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Method Article

Isolation, purification, and conditional immortalization of murine glomerular endothelial cells of microvascular phenotype

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A B S T R A C T

Glomerular endothelial cells (GEnC) are a specialized microvascular subset of endothelial cells that, when injured, result in many types of diseases within the kidney. Thus, techniques to study GEnC in a cell culture system are important to investigate mechanisms of GEnC injury. Studies of endothelial cell function in culture have predominately relied on using macrovascular endothelial cells from vascular areas other than the glomerulus. Over the last 15 years, glomerular endothelial cells lines have been created but were isolated by targeting cells expressing CD31. Some studies identified endothelial cells isolated from the microvasculature do not express CD31 and some suggest that CD31+ cells are phenotypically different than endothelial cells found in capillaries. Here we detail our method of isolation, purification, and conditional immortalization of mouse glomerular endothelial cells targeting endothelial cells that do not express CD31.

- This method allows for isolation, purification, and conditional immortalization of glomerular endothelial cells for continued passage of GEnCs beyond that of primary cell culture.
- This method can be used in genetically modified mice to investigate how a modification of a specific gene or protein affects the glomerular endothelium at the cellular level.

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Resource availability:	*N/A*

Required reagents and equipment

- Saturated Iron Oxide
  - Oxygen source
  - 2.6 g NaOH
  - 20 g of KNO_3
  - 9 g $FeSO_4$
  - Heating plate with spinning function and 2 spin bar
  - 500 ml Erlenmeyer flask
  - 10 ml pipet
  - Distilled Water
  - 0.9% NaCl
- Sterile instruments for procurement (2 forceps, 2 scissors, and 2 hemostats)
- Sterile razor blade
- Ethanol (EtOH)
- VetOne Euthanasia solution
- 20 ml sterile syringe
- 20 gage needles
- 1.5 mL capped tubes
- 35 mm^2 cell culture dishes (collagen coated and non-collagen coated)
- 15 mL conical tubes
- 50 mL conical tubes
- 25cm^2 cell culture flasks coated with collagen I
- Collagenase A
- DNAse I
- Phosphate buffer (1x PBS)
- Cell culture hood
- 37 °C incubator with rocker/shaker
- Hank's Balanced Salt Solution (HBSS)
- Isolation Buffer for Dynabeads (Calcium and magnesium free PBS with 0.1% BSA or FBS + 2 mM EDTA)
- Refrigerated centrifuge or chilled centrifuge to 4 °C
- Magnet for cell separation
- Sheep anti-rabbit Dynabeads (Fisher 11203D)
- Von Willebrand Factor (vWF) Rabbit anti-mouse antibody (Santa Cruz SC14014)
- Endothelial cell media
  - Dulbecco's Modified Eagle's Medium (DMEM)
  - Fetal Bovine Serum (FBS)

- Endothelial cell growth supplement constituted per manufacturer's instructions (Sigma E0760–15 mg)
- Mouse Interferon gamma (IFN-γ)

- Accutase
- Thermosensative SV40 T antigen (tsa58) infected cells
- Puromycin

Procedure

Description of protocol

Preparation of saturated iron oxide

Saturated Iron oxide is used for perfusing as the iron deposits in the glomerular basement membrane allowing for positive selection of glomeruli utilizing a magnet

(1) Bubble oxygen in 250 ml of distilled water for at least 10 min.
(2) Take 100 ml of oxygen saturated water and dissolve 2.6 g NaOH and 20 g of KNO_3.
(3) Using 100 g of the remaining oxygen saturated water, dissolve 9 g $FeSO_4$.
(4) Place container with dissolved NaOH and KNO_3 on the stir plate with spin vane. While stirring, pipet the $FeSO_4$ solution slowly into the NaOH and KNO_3 solution. The color should change from green to brown.
(5) After combining the two solutions, bring the solution to a boil and continue to boil for 30 min while stirring.
(6) While solution boils, weigh the 500 ml flask with the other spin bar. Record weight.
(7) Transfer the boiled solution to the weighed flask and store on a magnet at 4 °C for 2 days.
(8) After 2 days, decant the water while holding a large magnet to the bottom of the flask. Allow the iron oxide to settle and then carefully suction off the remaining water avoiding removal of the iron oxide on the bottom.
(9) Add 250 ml of distilled water to the iron oxide and mix for 2 min. Then decant water and repeat this step 4 times.
(10) After discarding the final wash, weigh the flask, stir bar and iron oxide.
(11) Subtract the flask and stir bar weight to find the final weight of iron oxide.
(12) Add enough 0.9% NaCl solution to achieve a final concentration of 50 mg of iron oxide/ml. This is a 10X stock solution.

Preparation of reagents for isolation of glomeruli

(1) Sterile 1X iron oxide solution and GEnC media should be prepared one day prior to isolation of glomeruli.
(2) Prepare a 1X stock of saturated iron oxide solution by diluting the 10X solution in 1X PBS to get necessary volume (at least 20 ml per mouse). Place in an autoclave safe vessel.
(3) Prepare endothelial cell media:
   a. DMEM + 75 mg/ml of endothelial cell growth supplement + 20% Fetal Bovine Serum + 100 mg/ml Streptomycin + 100 IU/ml Penicillin + 75 U/ml IFNγ
   b. Endothelial cell media can be stored at 4 °C and used within 1 month after making.
(4) Autoclave 1X iron oxide and instruments.
(5) Allow iron oxide to cool overnight at 4 °C and surgical instruments to cool at room temperature.

Isolation of glomeruli

(1) Prepare Collagenase A + DNase solution
   a. Collagenase A is utilized since it has several subtypes of collagenases which have broad activity on connective tissues while minimizing damage to the endothelial cells by having less trypsin-like activity.
   b. In 15 ml conical tube, add 0.01 g of Collagenase A and 2 ml of DNase I to 10 ml of PBS.
   c. Make 2 tubes of Collagenase A + DNase solution per mouse.

(2) Harvest mouse kidney tissue
   a. We utilized a 12 week old C57BL/6 male mouse obtained from Jackson Laboratory for cell isolation.
   b. Give intraperitoneal injection of Euthanasia Solution
      i. We make a 1–8 dilution of the stock Euthanasia solution in sterile saline for injection (48.75 mg pentobarbital per ml).
      ii. We use 0.05 to 0.08 ml of the 1:8 diluted Euthanasia solution per mice
         1. Smaller females (~20 g weight) receive 0.05 ml (~2.4 mg pentobarbital)
         2. Larger males (~35 g weight) get 0.08 ml (~3.9 mg pentobarbital)
         3. If anesthesia is not achieved after 5 min, additional 0.01 ml doses are given every 5 min until anesthesia is achieved.
   c. Transfer 20 ml of cooled iron oxide to 20 ml sterile syringe and place 20 gage needle on end. Keep on ice until needed.
   d. Once mouse becomes minimally responsive to stimuli, secure mouse in a Trendelenburg position to an aseptic work surface.
   e. Wipe down mouse underbelly with EtOH to minimize contamination.
   f. Retract abdominal skin and use scissors to remove skin from abdomen allowing for easy access to kidneys.
   g. Perform bilateral thoracotomy and remove anterior rib cage to expose heart.
   h. Using syringe loaded with iron oxide, quickly puncture left ventricle, cut superior vena cava/right atrium and inject 20 ml of ice cold iron oxide. The iron oxide will deposit in the glomerular basement membrane causing the kidneys to become pale with some black speckles. If the kidney does not become pale, another 20 ml perfusion can be performed.
   i. After injecting at least 20 ml of iron oxide, quickly localize the kidneys and place a hemostat across both main renal vessels and explant the kidneys.
   j. Place kidneys in sterile non-collagen coated cell culture dish with 5 ml PBS. Keep on ice and transfer to cell culture hood.

(3) Isolate glomeruli from kidney tissue
   a. In cell culture hood, finely mince kidneys into pieces using sterile forceps and razor blade.
   b. Place both kidneys into one 10 ml Collagenase and DNase solution.
   c. Incubate tissue in solution for 60 min at 37 °C and with shaker at 115 RPM.
   d. After 60 min, strain tissue-solution mix through a 100 µm cell strainer into a 50 ml conical tube.
   e. Wash with the filter with 5 ml HBSS and gently rub with sterile syringe plunger.
   f. Repeat step with a new 100 µm tube cell strainer.
   g. Centrifuge for 5 min at 1000 RPM and 4 °C.
   h. Remove supernatant and re-suspend pellet with 1.5 ml of sterile isolation buffer.
   i. Place 1.5 ml tube in magnet and allow solution to rest for at least a minute.
   j. Carefully, remove the fluid farthest away from the magnet.
   k. Remove tube from single tube magnet and re-suspend with 1.5 ml of sterile isolation buffer.
   l. Repeat steps i-k one more time.
      i. After this step, some of the fluid can be removed and placed on a microscope slide to verify the presence of glomeruli.

Isolation of glomerular endothelial cells

(4) Transfer the washed glomeruli to the remaining tube containing the collagenase A + DNase mixture and incubate at 37 °C for at least 60 min with shaker at 115 RPM for the second collagenase digestion.
(5) Prepare sterile von Willebrand factor (vWF) coated Dynabeads as per the manufacturers protocol.
(6) After the 60 min incubation, strain the glomeruli containing solution through a 40 µm cell strainer and wash with 5 ml of sterile isolation buffer.
(7) Transfer to a 15 ml conical vial and add vWF coated Dynabeads.
(8) Incubate digested glomeruli and vWF coated Dynabeads at 4 °C on a rocker for at least 30 min.

(9) Centrifuge tube for 5 min at 1000 rpm and 4 °C.
(10) Remove supernatant and re-suspend in 1.5 ml of Isolation buffer. Transfer to 1.5 ml tube.
(11) Place 1.5 ml tube in magnet. Wait 1 min for vWF coated Dynabeads to separate from the solution.
(12) Remove fluid phase, careful to not touch the side of the tube closest to the magnet as this can cause mechanical removal of GEnC and reduce cell yield.
(13) Remove tube from magnet and re-suspend with 1 ml of isolation buffer.
(14) Repeat steps 11–13 three more times (4 times total).
(15) After last wash, re-suspend in 1 ml of Isolation buffer.
(16) To dissociate the Dynabeads from the cells, add 500 µl of trypsin to the vWF coated Dynabead-cell mixture. Incubate 2 min at 37 °C.
(17) Transfer contents of the 1.5 ml tube containing the vWF coated Dynabead-cell mixture to a sterile, collagen coated 35 mm^2 dish. Add 500 µl of fresh culture media to dish.
(18) Wash 1.5 ml tube with 500 µl of media and transfer to dish.
(19) Place cells in 37 °C incubator with 5% CO_2.
(20) Every other day, remove old media and add 3 ml of fresh media.
(21) If cell yield is low:
    a. After second collagenase digestion and straining through 40 µm cell strainer, cells could be placed in a collagen coated flask and grown at 37 °C for a few days increase the number of isolated glomerular cells (podocytes and endothelial cells) prior to positive selection with vWF coated Dynabeads.
(22) After 7–10 days, the GEnCs should have multiplied and formed a monolayer of cells to cover approximately 80% of the surface of the dish.
(23) Once dish is $\geq$ 80% confluent, remove old media and wash cells by adding 2 ml of sterile PBS and gently swirling in dish. Remove and discard PBS.
(24) Add 1 ml of Accutase and place in 37 °C incubator until cells detach from dish (~5 min).
(25) To the dish of detached cells, add 2 ml of fresh media and gentle pipet to mix.
(26) Transfer 1 ml of detached cells into a collagen coated 25 cm^2 cell culture flask. This should be repeated with the remaining volume of detached cells allowing for a total of 3 flasks.
(27) Add 5 ml of fresh media to each flask and incubate the flask in 37 °C incubator with 5% CO_2.
(28) Remove the old media and replace with 6 ml of fresh media every other day.

Conditional immortalization

(29) Once the 3 flasks of GEnCs have multiplied for 1 day (endothelial cells should be $<$ 50% confluent), thaw a vial of the fibroblast infected with thermosensitive SV40 T antigen (tsa58) onto a collagen coated 75 cm^2 cell culture flask and cover with 10 ml of media containing DMEM + 10% FBS + puromycin at 2 µg/ml and incubate at 37 °C with 5% CO_2.
(30) Forty eight hours after plating tsa58 infected fibroblasts, remove DMEM+ FBS+ puromycin media and replace with 10 ml of endothelial cell media. The infected fibroblasts should be approximately 50% confluent and rapidly multiplying to ensure best yield of tsa58 virus.
(31) Once the 3 flasks of endothelial cells are approximately 65% confluent, remove media from fibroblasts infected with the tsa58 virus and place in a sterile 15 ml conical tube.
    a. The 75 cm^2 cell culture the flask containing the tsa58 infected cells can be discarded or the cells can be detached from flask using trypsin and frozen for later use.
(32) Filter the media containing the tsa58 virus by placing the media in a sterile 20 ml syringe with a 0.45 µm syringe top filter. This removes fibroblasts and cellular debris which can contaminate GEnC and affect purity of the cell line.
(33) Filter the virus media using the syringe into a new sterile 50 ml conical tube and combine with 10 ml of fresh endothelial cell media. Thoroughly mix the media by pipetting the media up and down several times.
(34) Remove and discard the media from the flasks of GEnCs. Add approximately 6 ml of the virus containing media to each flask of GEnCs.
(35) Incubate the GEnCs at 37 °C with 5% CO_2 and allow virus containing media to remain on GEnCs for 24 h.

(36) The next day, make puromycin containing endothelial cell media by adding 1.0 μg/ml of puromycin to freshly made endothelial cell media (will need ~22 μl of media).
(37) After 24 h of incubating the GEnCs with virus containing media, remove the virus containing media and discard. Add 6 ml of endothelial cell media containing puromycin at 1.0 μg/ml and incubate at 37 °C with 5% CO_2 for > 48 h.
   a. Of note, there will be a significant number of cells that die after puromycin exposure and only a minority of cells will remain.
(38) After at least 48 h, change media in flasks to endothelial cell media + puromycin at 0.5 μg/ml. Incubate cells at 33 °C with 5% CO_2 for at least 48 h.
(39) After at least 48 h, media on the now conditionally immortalized GEnCs can be changed to endothelial cell media without puromycin.
   a. At this stage, if the GEnCs are ≥ 80% confluent, the cells can be split into new 25 cm^2 cell culture flasks and excess can be frozen as stock for later use.
(40) Conditionally immortalized GEnCs can be passaged at 33 °C but need to be differentiated by being placed at 37 °C for 7–10 days prior to experimentation.
   a. Recommendations on maintaining stock
      i. When freezing cells, we add 10% DMSO per volume of cells to reduce crystal formation and improve cell viability and yield after freezing.
      ii. We store stocks of our cells at −80 °C and in liquid nitrogen.
      iii. We generally split our GEnCs when they are at a confluence of ~80% to prevent over growth.
         1. Our cells generally hit confluence of ~80% 3–5 days after plating a stock vial of cells but this can vary due to cell concentration and robustness after thawing.
      iv. Would recommend utilizing Accutase when detaching cells to reduce risk of cell surface marker digestion.
   b. Recommendations on differentiation:
      i. GEnCs planned for differentiation and experimentation should be given endothelial cell media without IFNγ to allow GEnCs to differentiate to a resting state.
      ii. Recommend the following number of cells and volume of media per growing vessel to prevent overgrowth of cells by days 5–7.
         1. 35 mm^2 dish: Approximately 15,000 cells with 3 ml of media.
         2. 25 cm^2 cell culture flasks: Approximately 100,000 with 6–7 ml of media.
            a. Of note, depending on health and robustness of cells, cells may grow faster or slower. As such, the number of cells per dish may need to be adjusted to obtain a confluence of ~80–90% by day of experimentation.
            b. Cells over 90% confluence may begin to de-differentiate or undergo apoptosis.

Protocol validation

After positive selection for glomerular cells with von Willebrand factor (vWF) coated Dynabeads, cells were observed over the next 14 days to confirm the phenotypic features of glomerular endothelial cells. By day 14, the cells formed the “cobblestone” pattern consistent with that of endothelial cells (Fig. 1B). After confirming the typical “cobblestone” appearance of endothelial cells, cells were stained for the microvascular endothelial cell marker vWF, endothelial cell marker CD31, and the podocyte marker Wilms Tumor marker 1 (WT1). Table 1 lists details of the markers used to validate cell culture phenotype. Staining for vWF, CD31, and WT1 was performed to ensure the markers present on glomerular endothelial cells were consistent with the microvascular phenotype described by Craig et al. (vWF positive, CD31 negative) [2]. All dishes were stained for actin (blue) to help identify the location of cells as well as help determine the relative percentage of cells staining positive for each marker. Podocytes from C57BL/6 isolated during our prior work [3] were used as a WT1 positive control and vWF negative control. Secondary antibody only controls for both vWF and WT1 was performed to ensure staining was not related to non-specific secondary antibody binding. Glomerular endothelial cells demonstrated positive staining for vWF (green) but not CD31 nor WT1, consistent with microvascular endothelial cells (Fig. 2). Purity was determined to be ≥ 90% given the relative ubiquitous vWF staining of the glomerular endothelial cells.

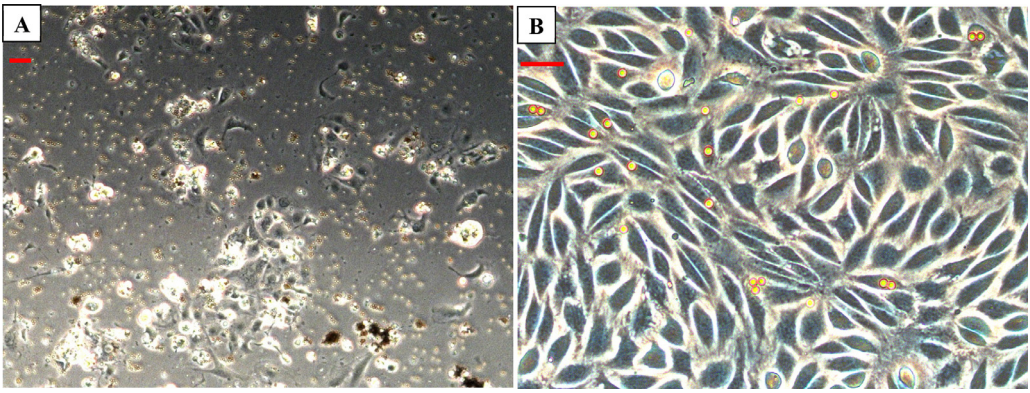

Fig. 1. Photographs taken of isolated cells prior to immortalization on a bright field microscope. (A) Cells on day 3 after isolation. Red-brown colored spheres are Dynabeads. Magnification 10x. (B) Cells on day 14 after isolation demonstrating the "cobblestone" pattern of endothelial cells. Yellow-orange colored spheres are Dynabeads. Magnification 20x. Red scale bars in upper left corner of each image represent 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Antibodies and primers used in method validation.

Antibodies for immunofluorescence		
vWF	Santa Cruz	Sc-14014
CD31	Thermo Fisher	HEC7 (#MA3100)
VE Cadherin	Abcam	Ab 33168
WT1	Santa Cruz	Sc-15421 (N-20)
Actin	Thermo Fisher	Alexa 635 Phalloidin (A34054)
DAPI	Thermo Fisher	Hoechst 33342
Primers for qPCR		
VE-cadherin	Forward: 5′-CACTGCTTTGGGAGCCTTC −3′	Reverse: 5′-GGGGCAGCGATTCATTTTTCT-3′
Dll4	Forward: 5′- TTCCAGGCAACCTTCTCCGA-3′	Reverse: 5′-GGAGTCCTGGCATCGTTGG-3′
Notch1	Forward: 5′- CCCTTGCTCTGCCTAACGC-3′	Reverse: 5′-ACTGCCGCTATTCTTGTCCC-3′
Antibodies for flow cytometry		
CD146	Miltenyi Biotec	130-102-230
CD31	BioLegend	105507
P-selectin	BD Horizons	564289

After confirming cells were microvascular endothelial cells from the glomerulus (GEnCs) as defined by the microscopic appearance and vWF positivity, cells were conditionally immortalized by infecting them with the thermosensitive SV40 T antigen as described above. GEnCs were incubated at 37 °C for 4 days to determine if vWF expression was altered by immortalization and if duration of differentiation affected vWF expression (Fig. 3A-C). Though the cells we prepared, stained, and imaged in an identical manner to the cells in Fig. 2, the relative intensity of vWF and actin was less. This was felt to be due to incomplete endothelial phenotype expression due to only 4 days of differentiation.

To verify the decreased intensity of the vWF staining was related to duration of differentiation and not due to the immortalization process, GEnCs were allowed to differentiate at 37 °C for 7 days prior to fixing and staining for immunofluorescence (Fig. 3D-F). By allowing the cells to differentiate at 37 °C for 7 days, the relative intensity of actin and vWF were comparable to cells imaged prior to immortalization (Fig. 2). Furthermore, this experiment confirmed that the immortalization process did not change the phenotype such that the cells continued to express vWF and not WT1. Lastly, it also demonstrated that the purity of GEnC was $\geq$ 90%.

In order to validate the GEnC were endothelial in origin and continued to express additional phenotypic markers characteristic of endothelial cells after differentiation, the conditionally

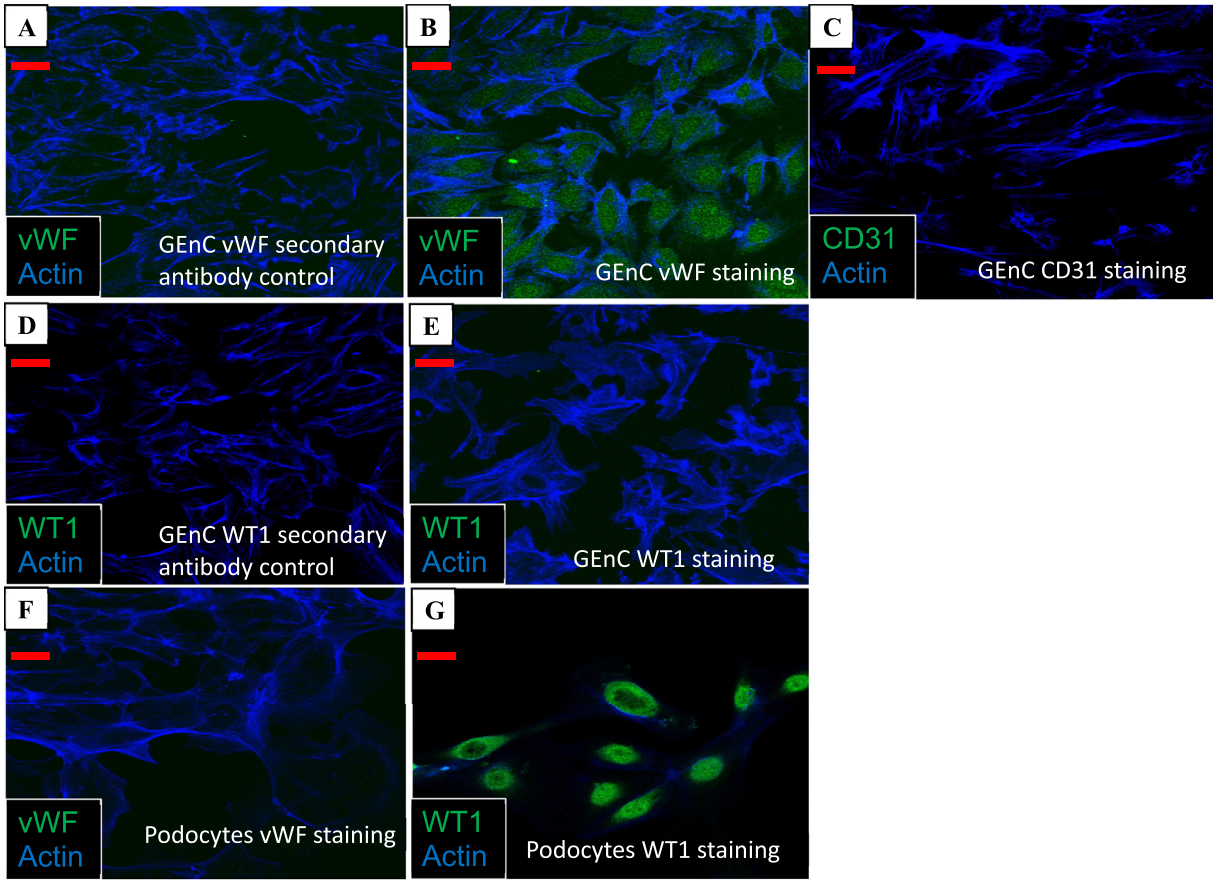

Fig. 2. Immunofluorescence of isolated cells prior to conditional immortalization. Isolated GEnC (A-E) were fixed with 4% paraformaldehyde and stained for von Willebrand Factor (vWF) (panel B), CD31 (panel C), or Wilms Tumor 1 (WT1) (panel E). All cells were stained for actin (blue) to help identify the presence of cells. Green in any of the images indicates positive staining. Secondary antibody controls (A and D) were isolated cells stained with only secondary antibody to ensure staining was specific for the primary antibody. Podocytes were also stained and imaged in parallel with the isolated GEnC to act as a negative control for vWF (F) and positive control for WT1 (G). Red scale bars in upper left corner of each image represent 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

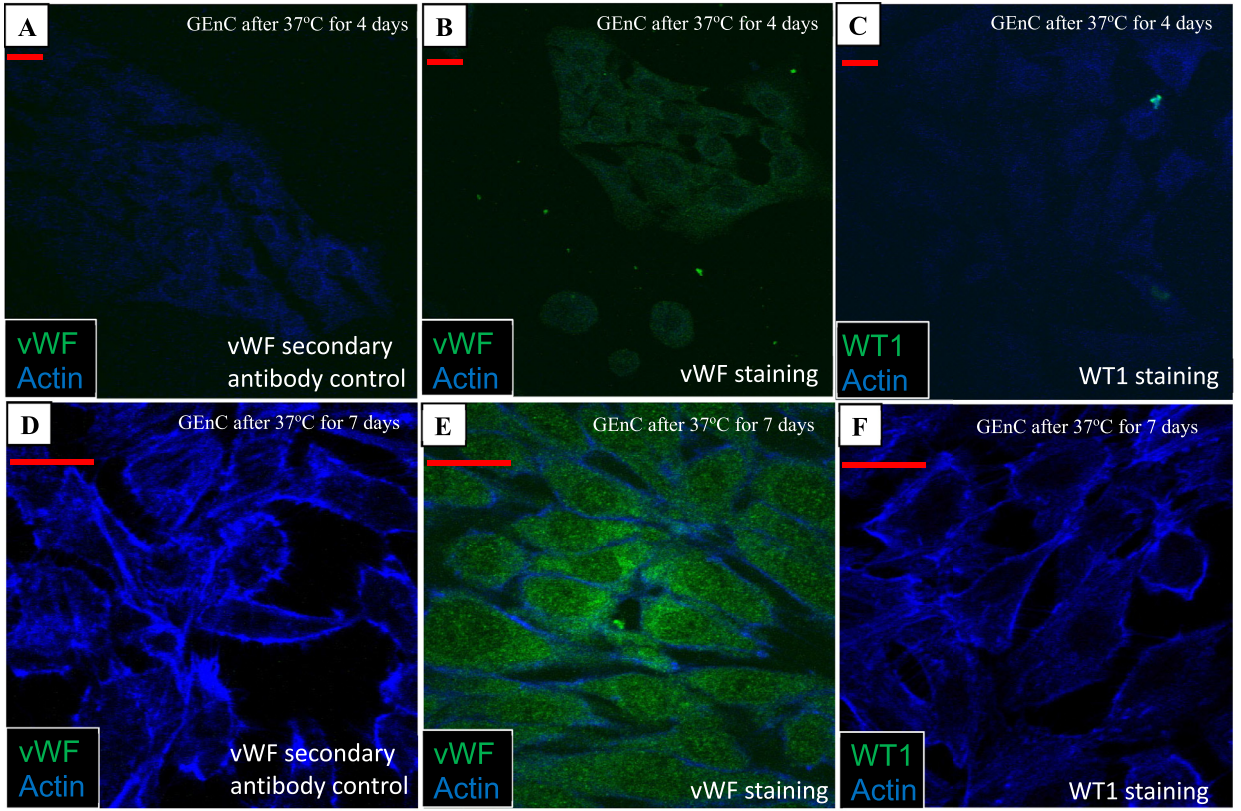

Fig. 3. Immunofluorescence of GEnCs after immortalization at 4 days versus 7 days of differentiation at 37 °C. Repeat immunofluorescence was performed to ensure induction of tsa58 and puromycin selection did not alter vWF or WT1 expression. GEnC were differentiated for either 4 days (A-C) or 7 days (D-F) at 37 °C, fixed with paraformaldehyde, and stained for vWF and WT1 utilizing the same protocol, antibody concentration, and microscopy settings as in Fig. 2. Actin (blue) was stained to localize cells. Secondary antibody controls were isolated GEnC stained with only secondary antibody. The GEnCs differentiated for 4 days (A-C) had relatively dim fluorescence for actin (A) and vWF (B) despite identical antibody concentrations and microscopy settings as that of GEnC differentiated for 7 days (D-F). The difference in fluorescence intensity was felt to be related to incomplete differentiation of the cells after only 4 days leading to sub-optimal expression of cell surface markers. Staining intensity for actin (D) and vWF (E) of GEnCs differentiated for 7 days at 37 °C was similar to that of GEnC prior to immortalization. Red scale bars in upper left corner of each image represent 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

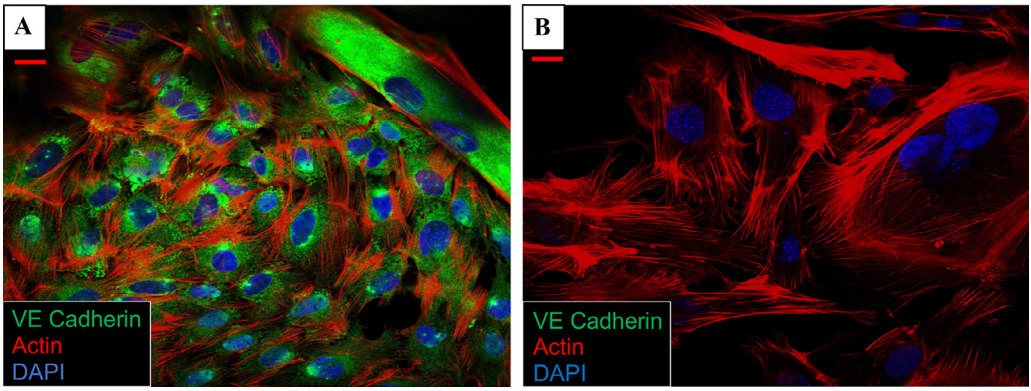

Fig. 4. GEnC (A and B) were differentiated for 7 days at 37 °C before being fixed with 4% paraformaldehyde and stained for VE-cadherin (panel A). All cells were stained for actin (red) and DAPI (blue) to help identify the presence of cells. Green in any of the images indicates positive staining for VE cadherin. Antibody control (panel B) were isolated cells stained with the goat anti-rabbit secondary antibody (the secondary antibody for VE cadherin) as well as actin and DAPI, to ensure the staining was specific for the primary antibody. Red scale bars in upper left corner of each image represent 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

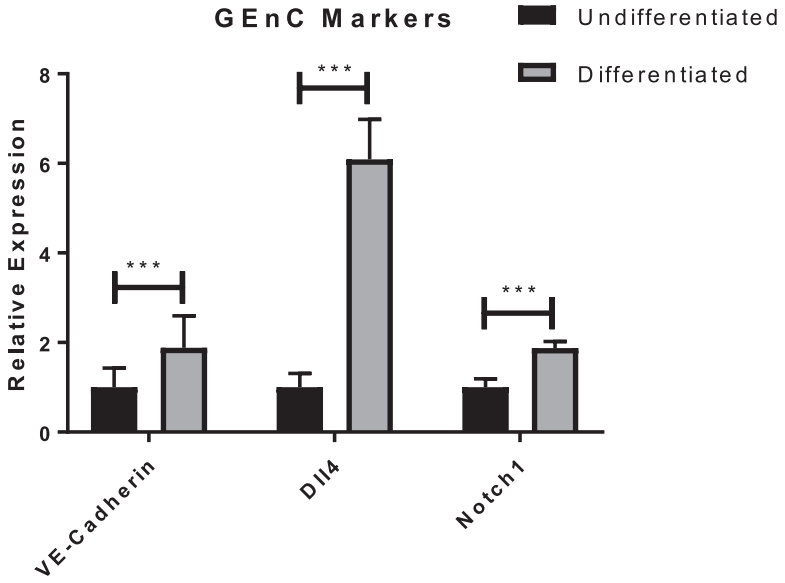

Fig. 5. GEnC were plated and kept at 33 °C ("Undifferentiated," black) or at 37 °C ("Differentiated," gray) for 7 days before undergoing RNA isolation and qPCR for the endothelial cells markers VE-cadherin, Dll4, and Notch1. Values are expressed as relative fold change compared to undifferentiated cells. Expression of all endothelial cell markers was significantly increased within 7 days of differentiation at 37 °C compared to those at 33 °C demonstrating the thermosensitive nature of the conditional immortalization (***$p < 0.0001$, $n = 9$).

immortalized GEnCs were allowed to differentiate at 37 °C for 7 days prior to fixing and staining for VE cadherin in addition to actin and DAPI (Fig. 4A and B). It again demonstrated the purity of the cells with ≥ 90% of cells staining positive for VE-cadherin.

As an additional method to verify the phenotype of GEnC cultures at 33 °C and at 37 °C, we performed RNA isolation and qPCR for endothelial cell markers VE-cadherin, Delta-like 4 (Dll4),

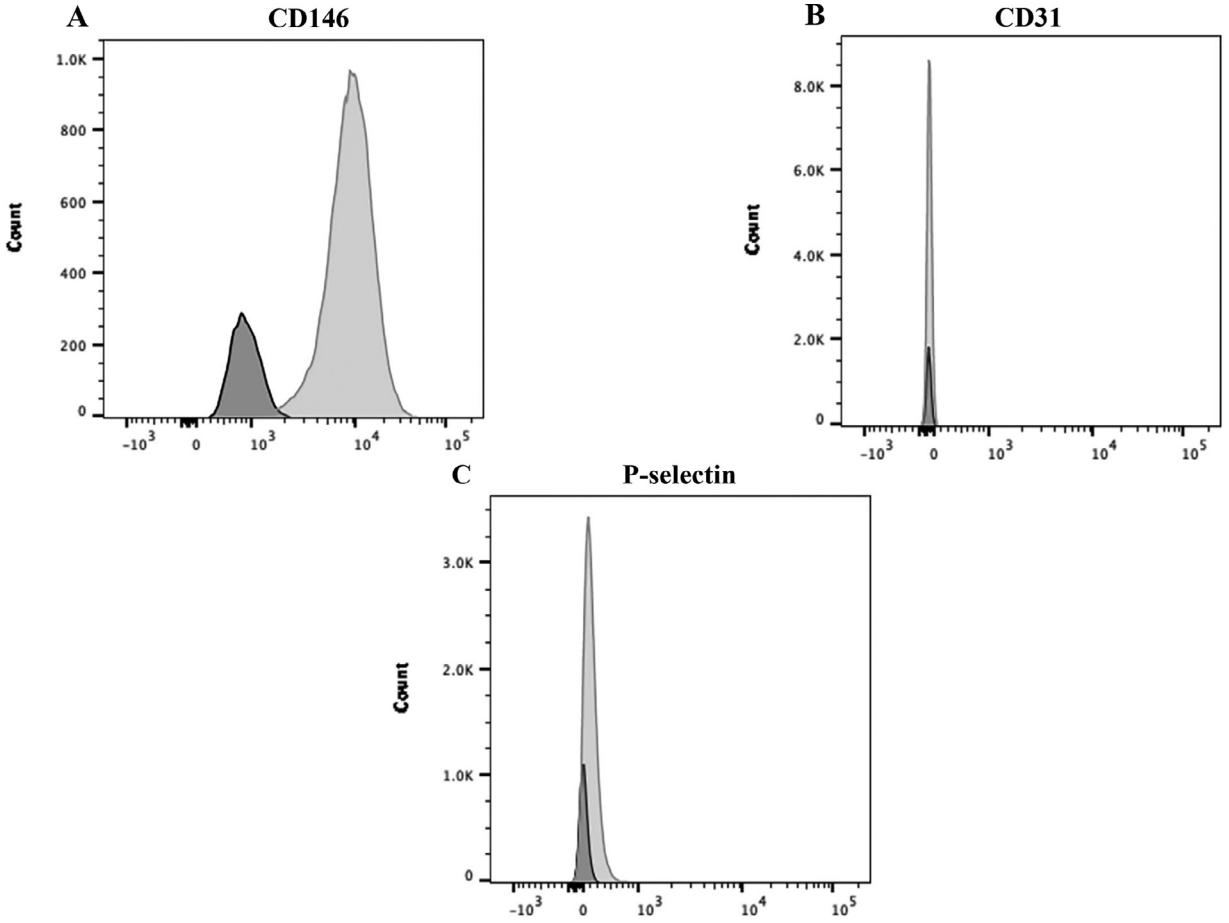

Fig. 6. Cells were differentiated at 37 °C for 7 days before undergoing flow cytometry for the endothelial cell markers CD146, CD31, and P-selectin (staining shown in light gray). Dark gray peaks are isotype controls. There continued to be no expression of CD31, minimal expression of P-selectin, but significant expression of CD146. These findings are consistent with previously reported characteristics of microvascular endothelial cells.

and Notch 1 (Fig. 5) [4,5]. We found that not only did GEnCs express these markers but that the differentiation at 37 °C caused a significant increase in the endothelial cell markers ($n = 9$, $p < 0.0001$).

Lastly, flow cytometry was performed on the immortalized GEnCs after being differentiated at 37 °C for 7 days (Fig. 6). In each graph, lighter gray peak are the GEnCs and darker gray peak are isotype controls. Our findings demonstrated the GEnC expressed the endothelial cell marker CD146 (Fig. 5A) but did not express CD31 (Fig. 5B) and had only minimal expression of P-selectin (Fig. 5C) consistent with an inactivated microvascular endothelial cell [6,7].

In this work, we describe a method to culture immortalized microvascular GEnC. We confirm the phenotype of microvascular GEnC by microscopic appearance and the presence of markers for microvascular endothelial cells (positivity for vWF, VE-cadherin, Dll4, Notch1, and CD146). Cultured GEnC were negative for CD31 and WT1, as expected for microvascular GEnC. This cell culture technique provides a useful experimental system to investigate the mechanisms of GEnC injury.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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