17. Costa, M. et al. Derivation of endothelial cells from human embryonic stem cells in fully defined medium enables identification of lysophosphatidic acid and platelet activating factor as regulators of eNOS localization. *Stem Cell Research*. **10** (1), 103–117 (2013).
18. Nguyen, M. T. X. et al. Differentiation of human embryonic stem cells to endothelial progenitor cells on laminins in defined and xeno-free systems. *Stem Cell Reports*. **7** (4), 802–816 (2016).
19. Ikuno, T. et al. Efficient and robust differentiation of endothelial cells from human induced pluripotent stem cells via lineage control with VEGF and cyclic AMP. *PLOS One*. **12** (3), e0173271 (2017).
20. Aoki, H. et al. Efficient differentiation and purification of human induced pluripotent stem cell-derived endothelial progenitor cells and expansion with the use of inhibitors of ROCK, TGF-β, and GSK3β. *Heliyon*. **6** (3), e03493 (2020).
21. Lian, X. et al. Efficient differentiation of human pluripotent stem cells to endothelial progenitors via small-molecule activation of Wnt signaling. *Stem Cell Reports*. **3** (5), 804–816 (2014).
22. Orlova, V. V. et al. Generation, expansion and functional analysis of endothelial cells and pericytes derived from human pluripotent stem cells. *Nature Protocols*. **9** (6), 1514–1531 (2014).
23. Kusuma, S., Gerecht, S. Derivation of endothelial cells and pericytes from human pluripotent stem cells. *Human Embryonic Stem Cell Protocols*. Edited by Turksen, K., 213–222, Humana Press. New York, NY (2014).
24. Bao, X., Lian, X., Palecek, S. P. Directed endothelial progenitor differentiation from human pluripotent stem cells via wnt activation under defined conditions. *Methods in Molecular Biology*. **1481**, 183–196 (2016).
25. Xu, M., He, J., Zhang, C., Xu, J., Wang, Y. Strategies for derivation of endothelial lineages from human stem cells. *Stem Cell Research & Therapy*. **10** (1), 200 (2019).
26. Arora, S., Yim, E. K. F., Toh, Y.-C. Environmental specification of pluripotent stem cell derived endothelial cells toward arterial and venous subtypes. *Frontiers in Bioengineering and Biotechnology*. **7**, 143 (2019).
27. Fang, J. S. et al. Shear-induced Notch-Cx37-p27 axis arrests endothelial cell cycle to enable arterial specification. *Nature Communications*. **8** (1), 2149 (2017).
28. Dalton, S. Linking the cell cycle to cell fate decisions. *Trends in Cell Biology*. **25** (10), 592–600 (2015).
29. Wolf, K., Hu, H., Isaji, T., Dardik, A. Molecular identity of arteries, veins, and lymphatics. *Journal of Vascular Surgery*. **69** (1), 253–262 (2019).
30. Rocha, S. F., Adams, R. H. Molecular differentiation and specialization of vascular beds. *Angiogenesis*. **12** (2), 139–147 (2009).
31. Goldie, L. C., Lucitti, J. L., Dickinson, M. E., Hirschi, K. K. Cell signaling directing the formation and function of hemogenic endothelium during murine embryogenesis. *Blood*. **112** (8),

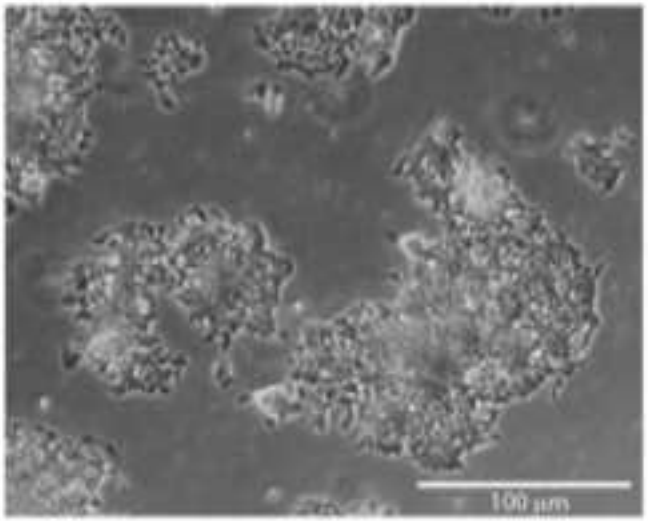
3194–3204 (2008).
32. Chanda, B., Ditadi, A., Iscove, N. N., Keller, G. Retinoic acid signaling is essential for embryonic hematopoietic stem cell development. *Cell*. **155** (1), 215–227 (2013).
33. Marcelo, K. L. et al. Hemogenic endothelial cell specification requires c-kit, notch signaling, and p27-mediated cell-cycle control. *Developmental Cell*. **27** (5), 504–515 (2013).
34. Dejana, E., Hirschi, K. K., Simons, M. The molecular basis of endothelial cell plasticity. *Nature Communications*. **8** (1), 14361 (2017).
35. Ottersbach, K. Endothelial-to-haematopoietic transition: an update on the process of making blood. *Biochemical Society Transactions*. **47** (2), 591–601 (2019).
36. Pauklin, S., Vallier, L. The cell-cycle state of stem cells determines cell fate propensity. *Cell*. **155** (1), 135–147 (2013).
37. Qiu, J., Nordling, S., Vasavada, H. H., Butcher, E. C., Hirschi, K. K. Retinoic acid promotes endothelial cell cycle early g1 state to enable human hemogenic endothelial cell specification. *Cell Reports*. **33** (9), 108465 (2020).
38. Sriram, G., Tan, J. Y., Islam, I., Rufaihah, A. J., Cao, T. Efficient differentiation of human embryonic stem cells to arterial and venous endothelial cells under feeder- and serum-free conditions. *Stem Cell Research & Therapy*. **6** (1), 261 (2015).
39. Ohta, R., Sugimura, R., Niwa, A., Saito, M. K. Hemogenic endothelium differentiation from human pluripotent stem cells in a feeder- and xeno-free defined condition. *Journal of Visualized Experiments: JoVE*. (148), 59823 (2019).
40. Galat, Y. et al. Cytokine-free directed differentiation of human pluripotent stem cells efficiently produces hemogenic endothelium with lymphoid potential. *Stem Cell Research & Therapy*. **8** (1), 67 (2017).
41. Sidney, L. E., Branch, M. J., Dunphy, S. E., Dua, H. S., Hopkinson, A. Concise review: Evidence for CD34 as a common marker for diverse progenitors. *Stem Cells (Dayton, Ohio)*. **32** (6), 1380–1389 (2014).

Figure 1

A



B



Day 0

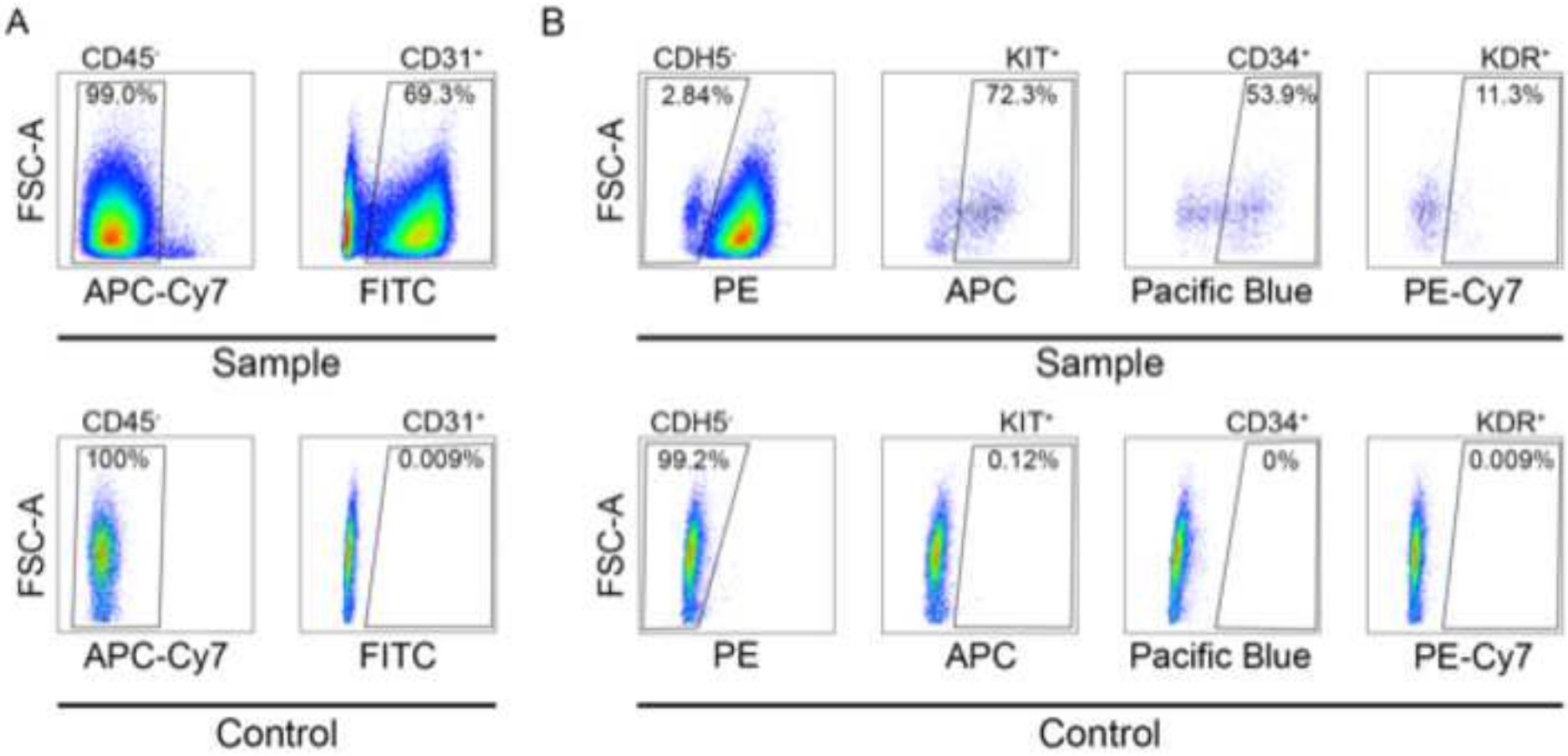


Figure 3

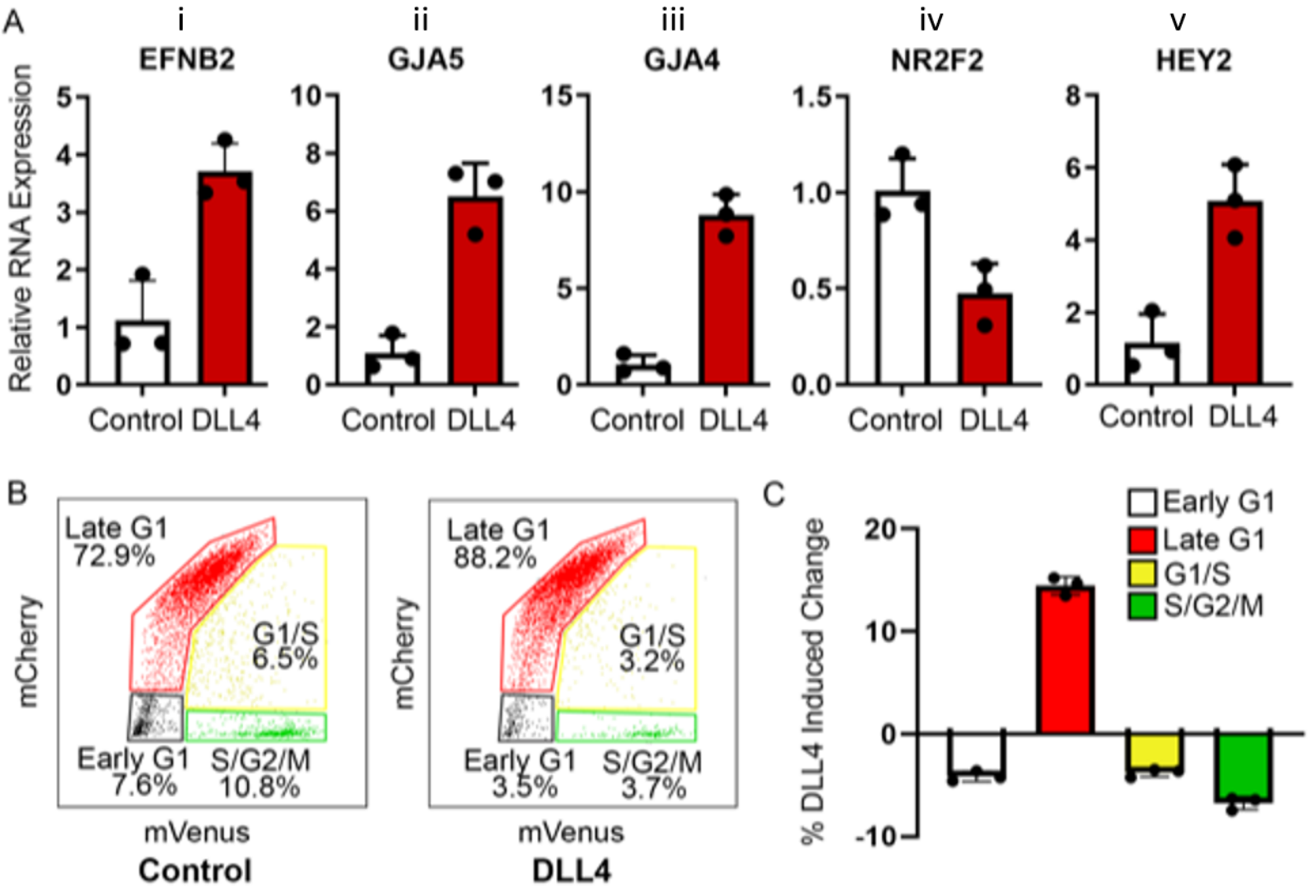
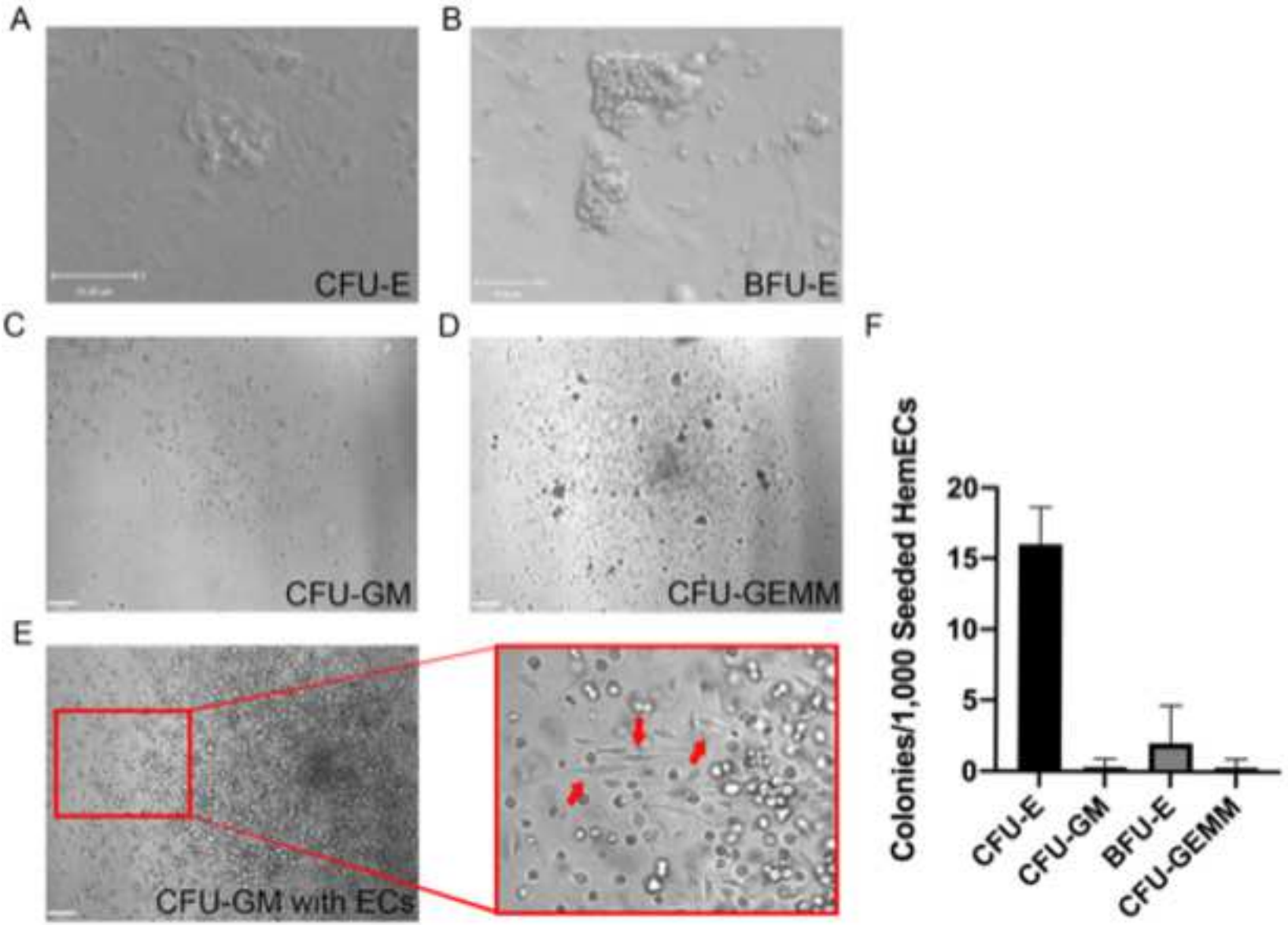


Figure 4



Table

Name	Forward	Reverse
EFNB2	TATGCAGAACTGCGATTTCCAA	TGGGTATAGTACCAGTCCTTGTC
EPHB4	CGCACCTACGAAGTGTGTGA	GTCCGCATCGCTCTCATAGTA
GJA5	CCGTGGTAGGCAAGGTCTG	ATCACACCGGAAATCAGCCTG
GJA4	ACACCCACCCTGGTCTAC	CACTGGCGACATAGGTGCC
HEY2	GCCCGCCCTTGTCAGTATC	CCAGGGTCGGTAAGGTTTATTG
NR2F2	GGACCACATACGGATCTTCCAA	ACATCAGACAGACCACAGGCAT

Name of Material/ Equipment	Brand Name	Company	Catalog Number
15 cm dishes		Corning	430599
35 mm dishes		Corning	430165
6-well plates		Corning	3516
Antimicrobial reagent	Normocin	Invitrogen	ant-nr-1
bFGF		R&D systems	233-FB-025
BMP4		BioLegend	595202
Bovine Serum Albumin (BSA)		Fisher Scientific	BP1600-1
Cell Detatchment Solution	Accutase	Stemcell Technologies	7920
Dimethyl Sulfoxide (DMSO)		Sigma Aldrich	D2650-100mL
Dispase		Stemcell Technologies	7913
DLL4		R&D systems	1506-D4/CF
DMEM:F12		Gibco	11320-033
Dulbecco's Phosphate Buffered Saline (PBS)		Gibco	14190144
Endothelial cell growth medium	EGM-2 Endothelial Cell Growth Medium-2 BulletKit (EGM-2)	Lonza	CC-3162
FACS tubes		Corning	352235
Fetal Bovine Serum (FBS)		Gemini Bio	100-106
Fibronectin		ThermoFisher Scientific	33016015
GSK3i/CHIR99021		Stemgent	04-0004-02
Hanks Balanced Salt Solution (HBSS)		Gibco	14175-095

Hydrochloric Acid (HCl)		Fisher Scientific	A144S-500
Matrix protein	Matrigel	Corning	356230
Methylcellulose-based medium	MethoCult H4435 Enriched	Stemcell Technologies	4435
Pluripotent stem cell differentiation medium	STEMdiff APEL 2	Stemcell Technologies	5270
Pluripotent stem cells: H1, H9, H9-FUCCI		WiCell	WA09 (H9), WA01 (H1)
Protein-Free Hybridoma Medium (PFMH)		Gibco	12040077
Retinoic Acid		Sigma Aldrich	R2625-50mg
Reverse transcription master mix	iScript Reverse Transcription Supermix	BioRad	1708840
RNA extraction kit	RNeasy Mini Kit	Qiagen	74104
Sodium Hydroxide (NaOH)		Fisher Scientific	SS255-1
Stem cell growth medium	mTeSR1	Stemcell Technologies	85850
SYBR Green master mix	iTaq Universal SYBR Green Master Mix	BioRad	1725121
Trypsin-EDTA		Gibco	25299956
$VEGF_{165}$ (VEGF-A)		PeproTech	100-20
α-CD31-FITC		BioLegend	303104
α-CD34-Pacific Blue		BioLegend	343512
α-CD45-APC/Cy7		BioLegend	304014
α-c-Kit-APC		BioLegend	313206

α-Flk-1-PE/Cy7	BioLegend	359911
α-VE-Cadherin-PE	BioLegend	348506

* Antibody fluorescent conjugates should be optimized based on the cell sorter used. Presented here are the

	Comments/Description
tissue culture treated	
tissue culture treated	
tissue culture treated	
use at 50 ng/mL	
use at 25 ng/mL	
recombinant human; use at 10 μg/mL	
polystyrene round bottom with filter cap	
use at 4 $\mu g/cm^2$	
10 mM stock; use at 5 μM	

Growth factor reduced. Refer to the Certificate of Analysis for the lot to determine the protein (Matrigel) concentration. This concentration is required to calculate the volume of Matrigel that contains 1 mg of protein.

human; H9-FUCCI were obtained from Dr. Ludovic Vallier's lab at Cambridge Stem Cell Institute

use at 0.5 μM

0.25%

use at 50 ng/mL

2 μg/mL*

2 μg/mL*

2 μg/mL*

2 μg/mL*

2 μg/mL*
2 μg/mL*
final concentrations utilized in this study.

We thank the reviewers and editorial staff for their suggestions, and summarize below the changes that we have made to address all concerns. We appreciate your input, which helped to significantly improve our manuscript, and hope that it will now be acceptable for publication in *JoVE*.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We checked through the manuscript and corrected all errors.

2. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Personal pronouns have been removed.

3. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials: e.g., Matrigel, Normocin, MethoCult, Qiagen, Bio-Rad, Stemcell Technologies), etc. We must maintain our scientific integrity and prevent the subsequent video from becoming a commercial advertisement.

The commercial language has been removed.

4. Please use standard abbreviations for time units preceded by a numeral. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks (Line: 140, 278, 288, 299, 306, 310, etc.).

The abbreviations have been changed to follow the suggested style.

5. Line 145: Please ensure that the term “triturating” used is appropriate in the context of this protocol.

Triturating has been replaced with “pipetting up and down”.

6. Line 449-463: Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Add this,” “Ensure that,” etc.).

The text has been edited to the imperative tense.

7. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We have reduced the highlighted essential sections of the protocol to 3 pages.

Reviewers' comments:

Reviewer #1:

Manuscript Summary: The manuscript from Nelson et al. established an easy-to-follow protocol to generate hemogenic endothelium from human pluripotent stem cells. As described in the discussion section, I agree with the significant advantage of this protocol over previously published one from Ohta et al. No requirement of spheroid formation, standard 37C 5% CO2 incubator, and one media (STEMdiff Apel 2) make this protocol more affordable for users than Ohta et al. To improve the visibility of the protocol, I would recommend the authors address the following points.

Major Concerns:

1. Morphological changes of colonies. Would help readers to grasp the idea of density and size of colonies if they see the representative pics lined with figure 1. E.g. days 0, 2, 5, and 8. In my experience, the density and size of colonies influenced the yield of hemogenic endothelium.

Thank you for this suggestion. Due to the importance of proper seeding density in order to obtain efficient differentiation, a representative image of the density and size of the colonies at day 1 of differentiation has been added to **revised Figure 1 (panel B)**. Additional images on subsequent days are shown in our recently published paper (Qiu et al. *Cell Reports* 2020)[1] that we refer the readers to.

2. Can the authors generate mature blood cells? The awaited application of this protocol includes the production of functional blood cells. As T-cells and B-cells take too long (>8 weeks) and require feeder cells (e.g. OP9-DLL4, MS5) in a healthy condition, I would be more satisfied to see the generation of mature myeloid cells as a bottom line. May the authors keep the culture after day 8 and see the expression of CD14, CD16, and CD33. Might take additional >14 days and constant media change though. SCF might be required for EHT. Both IL-3 and M-CSF might be required for monocyte differentiation. Still worth trying.

In related studies by our lab[1] utilizing the differentiation protocol described here, we used flow cytometry (Figures 1E, S1, and S2) to show that multi-lineage blood cells expressing lymphoid markers (CD20, CD3), myeloid markers (CD14, CD66b, CD41, CD235ab) and leukocyte markers (CD11b, CD45) are generated by day 8 of differentiation. However, as the focus of the protocol presented here is to describe a straight-forward method to generate hemogenic endothelial cells and study their molecular regulation, we did not try to optimize their further differentiation into mature blood cells because that was beyond the scope of our studies.

3. The yield of hemogenic endothelium. Could the authors explicit how many hemogenic endothelial cells can be isolated from 1 well of 6 well plates? Any difference between H1 and H9? Though both are good blood producer lines, just wanted to know the protocol works in independent lines.

The protocol works well in both H1 and H9 cell lines, but the H1 cell line generates hemogenic endothelial cells more efficiently compared to the H9 line. A note has been added after Protocol section 1.1. We have not measured the exact number of hemogenic endothelial cells that can be isolated from 1 well of a 6-well plate, but the expected yield would be 1.2% of total cells, based on our studies, to date.

[1] Qiu, J., Nordling, S., Vasavada, H.H., Butcher, E.C., Hirschi, K.K. Retinoic Acid Promotes Endothelial Cell Cycle Early G1 State to Enable Human Hemogenic Endothelial Cell Specification. *Cell Reports.* **33** (9) (2020).

4. The yield of CFU. Could the authors explicit the input cell number (FACS-sorted hemogenic endothelium?) and output the CFU number? Perhaps having a bar graph representation of each type of CFU per 1K HE cells would help visualization.

Thank you for this suggestion. We have incorporated a panel into **revised Figure 4 (panel F)** that displays the number of CFU obtained per 1000 plated hemogenic endothelial cells. This is also addressed in text in line 553.

5. Could the author comment on the application of MACS? Although FACS isolation of precise markers defines HE better, a routine harvest of HE cannot be capped by the availability and cost of a sorter. Especially for readers in a small institute… I would appreciate it if the authors comment on the hematopoietic capacity of CD34+ MACS isolated cells on day 8. They could adhere to fibronectin and undergo EHT eventually? Generate CFU-GEMM?

In this protocol, we describe a novel method for the identification and purification of hemogenic endothelial cells, a population of cells that expresses both endothelial and hematopoietic markers. Due to their complex phenotype, FACS isolation is required to purify hemogenic endothelial cells, and for this reason we have chosen not to discuss MACS sorting. We discuss in lines 649-651 that while CD34 is recognized as a hematopoietic marker, it is also expressed by other non-hematopoietic cell types[2]. Thus, while it is possible to sort cells at day 8 based on $CD34^+$ expression alone using MACS, a mixed population of cells would be obtained. Therefore, we expect that not all $CD34^+$ cells sorted using MACS would be capable of undergoing EHT and generating CFU-GEMM.

6. Semantic issue. What would the authors call this protocol? Elefanty & Stanley called hematopoietic organoids. Crooks called artificial thymic organoids. Even with some differences in protocols, both start from PSC aggregates go through primitive streak to hemogenic endothelium. The highlighted feature in both protocols is the invagination of primitive streak cells self-form certain 3D structures, thus called organoids. The same thing occurs in hematopoietic induction of PSC colonies, usually forming a sack-like structure. Probably occurs in the case of Nelson et al. too. It is up to authors, but having a memorable name would increase the visibility of this protocol to readers.

We have considered this interesting suggestion, and have chosen to keep the name of our protocol “Directed Differentiation of Hemogenic Endothelial Cells from Human Pluripotent Stem Cells.” Our focus was generating a serum- and murine feeder-free culture method for the differentiation of primordial and hemogenic endothelial cells from human pluripotent stem cells grown in a 2D monolayer. This method does not require or rely on the formation of embryoid bodies, and we did not observe the formation of organoids. Thus, we are not comfortable adding such information to our title.

[2] Sidney, L.E., Branch, M.J., Dunphy, S.E., Dua, H.S., Hopkinson, A. Concise Review: Evidence for CD34 as a Common Marker for Diverse Progenitors: CD34 as a Common Marker for Diverse Progenitors. *STEM CELLS*. **32** (6), 1380–1389 (2014).

Reviewer #2:
Manuscript Summary: This manuscript by Nelson and colleagues describes a detailed, useful and timely protocol for the directed differentiation of hemogenic endothelial cells form human PSC.

Major Concerns:
None.

Minor Concerns:
1. Title page: affiliation 4 seems to be missing

This information has been added.

2. The authors should indicate if they would expect their protocol to work equally well on induced pluripotent stem cells (iPSC).

We have initiated studies to determine whether this protocol can be used to generate hemogenic endothelial cells from human iPSCs. Results, to date, reveal that we are able to generate $CD31^+$ KDR^+ $c\text{-}Kit^+$ $CD34^+$ $VE\text{-}Cad^-$ $CD45^-$ cells from human iPS cells. However, as this work is ongoing and still preliminary, we prefer not to make any speculations in the manuscript.

3. Table 2: The sentence "Antibody fluorescent conjugates should be optimized based on cell sorter utilized. We used antibody at a final concentrations of 2 mg/mL" is given 6x. Maybe this is just a pdf formatting error, but if this refers to Table 2, consider adding an asterix referring to the individual antibodies and providing the sentence once.

Thank you for this suggestion; the change has been made.

4. Table of materials / Table 1: use at 5 0ng/mL --> there seems to be an extra space in between the number(s)

This typo had been edited.

Reviewer #3:
Manuscript Summary: The manuscript is well written with detailed explanation of the protocol. It would be a very useful protocol for scientists in the field. It is well presented.

Major Concerns:
1. The authors do not mention the volume of matrigel aliquoted into each tube.

The volume of the Matrigel aliquots will vary depending on the Matrigel lot. The researcher should refer to the certificate of analysis for the lot to identify the protein concentration. The volume containing 1mg of protein (Matrigel) can be calculated from this value. To clarify this point, a comment/description has been added to **revised Table 1** (List of reagents). In addition, to comply with publication guidelines, all direct references to Matrigel have been replaced with “matrix protein” in the manuscript text. The commercial product name can be found in **revised Table 1.**

2. The statement, "To passage cells:", should be on 2.4.1.

The statement “to passage cells” appears in Step 2.4, which is the parent heading for steps 2.4.1- 2.4.9. Steps 2.4.1- 2.4.9 are all steps necessary to passage cells; thus, we think this statement is more appropriately in Step 2.4.

3. What plate size are you using for differentiation?

Differentiation is performed in 6-well plates. A note has been added after Protocol Step 1.3 to clarify this point.

4. What coating is used for differentiation?

Differentiation is performed on Matrigel-coated plates. However, to comply with publication guidelines, all direct references to Matrigel in the main text have been replaced with “matrix protein.” The commercial product name can be located in **revised Table 1**. A note has been added after Protocol Step 1.3 to clarify that matrix protein-coated plates should be used for differentiation.

5. An approximate cell density for seeding would be desirable as too many cells would hinder optimum endothelial differentiation.

Thank you for catching this inadvertent omission. Small clumps of cells were seeded at a density of approximately 2 colonies per square centimeter, as described in Sriram et al.[3] This information has been added to Protocol Step 3.1. It is still advisable that the seeding density be evaluated and refined empirically, as needed.

6. What is the composition of the HE differentiation medium? This should be mentioned in the text.

We agree and the composition of the HE differentiation medium is provided in Protocol Step 1.10.

7. in Line 8.4, Where are the methocult cultures placed for 10 mins, at RT or 37 water bath?

The Methocult cultures are left at room temperature for 10 min. This has been added to Protocol Step 8.4. To comply with publication guidelines, all direct references to Methocult in the main text have been replaced with “methylcellulose-based medium”. The commercial product name can be located in **revised Table 1**.

8. Fig. 4C and 4D are not clear. include better pictures or at least show cytospins to show morphology of cells.

We agree that the images shown in Figs 4C and 4D are not ideal. However, our best higher quality images of CFU-GM and CFU-GEMM generated using this protocol have been recently published in a related manuscript[1] from our lab. We direct readers to this manuscript for additional CFU images in the **revised Figure 4 legend**.

---

[3] Sriram, G., Tan, J.Y., Islam, I., Rufaihah, A.J., Cao, T. Efficient differentiation of human embryonic stem cells to arterial and venous endothelial cells under feeder- and serum-free conditions. *Stem Cell Research & Therapy*. **6** (1), 261 (2015).

Reviewer #4:
Comments to Authors: In this study, the authors provide a novel method for the derivation of hemogenic endothelial cells from human embryonic stem cells within approximately one week using a murine feeder- and serum-free 2D culture system. This protocol yields a well-defined population of hemogenic endothelial cells (CD31+Flk-1+c-Kit+CD34+VE-Cadherin-CD45-). This study provides useful methods in detail. Some details still need to be improved during the revise.

Major comments:
1. The author claimed that the increased generation of hemogenic endothelial cells by DLL4 is associated with late G1 cell cycle arrest. It will be better that the authors provide more evidence to validate this.

We apologize for the confusion. The purpose of the DLL4 induction experiment in the present study was to demonstrate that the FACS-purified H9-Fucci $CD31^{+}CD45^{-}$ cells isolated at day 5 of differentiation are primordial in nature (able to be further specified to other endothelial cell types, such as arterial endothelial cells, as shown in Figure 3). This experiment was conducted independently of hemogenic specification. We have clarified this in the results section (lines 581-582) as well as in the **revised Figure 3 legend**.

2. In the Fig.2B, please provide flow cytometric gating strategy for CD45 and CD31, as shown in Fig.2A.

Since hemogenic endothelial cells are derived from primordial endothelial cells, the flow cytometric gating strategy for CD45 and CD31 in Fig 2B (representative flow cytometry gating strategy for hemogenic endothelial cells) is identical to that shown in Fig. 2A (representative flow cytometry gating strategy for primordial endothelial cells). A note has been added to **revised Figure 2 legend** to clarify this. This is also now mentioned in the text in lines 573-575.

3. Why CFU-E and BFU-E Fig.2B are not red?

We think the reviewer is referring to Figure 4, since Figure 2B illustrates representative flow cytometric gating for hemogenic endothelial cells. In the representative pictures of colony types in **revised Figure 4**, the cells are likely at an early stage of development and not yet hemoglobinized.

Minor comments:
1. In the step 3.1, please ensure how many or the numbers of small clumps were seed? Or provide more detail information how to evaluate the seeding density of small clumps.

Thank you for catching this inadvertent omission. Small clumps of cells were seeded at a density of approximately 2 colonies per square centimeter, as described in Sriram et al.[3]. This information has been added to Protocol Step 3.1. It is still advisable that the seed density be evaluated and refined empirically, as needed.

2. Please describe the differentiation protocol by using more accurate words, ie. don't use sentence like "If the media has become yellow" in the part of 3.5 and 3.6.

This phrasing has been removed from the protocol.

3. The "+" and "-" Fig.2 should be superscript.

This edit has been made.

Reviewer #5:
Manuscript Summary: The manuscript of Nelson et al, "Directed Differentiation of Hemogenic Endothelial Cells from Human Pluripotent Stem Cells" provides a detailed, step by step, description of the method.

Major Concerns:
1. Please discuss the reason for selection VE-Cadherin negative cells for hematopoietic differentiation: "To verify the hematopoietic potential of hemogenic endothelial cells, the CD31+ Flk-1+ c-Kit+554 CD34+ VE-Cadherin-CD45- endothelial cells isolated through FACS". Some other authors believe that VE-Cadherin is an essential marker of hemogenic endothelium (VE-Cadherin+, CD31+, CD34+, CD73-, CD43-), see, for instance, the review in Blood "Hematopoietic specification from human pluripotent stem cells". 2013 12;122(25):4035-46. 10.1182/blood-2013-07-474825

We refer the reviewer to Figure 2D of our companion publication in *Cell Reports* (Qiu, J., Nordling, S., Vasavada, H.H., Butcher, E.C., Hirschi, K.K. Retinoic Acid Promotes Endothelial Cell Cycle Early G1 State to Enable Human Hemogenic Endothelial Cell Specification. *Cell Reports.* **33** (9) (2020)). In this study, although both $CD31^{+}CD45^{-}VE\text{-}Cad^{+}$ and $CD31^{+}CD45^{-}VE\text{-}Cad^{-}$ cells generated hematopoietic colonies, we found significantly greater hematopoietic potential among the $CD31^{+}CD45^{-}VE\text{-}Cad^{-}$ cells; thus, we used absence of VE-Cad to enrich for endothelial cells with blood-forming potential. Furthermore, it is important to note that hemogenic endothelial cells exist on a continuum of gene expression, and they must lose expression of the endothelial specific genes such as VE-Cadherin to generate hematopoietic cells.

Minor Concerns:
1. Please indicate if the lymphoid component was not attainable with described method or it was not tested?

In related studies by our lab[1] utilizing the differentiation protocol described here, we show (Figures 1E, S1, and S2) through flow cytometry that multi-lineage blood cells expressing lymphoid markers (CD20, CD3), myeloid markers (CD14, CD66b, CD41, CD235ab) and leukocyte markers (CD11b, CD45) are generated by day 8 of differentiation.

2. Please provide a reference to a closely related study on the differentiation of human pluripotent stem cells to hemogenic endothelium with GSK3 inhibitor (CHIR99021). Stem Cell Res Ther 2017 Mar 17;8(1):67 DOI: 10.1186/s13287-017-0519-0

Thank you for directing us to this reference. We have incorporated it into our **revised Discussion** (lines 639-648).

ELSEVIER LICENSE
TERMS AND CONDITIONS

Feb 08, 2021

This Agreement between University of Virginia -- Elizabeth Nelson ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	5004381478856
License date	Feb 08, 2021
Licensed Content Publisher	Elsevier
Licensed Content Publication	CELL REPORTS
Licensed Content Title	Retinoic Acid Promotes Endothelial Cell Cycle Early G1 State to Enable Human Hemogenic Endothelial Cell Specification
Licensed Content Author	Jingyao Qiu,Sofia Nordling,Hema H. Vasavada,Eugene C. Butcher,Karen K. Hirschi
Licensed Content Date	Dec 1, 2020
Licensed Content Volume	33
Licensed Content Issue	9

Licensed Content Pages	1
Start Page	108465
End Page	0
Type of Use	reuse in a journal/magazine
Requestor type	academic/educational institute
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	2
Format	electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Title of new article	Directed Differentiation of Hemogenic Endothelial Cells from Human Pluripotent Stem Cells
Lead author	Elizabeth A. Nelson
Title of targeted journal	JoVE
Publisher	JoVE
Expected publication date	Mar 2021
Portions	Adaptation of Figure 1A and adaptation of Figure 2F

	University of Virginia PO Box 800732
Requestor Location	CHARLOTTESVILLE, VA 22908 United States Attn: University of Virginia
Publisher Tax ID	98-0397604
Billing Type	Credit Card
Credit card info	Visa ending in 1157
Credit card expiration	08/2021
Total	64.40 USD

Terms and Conditions

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE

SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier's permissions helpdesk here). No modifications can be made to any Lancet figures/tables and they must be reproduced in full.

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment

terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.

16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

17. **For journal authors:** the following clauses are applicable in addition to the above:

Preprints:

A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).

Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).

If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.

Accepted Author Manuscripts: An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.

Authors can share their accepted author manuscript:

- immediately
    - via their non-commercial person homepage or blog
    - by updating a preprint in arXiv or RePEc with the accepted manuscript
    - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
    - directly by providing copies to their students or to research collaborators for their personal use
    - for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement
- After the embargo period
    - via non-commercial hosting platforms such as their institutional repository
    - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

Published journal article (JPA): A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing, formatting, (if relevant) pagination and online enrichment.

Policies for sharing publishing journal articles differ for subscription and gold open access articles:

Subscription Articles: If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.

Theses and dissertations which contain embedded PJAs as part of the formal submission can

be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.

Gold Open Access Articles: May be shared according to the author-selected end-user license and should contain a CrossMark logo, the end user license, and a DOI link to the formal publication on ScienceDirect.

Please refer to Elsevier's posting policy for further information.

18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.

19. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

Elsevier Open Access Terms and Conditions

You can publish open access with Elsevier in hundreds of open access journals or in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author's choice of Creative Commons user license. See our open access license policy for more information.

Terms & Conditions applicable to all Open Access articles published with Elsevier:

Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation. If any changes have been made, such changes must be clearly indicated.

The author(s) must be appropriately credited and we ask that you include the end user license and a DOI link to the formal publication on ScienceDirect.

If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.

Additional Terms & Conditions applicable to each Creative Commons user license:

CC BY: The CC-BY license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by/4.0.

CC BY NC SA: The CC BY-NC-SA license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. Further, any new works must be made available on the same conditions. The full details of the license are available at http://creativecommons.org/licenses/by-nc-sa/4.0.

CC BY NC ND: The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by-nc-nd/4.0. Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee.

Commercial reuse includes:

- Associating advertising with the full text of the Article
- Charging fees for document delivery or access
- Article aggregation
- Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

20. **Other Conditions**:

v1.10

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.