Novel Strategy for Isolation of Mice Bone Marrow – Derived Endothelial Cells (BMECs)

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Method

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Abstract

Background: In the bone marrow microenvironment, endothelial cells (ECs) are individual cells that form part of the sinusoidal blood vessels called "bone marrow endothelial niche." They account for less than two percent of the bone marrow cells. They may play critical functions in generating growth and inhibitory factors that promote the recovery of the hematopoietic stem cells.

Methods: Two steps approach for isolation of bone marrow endothelial cells from mice. In brief, the bone marrow extracted from the mice long bones and culture overnight with DMEM supplemented with 20% FBS and antibiotics. The floating cells discarded, and the adhered section detaches with accutase and bone marrow endothelial cells selected using CD31 microbeads. The isolated bone marrow endothelial cells were cultured in dish pre-coated with rat-tail collagen type 1 with endothelial growth factor media. The cells were verified by confocal microscopy for morphology and tube formation by matrigel assay. We validate the purity of the cells by flow cytometry, RT-qPCR, immunofluorescence staining, and immunoblotting. Finally, we induced the bone marrow endothelial cells with recombinant tumor necrosis factor-alpha.

Results: Our findings prove that the cell isolated are characteristic of bone marrow endothelial cells and response to tumor necrosis factor-alpha by an increase in proliferation and enhance the expression of adhesion molecules.

Conclusions: This method simplifies the extraction of primary bone marrow endothelial cells for in vitro studies. The significance of this method will provide an excellent opportunity for stem cell research of endothelial cells function and dysregulation in vitro studies of mice model.

Introduction

The endothelial cells are part of the sinusoid-vascular niche in the bone marrow microenvironment. They play a significant role in producing growth and inhibitory cytokine that regulates the function of the hematopoietic stem cells. Also, they display adhesion molecules that interconnecting the hematopoietic progenitor cells. In the bone marrow microenvironment, they line the lumen of the sinusoidal-vascular niche. In response to inflammatory stimuli, they increase in proliferation to maintain the integrity of the blood vessels. The inflammatory response releases cytokines that promote their activation, such as tumor necrosis factor, interleukin 6. These cytokines, in turn, increase the expression of the endothelial adhesion molecule, such as; E-selectin, Vascular adhesion molecules (VCAM − 1), and intracellular adhesion molecules (ICAM-1) respectively. (1–3). Recent studies have reported that the endothelial niche is divided into the sinusoidal place and vascular niche. They are both recognized by the positive marker for vascular endothelial adhesion molecule (VE-cadherin) and CD31, but the sinusoidal slot expresses positive attributes for Vascular endothelial growth receptor factor 3 (VEGFR-3)(4–7). Besides, most studies have documented that endothelial cells express a positive marker such as; von Willebrand factor (VWF), intracellular adhesion molecule 1 (ICAM-1 /CD106), vascular adhesion molecule 1 (VCAM-

1/CD105), E-selectin, BMA120, and endothelial selective adhesion molecule (ESAM). (8–10).Endothelial cells dysfunction within the bone marrow environment due to chemotherapeutic agents or radiotherapy may result in the deletion of VEGFR2 in adult mice. It may prevent the renewal of the sinusoidal endothelial and the recovery of hematopoietic stem cells. (11). The isolation of endothelial cells from the bone marrow could use to evaluate the functions and malfunctions of the endothelial cell in vitro studies. There are different types of endothelial cells, such as human umbilical vein endothelial cells (HUVECs), and mouse brain-derived endothelial cells (MBDECs). The existence of endothelial cells in different locations exhibits similar functions, such as recruitment of progenitor cells in response to inflammatory stimuli and capillary-like lumen tube formation (12). Although one of the recommended methods to evaluate endothelial cells is by flow cytometry analysis. Still, the conviction of real endothelial cells has proven difficult due to the lack of a particular monoclonal antibody against the cells. In this study, we developed a strategic method for isolation of mouse bone marrow endothelial cells by cell adhesion method followed by single-cell suspension with magnetic MLECs beads incubation and bounded cell isolation. This protocol can use to evaluate the function and dysfunction of bone marrow-derived endothelial cells in vitro studies. We will also highlight factors that need to take into consideration when using this method.

Materials And Methods

MATERIALS:

1. Dulbecco's phosphate buffer solution (DPBS) potassium chloride (KCl) 0.2 g/L, sodium chloride (NaCl) 8.0 g/L, sodium phosphate Dibasic (Na_2HPO_4) 1.15 g/L potassium phosphate monobasic (KH_2PO_4) 0.2 g/L in Milli-Q water [pH 7.2–7.6 adjusted with hydrochloride acid (HCl) sterilized and store at 4^0C.
2. 0.5molar Ethylenediaminetetraacetic acid (EDTA) sterile by autoclave store at 4^0C
3. Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) (gibco, Thermo Fisher Scientific).
4. 0.5 ml nest micro-centrifuge tube and 1.5 ml Eppendorf centrifuge tube, 18G needle and syringe, 70um cell strainer (Biologix group limited), Sterile 15 ml and 50 ml conical tube and 70% ethanol
5. Accutase solution (absin, Biochemical Company).
6. 10 mm cell culture dish, 12–48 well plates
7. Endothelial cells medium (EBM-2), [Lonza, Clonetics®] containing growth factors: 25 ml Fetal bovine serum (FBS), 0.2 ml hydrocortisone 2 ml, and 0.5 ml of Human fibroblast growth factor (hFGF), vascular endothelial growth factor (VEGF), Human epidermal growth factor (hEGF) ascorbic acid Heparin, Gentamicin/Amphotericin.
8. 10,000uints/ml) /streptomycin 10,000ug/ml (gibco, Thermo Fisher Scientific).
9. Micro-dissecting board, Surgical scissors, sterile gloves, and Gauze.
10. Rat tail collagen type 1 3 mg/ml (Catalog #A10483-01) plus 0.2M sterile HCL

11. CD 31 microbeads, MS column (MACS, Miltenyi Biotec).

Equipment:

1. Laminar flow hook. (Thermo Fisher Scientific).
2. High and low-speed centrifuge
3. Confocal microscopy. (Nikon Eclipse Ti microscopy, Tokyo, Japan).
4. Corning® Matrigel® Basement membrane matrix, Phenol Red-free. *LDEV-free
5. Flow cytometry (BD LSRFortessa ™)
6. 5% CO2 humidified incubator
7. Autoclave machine
8. Roche real-time quantitative machine
9. Bio-Rad agarose electrophoresis machine
10. Magnetic separation column.

Pre-requisite solution:

1. 1 mm Ethylenediamine tetra acetic acid (EDTA)/DBPS (100 ul of 0.5M EDTA in 50 ml DPBS)
2. Dulbecco's Modified Eagle Medium (DMEM) plus 20% fetal bovine serum (FBS) ,and 500 ul of penicillin (10,000uints/ml) /streptomycin 10,000ug/ml
3. 50ug/cm^2 rat tail collagen type 1 in 0.02M HCl, pre-coat the cell culture dish overnight in the incubator at 37^0 C.
4. Prepare an appropriate volume of the endothelial cell growth media containing the factor, minimum 50 ml store at 4^0 C for 1 month.
5. Harvesting buffer solution (2% FBS/1 mm EDTA/DBPS) store at ore at 4^0 C for 1 month.

Animal model:

The wild type C57BL/6 mice (age 8-12weeks) obtained from the Jackson laboratory. The mice housed per the guidelines of Xuzhou Medical University, Jiangsu Province, China. The use committee approved the animal studies of Xuzhou Medical University (Xuzhou, China) and the National Institute of Health guide for the care and use of laboratory animals, Jiangsu province, China. **(Acceptance number: XZMC20130226).**

DAY 1

Step-by step process of bone marrow extraction:

1. Mice aged 8–12 weeks terminated by cervical dislocation, and the whole mice soaked in 70% ethanol for 2-5minutes, then placed on the sterile dissecting board on the laminar flow hook. The

long bones of the femora and tibias pulled off with micro-dissecting scissors placed in sterile Dulbecco's phosphate buffer solution (DPBS).

2. The muscles were detached from the bones by forceps, and the bones scrubbed to remove any residual soft tissues. The bones were washed twice with DPBS solution containing 1mmEDTA. One edge of the long bones cut –off place in a new dish containing the DPBS/EDTA solution.
3. An 18G needle pushed to the bottom of the 0.5 ml nest microcentrifuge tube. The cut edge of the long bone inverted downwards in the 0.5 ml nest microcentrifuge (maximum of 2 tibias and 2 femora) and the lid closed.
4. The nest of the 0.5 ml micro-centrifuge tube transfer into a 1.5 ml centrifuge tube, sealed with Parafilm and centrifuge at 15,000 g for 30 seconds. The nest 0.5 ml microcentrifuge discarded and the visible pellet at the bottom of the 1.5 ml Eppendorf tubes suspended with sterile DPBS/EDTA solution.
5. The suspended bone marrow cells with the PBS/EDTA solution, filtered with 70um cell strainer (Biologix group limited) and centrifuge at 300 g for 5 minutes at room temperature. The pellet resuspended in DMEM supplemented with 20% fetal bovine serum (FBS), and 500 ul of penicillin (10,000uints/ml) /streptomycin 10,000ug/ml.
6. The cells plated in a sterile 10 mm dish with a minimum of 10^9 cells/plate and incubated in a 5% CO_2 humidified incubator overnight.

DAY 2

Step-by-step Process of isolating bone marrow-derived endothelial cells:

1. After overnight incubation, the floating cells poured off. The adhered cells were washed with pre-warmed DPBS twice and detached with appropriate accutase solution for 15 minutes at room temperature.
2. The detachment stopped by adding the harvesting buffer PBS, 2% FBS, 1 mm Ethylenediaminetetraacetic acid (EDTA), and penicillin/streptomycin) with repeated pipetting to detached the remaining cells. The detach cells transferred into 15 ml tubes, centrifuge at 300 g for 10 minutes at room temperature. The total cell number determined by hemocytometer or automated cell counting machine.
3. The pellet cells resuspended with the harvesting buffer solution CD31 microbeads by manufacturer's protocols and stored at 4^0 C for 15 minutes.
4. The cells resuspended with 10 ml of harvesting buffer solution, then centrifuge at 300 g for 10 minutes at room temperature to wash off the excess beads from the cells after 15 minutes incubation.
5. The MS column attached to the magnetic washed once with the harvesting buffer, bone marrow cells pass through the column. The magnetic cells were washed three times with the harvesting buffer followed by a single wash with endothelial cell medium containing the factors.

6. The magnetic cells pushed into new sterile tubes with the endothelial cell media containing the additional growth factors, the total cells count determined by hemocytometer or automated cell counting machine.
7. The plate pre-coated with rat-tail collagen type 1, wash with sterile DPBS solution twice followed one wash with the endothelial cells growth media. Appropriate cell number seeded into the coated dishes and incubate into the incubator.

CHARACTERIZATION OF PRIMARY BONE MARROW-DERIVED ENDOTHELIAL CELLS (BMDECS).

Bone endothelial cell structure visualization

The cultured cells sequentially observed every day for the capillary-like structure appearance of endothelial cells with confocal microscopy and photographed. (Nikon Eclipse Ti microscopy, Tokyo, Japan).

Matrigel capillary tube formation assay of BMDECs

Matrigel was allowed to thaw on ice overnight according to the manufacture's protocols. Prechilled 24 well plates were coated on ice with 200 ul Matrigel per well. Gels were incubated for 30 minutes for solidification at 37 ^{0} C. BMDECs 10^5 cells, resuspended in 500 ul of the pre-cold endothelial cell medium supplemented with growth factors and cell plated on the gels. The plated cells were incubated in the humidifier at 37^{0} C for 4–7 days. The morphological changes are periodically monitored and photographed.

Identification of bone marrow endothelial cells by flow cytometry analysis

The selected CD31 positive cells analyzed by flow cytometry with a panel of antibodies; [CD31 (PE. anti-mouse, eBioscience™), CD45 (FITC anti-mouse, eBioscience™) CD106 (PE, Rat-anti-mouse, BD-Pharmingen™), CD144 (APC, anti-mouse, eBioscience™) and ESAM (APC, anti-mouse, Biolegend®) antibodies]. 500,000cells/ml were collected per tube, washed with PBS, centrifuge at 400 g for 5 minutes at room temperature (x2), incubated with the recommended dilution of antibodies, store at 4^{0} C for one hour, and analyzed by flow cytometry (BD LSRFortessa ™) within 24 hours. The lower threshold uses to exclude debris and the live cells with gating (20,000 cells) according to FSC x SSC, followed by sections containing the antibodies. The data retrieve from the flow cytometry software and analyze by flow Jo software version 7.6.2.

Characterization of primary bone marrow-derived endothelial cells by Real-time quantitative PCR (RT-qPCR)

To verify the molecular expression of the bone marrow endothelial cells, total RNA extracted from the cells after 7 days of incubation and the negative cells immediately after isolation using Trizol reagents

(TIANGEN Cat#dp424). The cDNA is synthesized by using 5X All in one RT Master mix (Cat.No.G492) and kept at -20 ^{0}C. Primers sequences and probe are shown in (Table 1). For RT-qPCR, the synthesized cDNA samples 10 ng were amplified with the SYBR green master mix in a final volume of 20 ul, as described in our previously published article(13). The mean threshold values are used to evaluate the molecular gene expression with normalization with mouse beta-actin.

Validation of bone marrow endothelial cells by immunoblotting analysis

To examine the molecular expression of bone marrow endothelial cells. The cells were placed in a plate pre-coated with rat tail collagen type 1 washed with cold PBS, and a herpes-chap lysis buffer containing the protease inhibitors pour into the dish, incubate for 10 minutes, and adherent cells scraped off with cell scraper. The lysed cells were centrifuged at 20,000 g for 30 minutes at 4^0 C. While For the non-endothelial cells (CD31 negative), the protein isolated immediately after isolation and protein stored at -20^{0}C until ready for use. The cell supernatants run on SDS-PAGE 8–12% gel (BIO-RAD; Hercules, CA). The proteins were transferred to the P 0.45 PVDF blotting membrane (Amersham™Hybond™ Germany) by the wet transfer method. Primary and secondary antibodies are shown in (Table 2).

Characterization of BMDECs by immunofluorescence staining

To certify the bone marrow endothelial cells for the expression of **CD31** (PECAM-1), VE-cadherin (CD144), and ICAM-1 with passage zero determine by direct immunofluorescence staining. The CD31 positive cells were plated into pre-coated 48-well with rat tail collagen type 1 for 5–7 days, as described above. After 60–70% of the confluence, the medium removed, cells fixed with 4% paraformaldehyde (PF for 20 minutes at RT, followed by two wash 3 minutes apart with PBS. The cells were permeabilized with 100% cold methanol at room temperature for 20 minutes, rinse with PBS three times and blocked with 1% BSA/PBS for 1 hour at room temperature. Cells incubated with the recommended dilution of primary antibodies overnight at 4^{0}C. The cells were cleaned twice with PBS and counterstained with Hoechst for 5 minutes at 37^{0}C. The cells were washed with PBS and imaging acquired using inverted Nikon microscopy (Nikon Eclipse Ti microscopy, Tokyo, Japan). Primary and secondary antibodies are shown in (Table 2).

INDUCTION OF PRIMARY BONE MARROW DERIVED ENDOTHELIAL CELLS BY RECOMBINANT TUMOR NECROSIS FACTOR ALPHA (TNF-α).

Assessment of primary bone marrow-derived endothelial cell proliferation

To examine the cell proliferation of primary bone marrow-derived endothelial cells, 10^{5}cells were seed in 48 well plates incubated for 7 days. After 7 days, the BMDECs was stimulating with recombinant TNF-α (10 ng/ml) and the control with 1% FBS with DPBS for 48hrs, the media containing the recombinant cytokine and FBS/DPBS were replaced with new endothelial media and incubated for another 7 days. The cells were harvest by trypsin/ETDA (VICMED 0.25% 0.02%), centrifuge at 350 g for 5 minutes, wash 2x with PBS, and fixed with 70% cold –ethanol at -20^{0}C for 1hour. The fixed cells centrifuge as above followed by washed with FACS buffer incubated with Ki-67 (FITC, anti-mouse, Biolegend®) and F4/80

(APC, anti-mouse as isotype control Biolegend®) for 30 minutes at room temperature. The data acquired by flow cytometry. For the cell count, the cells were stain with trypan blue and live cells counted by hemocytometer.

The initiation of bone marrow endothelial cells by recombinant tumor necrosis factor-alpha

The molecular expression of markers specific for bone marrow endothelial cells verifies by; real-time quantitative polymerase chain reaction (RT-qPCR) or immunoblotting and immunofluorescence staining. The cells were cultured for 7 days to form a confluence, then stimulated with TNF-A at (10 ng/ml) and vehicle control (2% fetal bovine serum in PBS) for 48hrs. Cells were then harvested for RT-qPCR, western blotting analysis and stained for immunofluorescence, all samples done in triplicate, and results expressed in mean with standard deviation.

Statistical analysis

All data were statistically analyzed using Graph Prism 6 and paired two-tailed student's test used for comparison mean ± standard deviation. P-values ≤ 0.05 are considered statistically significant.

Results

Morphological observation and tube formation of bone marrow endothelial cells: In brief, after isolation of the bone marrow endothelial cells, as shown in Fig. 1. The 12-well plates pre-coated with 50 µg/L with rat tail collagen type 1 overnight in the humidifier incubate at 37^0C. The pre-coated dishes were washed twice with sterile DPBS and rinse once with endothelial cells growth media. The isolated cells culture in the 12-well plates and observed daily for the appearance of the cobblestone with the confocal microscopy. On the third day, the ameboid-like shape is observed and typical cobblestone of bone marrow endothelial on day 7 as shown in Fig. 2B & C. For bone marrow endothelial cells tube formation, the 24 well Prechilled on ice and pre-coated with matrigel solution. The pre-coated well incubated at 37^0C for 30 minutes. 100, 000 cells culture into the matrigel basement membrane and observed daily for the capillary-like tube formation of the cells. The tube formation of the bone marrow endothelial cells observed on a fourth day, as shown in Fig. 2D. The in vitro capillary-like structure is characteristic of bone marrow endothelial cells.

Flow cytometry analysis of bone marrow endothelial cells: Briefly, the floating cells discarded after overnight incubation, and the adhered cells detached with accutase solution. The resuspended cells centrifuge and the bone marrow endothelial cells were selected with CD31 microbeads. The CD31 positive cells stain for surface markers specific for bone marrow endothelial cells and the unstained cells as an isotype control. The bone marrow endothelial cells express > 98% of CD45 negative and positive expression for; CD31 (83.70 ± 0.0999), CD106 (85.46 ± 1.925) CD144 (63.35 ± 0.707), ESAM (68.65 ± 0.6364) respectively compared to the control as shown in Fig. 3(A-E). This data shows that the isolated cells are bone marrow endothelial cells, which can be used in vitro studies of endothelial cells function and malfunction in mice model.

Real-time quantitative PCR and immunoblotting: The fold change of the relative messenger RNA for bone marrow endothelial cells is more significant than the non-endothelial cells for ICAM-1, VCAM-1 ESAM, and VE-cadherin, respectively, as shown in figure (4A-D). Furthermore, the immunoblotting shows an increase in the expression of E-selectin, VE-cadherin, PECAM-1, ICAM-1, and VCAM-1, respectively, as shown in Fig. 4E.

Certification of primary bone marrow-derived endothelial cells by immunofluorescence staining: To certified the purity of the primary bone marrow-derived endothelial cells, the cells were cultured in a 48-well plate, after 60–70% of the confluence is achieve then tested against anti- CD31 (PECAM-1), anti-VE-cadherin (CD144) and anti-CD106 (ICAM-1) respectively. The cell shows the expression of three EC markers; PECAM-1, VE-cadherin, and ICAM-1, respectively, as shown in Fig. 5 (A-C). The mean fluorescence was quantified using Image J software on three experimental repeats, which reveals that expression of the EC-markers in order PECAM-1 > VE-cadherin > ICAM-1 as shown in Fig. 5(D).

The response of bone marrow endothelial cells to tumor necrosis factor-alpha: Briefly, the bone marrow endothelial cells culture for seven days and treated with recombinant tumor necrosis factor-alpha (10 ng/ml) for 48 hours and untreated cells with 2% FBS in PBS solution. Delta-delta threshold values determined the relative mRNA expression. The bone marrow endothelial cells response to tumor necrosis factor-alpha with the increase in fold change of; ICAM-1 (110.6 ± 19.8) and VCAM-1 (3.972 ± 0.6093), VE-cadherin (11.46 ± 2.034), and ESAM (3.556 ± 0.4622) respectively as shown in Fig. 6(A-D $p < 0.05$) versus the control. The immunoblotting results also confirmed an increase in protein expression of ICAM-1 and VCAM-1, respectively, as shown in Fig. 6 (E-J, $P < 0.05$). However, the protein expression of the VE-cadherin molecule decrease in response to TNF-α. The immunofluorescence staining also confirmed an increase in protein expression of ICAM-1 and decrease protein expression of VE-cadherin compared to the control, as shown in Fig. 7 (C-E, $P < 0.05$).Also, the bone marrow endothelial cells respond to TNF-α by increases in number, as shown in Fig. 7(A-B $p < 0.05$). This data indicates that TN-α enhances the proliferation and growth of bone marrow endothelial cells in vitro studies.

Discussion

In this study, we outline the step-by-step process of extracting bone marrow endothelial cells from mice long bones. Since bone marrow, endothelial cells are a critical component of the sinusoidal-vascular niche in the bone marrow microenvironment. There are no established cell lines for in vitro studies of bone marrow endothelial cells, which respond to inflammatory stimuli and promote hematopoietic stem cell regeneration. The morphological identification of the endothelial cells is a critical step in the process of demonstrating a functional vascular network that is associate with proliferation and differentiation, followed by elongation and assemble to a capillary-like a lumen/linear cord-like vessels forming an infusible vascular tube. (14–16) The amboidal –like the shape of the primary bone marrow-derived endothelial cells seen on the third-day post-culture, atypical cobblestone structure of the cells appeared on day 7, and the perfusable capillary-like lumen forming tube confirmed with Matrigel assay. The bone marrow microenvironment consists of three vascular networks; arterioles, transitional, and sinusoids. The

endothelial cells are enclosed to sinusoid- vascular that form the endothelial –vascular niche in the hematopoietic stem cell that supports the maintenance and retaining the function of the stem cell niches. Besides, within the perivascular slots of the HSC they are interconnected by endothelial adherent molecules, including; VE-cadherin and ESAM as well as endothelial immunoglobulin-like adhesion molecules including; PECAM-1 (CD31), E- selectin, ICAM-1 (CD106) and VCAM-1 (CD105)(5,9,17,18). Therefore, validation of primary bone marrow-derived endothelial cells using these molecules will be essential in the isolation of pure endothelial cells from mice bone marrow. The cells isolated demonstrate the characteristic expression of these adhesion molecules. We further verified by the expression of the relative messenger RNA and the protein expression of these adhesion molecules, respectively. Also, primary bone marrow-derived endothelial cells were certified by immunofluorescence staining and show features of these adhesion molecules in sequential order PECAM-1, VE-cadherin and ICAM-1 respectively. Finally, we evaluated the response of bone marrow endothelial cells with recombinant tumor necrosis factor-alpha. Our results show that bone marrow endothelial cells increase in proliferation and expression of adhesion molecules, respectively. Also, the protein expression of vascular endothelial cadherin decrease with the change in the typical shape of the cells (19–22).

The importance and future application of bone marrow endothelial cells: The method can be used in vitro study to evaluate the functions and dysregulation of bone marrow endothelial cells for stem cell research of mice model. The significance of this method will provide an excellent opportunity to evaluate the endothelial-vascular niche in the hematopoietic stem. This technique will improve the practicality of endothelial stem cell research.

Conclusion

We established a method for isolation of primary bone marrow-derived endothelial cells from mice bone marrow, which can be used to evaluate the function of endothelial cells in vitro studies. We suggest the following factors must be considered when isolating these cells.

PRE-REQUISITES PROCESSING

1. Before isolation, boil all instruments, air dry in the oven for at least 30 minutes, and add Gauze to autoclave.
2. Prepare fresh DPBS solution with PH 7.2–7.6, 0.5 molar EDTA and sterilize
3. Sterilize 0.5 ml tube and 1.5 ml tubes for density centrifugation of the bone marrow cell from the mice bones.
4. Prepare sterile 1mmEDTA/DPBS and harvesting buffer solution kept at 4^0C for 30 days only.
5. Pre-coat the dish with rat tail collagen overnight at 37^0C in the incubator, wash three times with sterile DPBS, air dry for 30 seconds before pouring the endothelial cell growth media

ISOLATION

1. Try to spend less time on harvesting the bones from the mice, wash bones with sterile DPBS, and a final wash with DPBS containing 1mmEDTA to prevent blood clotting and clumping of the cells.
2. Use a 5 ml syringe needle to perforate the center of the 0.5 ml tube and invert the cut edge of the bones (maximum 4 2 tibias, 2 femora), suspend the pellet with DPBS/1mmEDTA.
3. Incubate the bone marrow cells, not more 24 hours.
4. Change medium after 3–4 days to maintain the cell numbers.

LIMITATION

1. To get enough cells its involve a large number of mice (age 6–12 weeks), especially for immunoblotting techniques.
2. The cell passage number should not exceed two passages (Passage 0 and 1 recommended). More than 3 passages, the cells are prone to fungal infection and cell drift.
3. Contamination of primary bone marrow-derived endothelial cells is common with macrophages, fibroblasts, if incubated cells for than 24 hr. To avoid that, try to harvest cells less than 24 ours incubation and repeats passage for the cells twice via the column.
4. The endothelial growth media is susceptible to infection, have an excellent working sterile environment key to get optimal results. Density centrifugation minimizes the risk of contamination.
5. The primary bone marrow-derived endothelial have difficulties attached to the glass slide for immunofluorescence staining for this procedure using 48 or 96 wells to get optimal results.

List Of Abbreviations

- Bone marrow-derived endothelial cells (BMDECs)
- Bone marrow endothelial cells (BMECs)
- Endothelial cells (ECs)
- Intracellular adhesion molecule- 1 (ICAM-1)
- Vascular adhesion molecule-1(VCAM-1)
- Vascular endothelial selectin adhesion molecules (ESAM)
- Vascular endothelial cadherin molecule (VE-cadherin)
- Ethylenediaminetetraacetic acid (EDTA
- Dulbecco's phosphate buffer solution (DPBS

Declarations

Ethical approval for animal studies

The use committee of Xuzhou Medical University (Xuzhou, China) and the National Institute of Health guide for the care and use of laboratory animals, Jiangsu province, China approved the studies with acceptance number: **XZMC20130226.**

Consent for participants

Not applicable

Consent for publication

not applicable.

Data availability

All relevant data in the study included in the article; further inquiries can be directed to the corresponding authors.

Conflict of interest

The authors declared that this study was not conducted for financial gain or any commercial part that could be construed as a potential conflict of interest

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Author's contribution

A.O.S planned the experiment and worked with S. Y. A through the experimental procedure. Results analysis done by A. O. S along with S. Y. A and W. J., The corresponding authors, give expert advice and provide technical support throughout the experiment.

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Tables

Table 1: Quantitative PCR primers sequences and immunoblotting antibodies

TARGET GENES	FORWARD 5' ⟶ 3.'	REVERSE 3' ⟶ 5.'
BETA ACTIN	ATGTGGATCAGCAAGCAGGA	AAGGGTGTAAAACGCAGCTCA
ESAM	CCTGGTCAGTAGCCTGGTTC	CCTGGTCAGTAGCCTGGTTC
VE-CADHERIN	TTTGCCCAGCCCTACGAACC	ACCGCCGTCATTGTCTGCCT
ICAM-1	GGCATTGTTCTCTAATGTCTCCG	GGCATTGTTCTCTAATGTCTCCG
VCAM-1	GGCATTGTTCTCTAATGTCTCCG	CTCCTTCACACACATAGACTCC

Table 2
Western blot and immunofluorescence staining antibodies

ANTIGEN	Ab SPECIES	COMPANY	CAT. NUMBER
Rabbit Ig-HRP	GOAT	Absin	AS001
Mouse Ig-HRP	GOAT		
GAPDH	Rabbit-polyclonal	Service bio	GB1100
E-SELECTIN	Mouse monoclonal	Santa Cruza Biotechnology	G2718
VE-cadherin (CD144)	Rabbit-polyclonal	Affinity Bioscience	AF6265
PECAM-1 (CD31)	Rabbit-polyclonal	Affinity Bioscience	AF6191
ICAM-1 (CD106)	Rabbit-polyclonal	Affinity Bioscience	AF6088
VCAM-1 (CD105)	Rabbit-polyclonal	Affinity Bioscience	DF6082

Figures

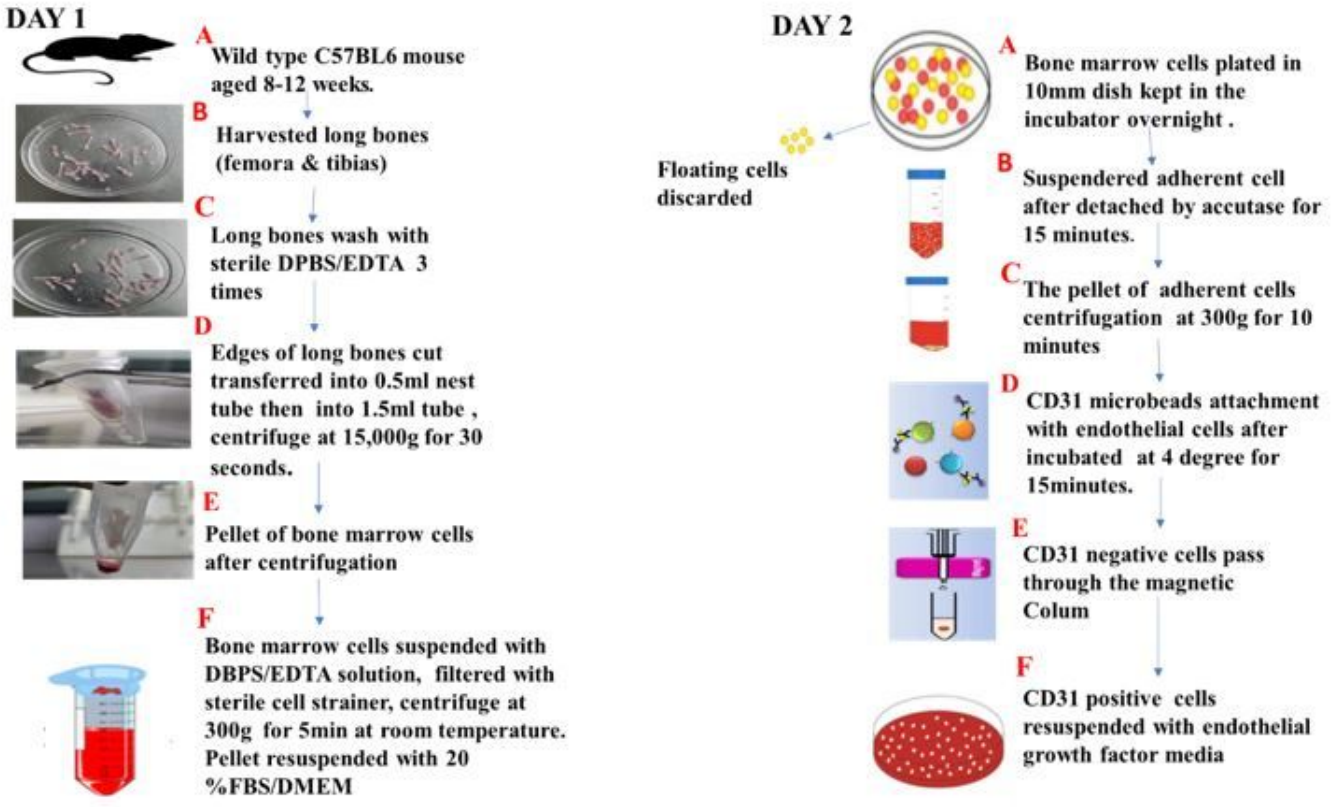

Figure 1

Step-by-step process of isolating bone marrow endothelial cells: DAY 1: (A) the mice were killed by cervical dislocations soak in 70% ethanol for 2 minutes. (B) Long bones of femur and tibia harvested placed in sterile dish after dissecting the muscle, tendon, and ligaments. (C) The long bones were washed with DPBS/EDTA solution three times. (D) One end of the long bone cut-off with dissecting forceps and inserted into the 0.5ml nest microtubes, centrifuge at 15,000 for 30seconds. (E) The pellet of the bone marrow after centrifugation (F) The bone marrow pellet suspended with DPBS/EDTA solution, filtered with a cell strainer and centrifuge at 300g for 5 minutes at room temperature. DAY 2: (A) the bone marrow cells resuspended with DMEM supplemented with 20% FBS plus antibiotics and incubate in the incubator overnight. Floating cells discarded and adhered cells washed twice with DPBS/EDTA solution. (B) Accutase solution poured into the dish to detach the attached cells for 15 minutes at room temperature. (C) The cells resuspended with DPBS.EDTA solution and centrifuge at 300g for 10 minutes. (D) The resuspended bone marrow cells incubated at 40 C with the microbeads. (E) The beads pass via the column and wash three times. (F) The magnetic cells resuspended with endothelial media.

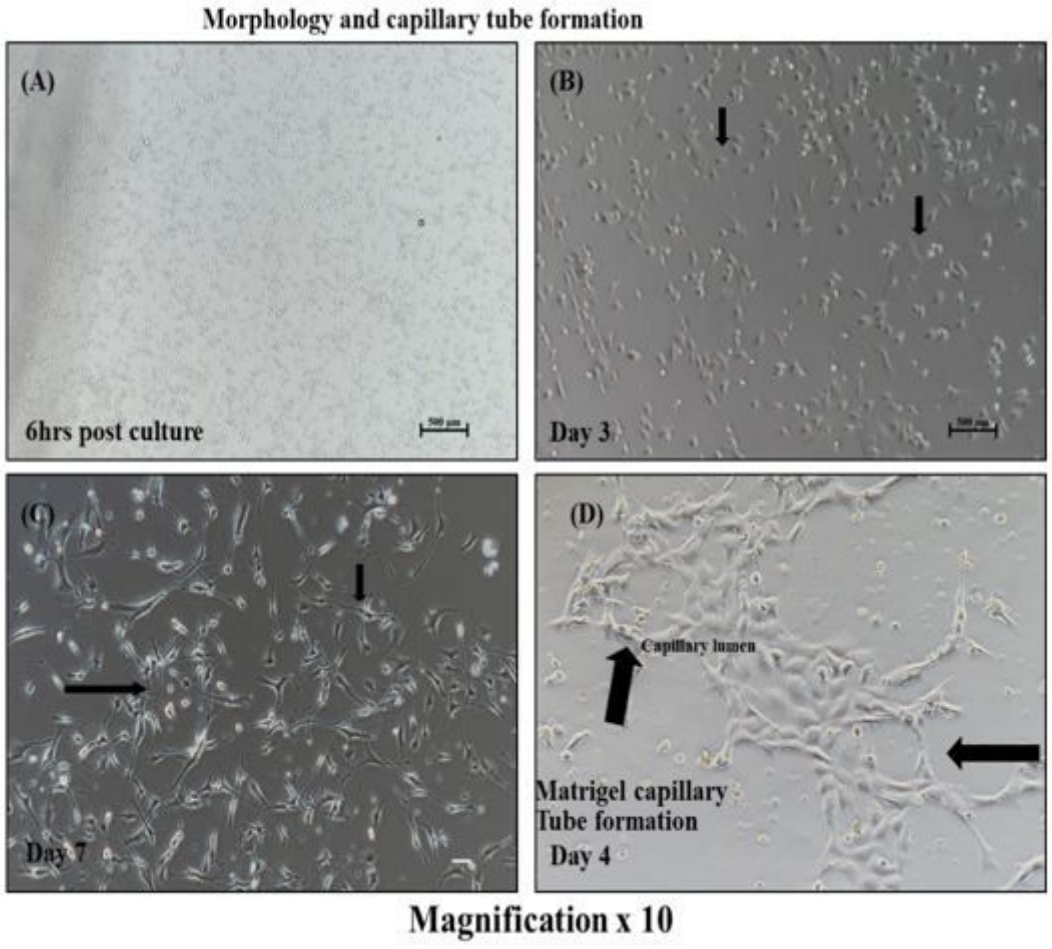

Figure 2

Morphology and tube formation of bone marrow endothelial cells; (A) Appearance of the cells after 6 hours of isolation (B) visualization of the ameboid-like structure of the bone marrow endothelial cells (C)

Full visualization of the cobblestone of the bone marrow endothelial cells. The arrows point toward the cobblestone appearance of BMDECs (D) Bone marrow endothelial cells tube formation by Matrigel assay and the arrows point to the capillary-like tubules of the cells on day 4 post cultured.

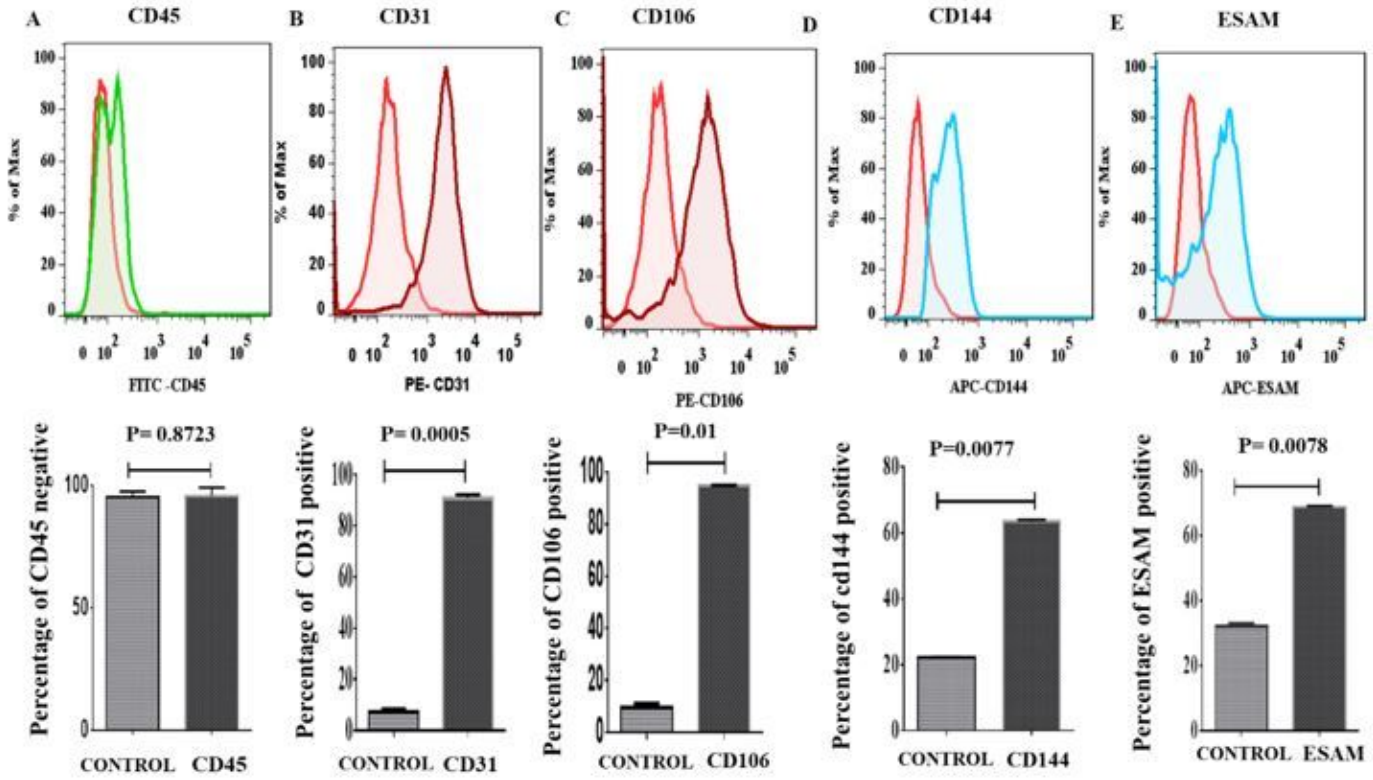

Figure 3

Characterization of primary bone marrow-derived endothelial cells by flow cytometry: (A) the primary BMDECs were negative for CD 45 (98.2%). (B) CD 31 (91.5%) (C) CD106 (94.9.46%), (D) CD144 (63.35%) and (E) Endothelial selective adhesion molecules (ESAM) (68.65 %). The red tented histograms represent the unstained cells as the isotype control, and the colored histograms indicate the antigen staining of the endothelial cells surface markers. The bar chart indicates the mean ± SD using the student's t- the test of two experimental repeats when compared to the isotype control (n =2) and p-value ≤ 0.05 and considered statistically significant. For CD 45, there is no statistical significance between the isotype control and the stained cells, which verify the purity of the endothelial cells as primary bone marrow-derived endothelial cells express CD45 negative.

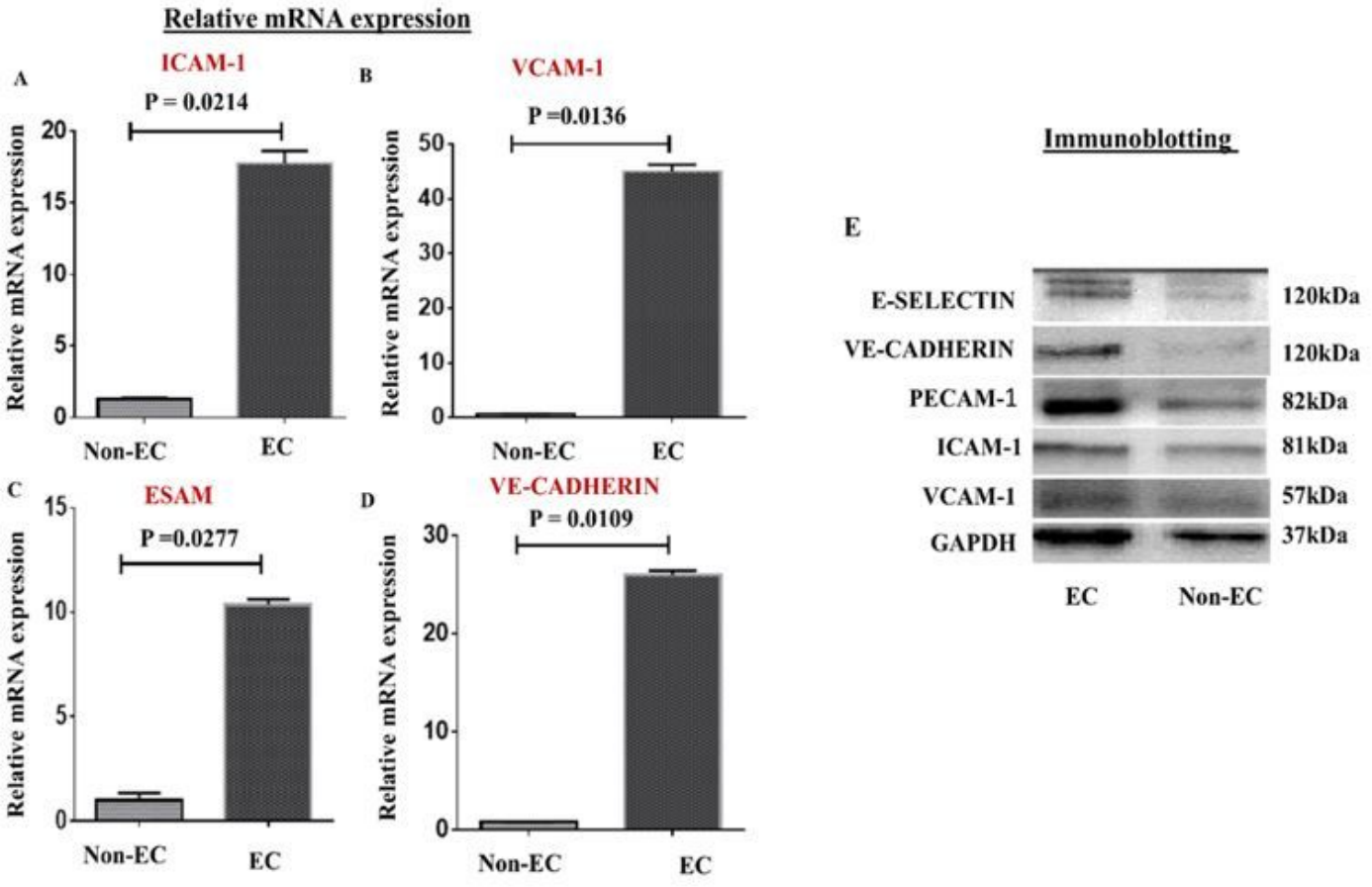

Figure 4

Characterization of primary bone marrow-derived endothelial cells by RT-QPCR and immunoblotting: (A-D) represents the relative mRNA expression of primary bone marrow-derived endothelial cells. The bar chart indicated the fold of the gene expression of primary BMDECs compared to the non-endothelial cells. The fold change is quantified by the Pitfalls method, followed by a student's test comparison between the Non-endothelial cells and endothelial cells. P-value ≤0.05 considered being statistically significant. (E) The immunoblotting analysis of primary bone marrow-derived endothelial after 7 days of cultured, which further substantiates the purity of endothelial cells (n= 3 indicates independent experiments).

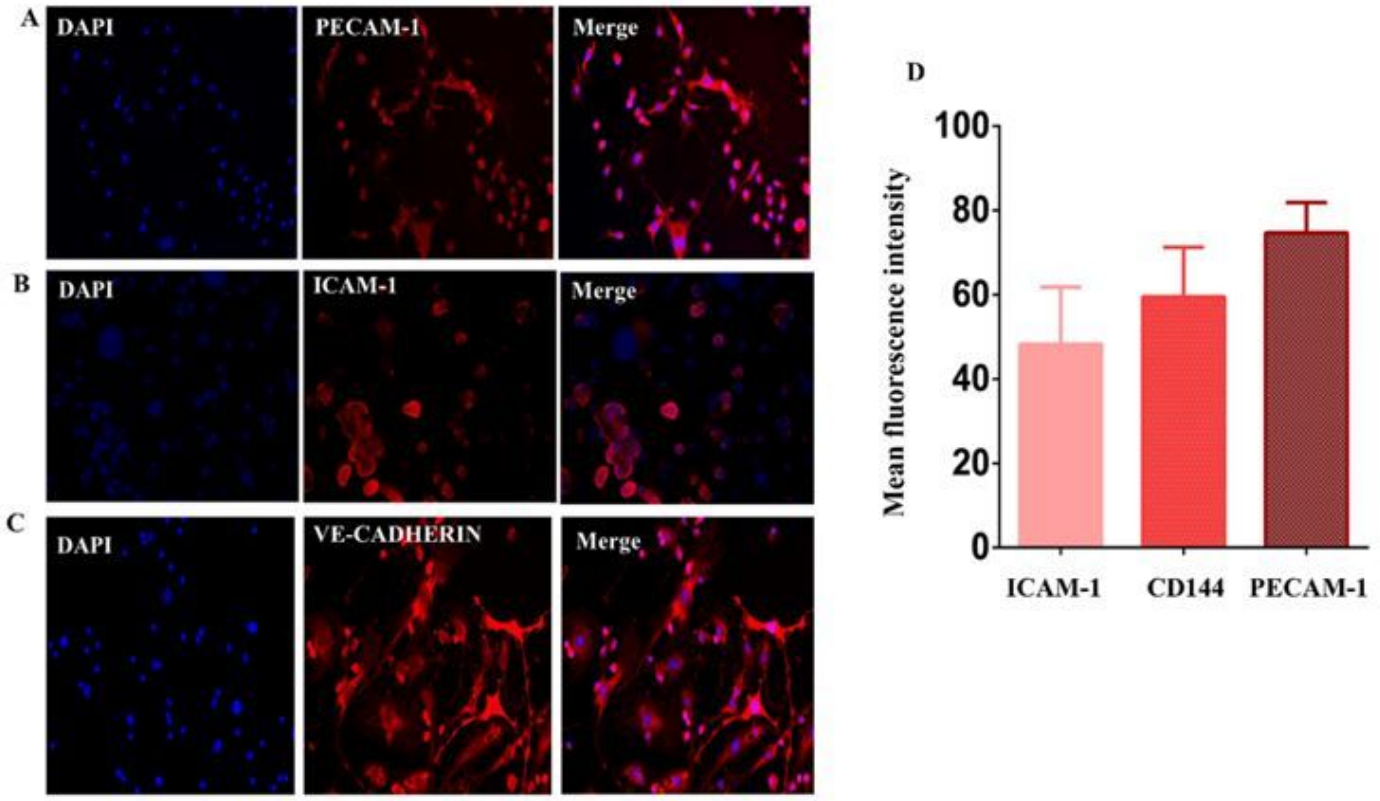

Figure 5

Identification of primary bone marrow-derived endothelial cells by immunofluorescence staining: (A) anti-CD31 (PECAM-1), (B) anti-CD106 (ICAM-1) and (C) anti-CD144 (VE-cadherin) the cell shows the expression of the three EC markers. (D) The mean fluorescence for the three EC-marker was relatively quantified by image J, which shows the order of the three EC-markers (PECAM-1> VE-cadherin > ICAM-1) n = 3, Scale bar 50um.

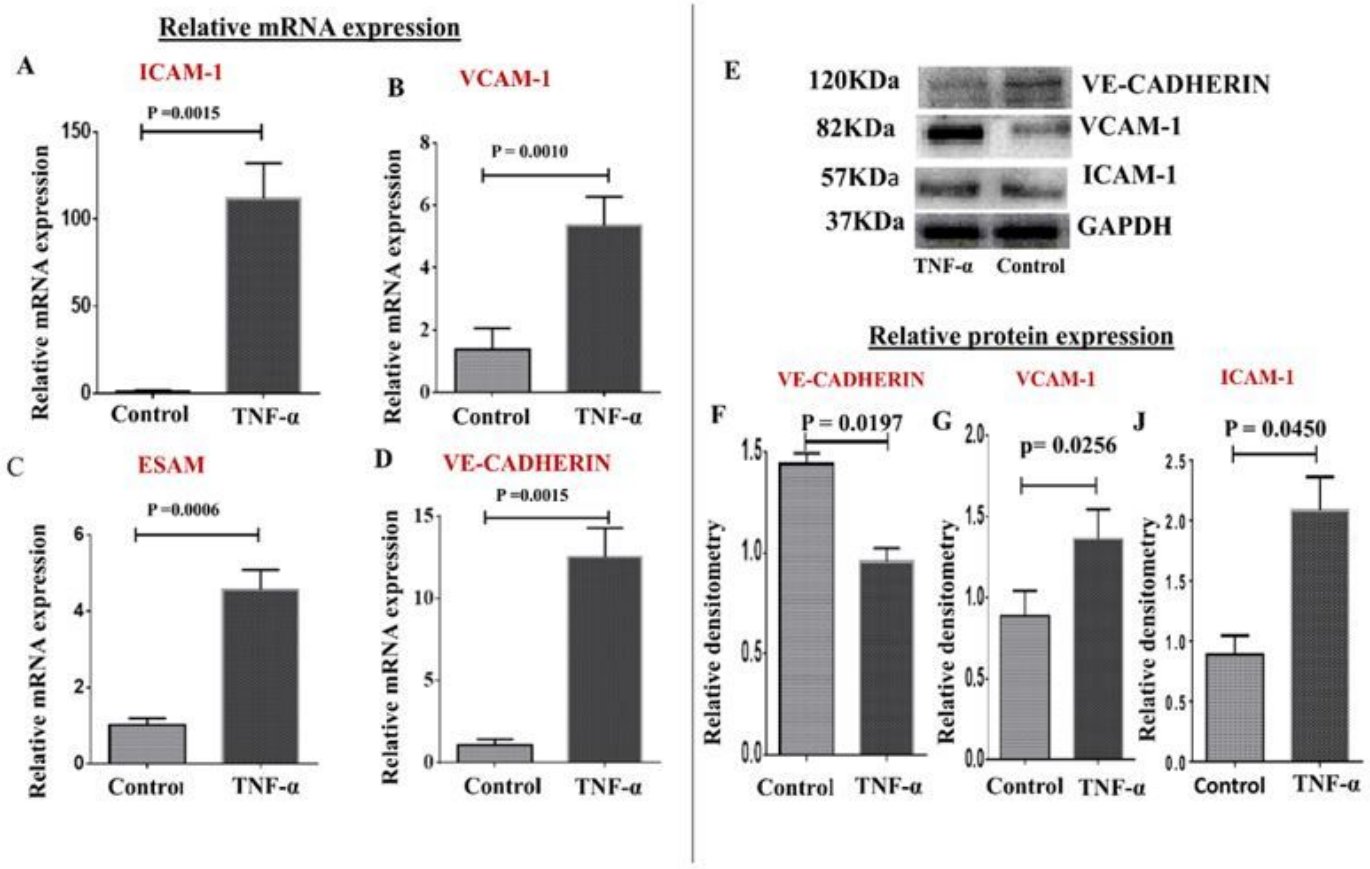

Figure 6

induction of primary bone marrow-derived endothelial cells by tumor necrosis factor-alpha and quantify by RT-qPCR and immunoblotting. (A-B) the relative mRNA expression molecule of intracellular adhesion molecule -1 (ICAM-1) and vascular adhesion molecule -1 (VCAM-1). (C-D) the mRNA expression of endothelial junction adherent molecules (ESAM and VE-cadherin) using beta-actin as a reference gene. (E) Western blot imaging of proteins. (F-H) the relative densitometry of VE-cadherin, VCAM-1, and ICAM-1 using GAPDH as endogenous gene image J software. The bar chart indicates the mean± SD by student's t-test, (n = 3 independent) experiment p-vale ≤ 0.05 considered statistically significant.

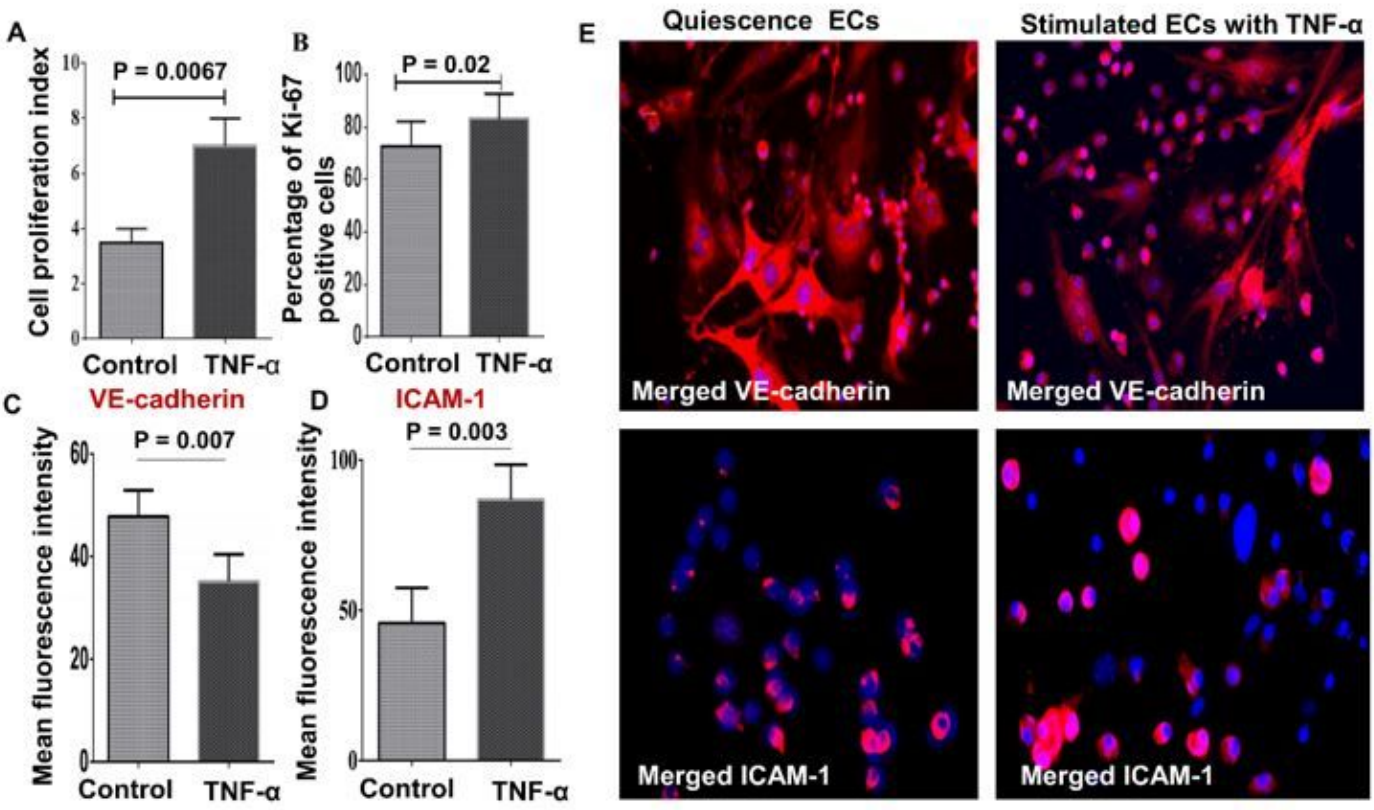

Figure 7

(A-B) Bone marrow endothelial cell proliferation Ki-67 by flow cytometry analysis and hemocytometer (C-D) The mean fluorescence of VE-cadherin and ICAM-1 in both quiescence and stimulated ECs quantified by image J presented in a bar chart with mean± SD (n = 3). (E) The merged immunofluorescence staining of both quiescence EC and treated endothelial cells with TNF-α. Scale bar = 50μm. n = 3.