# Efficient Differentiation of Human Induced Pluripotent Stem Cells into Endothelial Cells under Xenogeneic-free Conditions for Vascular Tissue Engineering

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**SUPPLEMENTARY MATERIAL**

**Table S1. Representative approaches for deriving endothelial cells from hiPSCs**

| Publication | Outcome | Xenogeneic Reagents |
|---|---|---|
| Patsch et al., 2015 [1] | hiPSC-ECs derived via cell monolayer-based method | mTeSR1 medium, Matrigel, Stempro-34 |
| Lian et al., 2014 [2] | hiPSC-derived endothelial progenitor cells and hiPSC-ECs via cell monolayer-based method | mTeSR1 medium, Matrigel, advanced DMEM/F12, EGM-2 medium |
| Prasain et al., 2014 [3] | hiPSC-derived endothelial colony forming cells via cell monolayer-based method | mTeSR1 medium, Matrigel, rat collagen, EGM-2 medium |
| Sivarapatna et al., 2015 [4] | hiPSC-ECs derived via embryoid body-based method | mTeSR1 medium, Matrigel, FBS, porcine gelatin |
| Qian et al., 2017 [5] | human pluripotent stem cell-derived, blood-brain barrier endothelial cells via cell monolayer-based method | mTeSR1 medium, Matrigel |

**Table S2. Components of culture media**

| medium | components |
|---|---|
| **Xenogeneic hiPSC culture medium** | mTeSR1 medium (STEMCELL Technologies, 85850) |
| **Xenogeneic-free hiPSC culture medium** | Essential 8™ Medium (E8 medium; ThermoFisher, A1517001) |
| **Xenogeneic mesoderm induction medium** | 47.5% (v/v) Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12, ThermoFisher, 11320033) and 47.5% (v/v) Neurobasal Medium (ThermoFisher, 21103049) supplemented with:<br>• 2% (v/v) B-27 Supplement minus vitamin A (ThermoFisher, 12587010)<br>• 1% (v/v) N-2 Supplement (ThermoFisher, 17502048)<br>• 1% (v/v) L-glutamine (2mM, ThermoFisher, 25030081)<br>• 1% (v/v) penicillin/streptomycin (pen/strep; ThermoFisher, 15140-122)<br>• 0.048 mM β-Mercaptoethanol (Sigma-Aldrich, M3148)<br>• 6 µM CHIR99021 (Selleckchem, s2924)<br>• 25 ng/mL animal-free recombinant human bone morphogenetic factor (BMP4, Peprotech, AF-120-05-ET; reconstituted in Dulbecco's Phosphate-Buffered Saline [PBS, ThermoFisher, 14190-144] containing 0.1% [w/v] bovine serum albumin [BSA, Sigma-Aldrich, A9647])<br>Sterilized with filtration, stored at 4°C up to one month |
| **Xenogeneic-free mesoderm induction medium** | 47.5% (v/v) CTS KnockOut DMEM/F-12 (ThermoFisher, A1370801) and 47.5% (v/v) CTS Neurobasal Medium (ThermoFisher, A1371201) supplemented with:<br>• 2% (v/v) xenogeneic-free, CTS B-27 Supplement minus vitamin A (ThermoFisher, A3353501)<br>• 1% (v/v) xenogeneic-free, CTS N-2 Supplement (ThermoFisher, A1370701)<br>• 1% (v/v) L-glutamine (2mM, ThermoFisher, 25030081)<br>• 1% (v/v) pen/strep (ThermoFisher, 15140-122)<br>• 0.048 mM β-Mercaptoethanol (Sigma-Aldrich, M3148)<br>• 6 µM CHIR99021 (Selleckchem, s2924) |

| | |
|---|---|
| | • 25 ng/mL animal-free recombinant human BMP4 (Peprotech, AF-120-05-ET; reconstituted in PBS containing 0.1% [w/v] human serum albumin [HSA, Propsecbio, PRO-354])<br>Sterilized with filtration, stored at 4°C up to one month |
| **Xenogeneic EC induction medium** | Stempro-34 SFM basal medium (ThermoFisher, 10639011) supplemented with:<br>• Stempro-Nutrient Supplement (ThermoFisher, 10639011)<br>• 1% (v/v) L-glutamine (2mM, ThermoFisher, 25030081)<br>• 1% (v/v) pen/strep (ThermoFisher, 15140-122)<br>• 200 ng/mL recombinant human recombinant human vascular endothelial growth factor (VEGF-A165, Peprotech, AF-100-20; reconstituted in PBS containing 0.1% [w/v] BSA)<br>• 2 µM Forskolin (Abcam, ab120058) |
| **Xenogeneic-free EC induction medium** | Xenogeneic-free Stemspan H3000 (STEMCELL Technologies, 09850) supplemented with:<br>• 1% (v/v) L-glutamine (2mM, ThermoFisher, 25030081)<br>• 1% (v/v) pen/strep (ThermoFisher, 15140-122)<br>• 200 ng/mL animal-free recombinant human VEGF-A 165 (Peprotech, AF-100-20; reconstituted in PBS containing 0.1% [w/v] HSA)<br>• 2 µM Forskolin (Abcam, ab120058) |
| **Xenogeneic primary EC expansion medium** | Complete EGM-2 Endothelial Cell Growth Medium-2 bullet kit (Lonza, CC-3162), containing EBM-2 Basal Medium supplemented with:<br>• 2% (v/v) fetal bovine serum (FBS, provided by EGM-2 bullet kit)<br>• 0.04% (v/v) Hydrocortisone (provided by EGM-2 bullet kit)<br>• 0.4% (v/v) human fibroblast growth factor 2 (FGF2, provided by EGM-2 bullet kit)<br>• 0.1% (v/v) human insulin-like growth factor-1 (R3-IGF-1, provided by EGM-2 bullet kit)<br>• 0.1% (v/v) ascorbic acid (provided by EGM-2 bullet kit)<br>• 0.1% (v/v) human epidermal growth factor (EGF, provided by EGM-2 bullet kit)<br>• 0.1% (v/v) gentamicin-amphotericin-B (GA-100, provided by EGM-2 bullet kit)<br>• 0.1% (v/v) heparin (provided by EGM-2 bullet kit)<br>• 0.1% (v/v) recombinant human vascular endothelial growth factor (VEGF, provided by EGM-2 bullet kit) |

| | |
|---|---|
| **Xenogeneic-free primary EC expansion medium** | EBM-2 basal medium (Lonza, CC-3156) supplemented with:<br>• 5% (v/v) pooled human serum (HuS; Seracare, 1830-0003)<br>• 10 ng/mL animal-free recombinant human FGF2 (Peprotech, AF-100-18C; reconstituted in PBS containing 0.1% [w/v] HSA)<br>• 0.5 ng/mL animal-free recombinant human VEGF-A 165 (Peprotech, AF-100-20; reconstituted in PBS containing 0.1% [w/v] HSA)<br>• 20 ng/mL animal-free recombinant human IGF1 (Peprotech, AF-100-11; reconstituted in PBS containing 0.1% [w/v] HSA)<br>• 5 ng/mL animal-free recombinant human EGF (Peprotech, AF-100-15; reconstituted in PBS containing 0.1% [w/v] HSA)<br>• 1% (v/v) pen/strep (ThermoFisher, 15140-122) |
| **Xenogeneic hiPSC-EC expansion medium** | EBM-2 Basal Medium from EGM-2 bullet kit (Lonza, CC-3162) supplemented with:<br>• 2% (v/v) fetal bovine serum (FBS, provided by EGM-2 bullet kit)<br>• 0.04% (v/v) Hydrocortisone (provided by EGM-2 bullet kit)<br>• 0.4% (v/v) human FGF2 (provided by EGM-2 bullet kit)<br>• 0.1% (v/v) human R3-IGF-1 (provided by EGM-2 bullet kit)<br>• 0.1% (v/v) ascorbic acid (provided by EGM-2 bullet kit)<br>• 0.1% (v/v) human EGF (provided by EGM-2 bullet kit)<br>• 0.1% (v/v) gentamicin-amphotericin-B (GA-100, provided by EGM-2 bullet kit)<br>• 0.1% (v/v) heparin (provided by EGM-2 bullet kit)<br>• 0.1% (v/v) recombinant human VEGF (provided by EGM-2 bullet kit)<br>• 50 ng/mL recombinant human VEGF-A 165 (Peprotech, AF-100-20; reconstituted in PBS containing 0.1% [w/v] BSA) |
| **Xenogeneic-free hiPSC-EC expansion medium** | EBM-2 basal medium (Lonza, CC-3156) supplemented with:<br>• 5% (v/v) pooled human serum (HuS; Seracare, 1830-0003)<br>• 10 ng/mL animal-free recombinant human FGF2 (Peprotech, AF-100-18C; animal-free reconstituted in PBS containing 0.1% [w/v] HSA)<br>• 50 ng/mL animal-free recombinant human VEGF-A 165 (Peprotech, AF-100-20; reconstituted in PBS containing 0.1% [w/v] HSA)<br>• 20 ng/mL animal-free recombinant human IGF1 (Peprotech, AF-100-11; reconstituted in PBS containing 0.1% [w/v] HSA) |

| | |
|---|---|
| | - 5 ng/mL animal-free recombinant human EGF (Peprotech, AF-100-15; reconstituted in PBS containing 0.1% [w/v] HSA)<br>- 1% (v/v) pen/strep (ThermoFisher) |

**Table S3. List of Primers for qRT-PCR.**

| Gene | Primer sequence |
|---|---|
| GAPDH-F | TGTTGCCATCAATGACCCCTT |
| GAPDH-R | CTCCACGACGTACTCAGCG |
| CD31-F | TGCAGTGGTTATCATCGGAGTG |
| CD31-R | CGTTGTTGGAGTTCAGAAGTG |
| VECAD-F | GCACCAGTTTGGCCAATATA |
| VECAD-R | GGGTTTTTGCATAATAAGCAGG |
| eNOS-F | GGAACCTGTGTGACCCTCA |
| eNOS-R | CGAGGTGGTCCGGGTATCC |
| KDR-F | TGCCTCAGAAGAGCTGAAAAC |
| KDR-R | CACAGACTCCCTGCTTTTGCT |
| OCT4-F | GCAGCTCGGAAGGCAGAT |
| OCT4-R | TGGATTTTAAAAGGCAGAAGACTTG |
| KLF2-F | ACTTTCGCCAGCCCGTGC |
| KLF2-R | AGTCCAGCACGCTGTTGAG |
| KLF4-F | CGAACCCACACAGGTGAGAA |
| KLF4-R | TACGGTAGTGCCTGGTCAGTTC |
| VCAM-1-F | CCTGAGCCCTGTGAGTTTTG |
| VCAM-1-R | GGGTACACGCTAGGAACCTT |
| ICAM-1-F | ACCATCTACAGCTTTCCGGC |
| ICAM-1-R | CAATCCCTCTCGTCCAGTCG |

**Table S4. List of antibodies for immunostaining.**

| Antibody | Vendor | Product Identifier |
|---|---|---|
| CD31 (flow cytometric analysis) | BD Pharmingen | 555445 |
| VE-cadherin (flow cytometric analysis) | Santa Cruz | sc-9989 |
| CD31 (immunostaining) | Abcam | ab28364 |
| VE-cadherin (immunostaining) | Santa Cruz | sc-6458 |
| eNOS | Abcam | ab5589 |
| vWF | Abcam | ab6994 |
| OCT4 | Abcam | ab18976 |
| collagen type IV | Abcam | ab6586 |
| laminin beta 1 | Lifespan Bio Sciences | LS-C190219 |
| Alexa 488 goat anti-mouse IgG | ThermoFisher | A11029 |
| Alexa 488 goat anti-rabbit IgG | ThermoFisher | A11008 |
| Alexa 555 goat anti-mouse IgG | ThermoFisher | A21426 |
| Alexa 555 goat anti-rabbit IgG | ThermoFisher | A21428 |
| Mouse IgG Isotype Control, FITC | ThermoFisher | 31505 |

**Supplementary Figures**
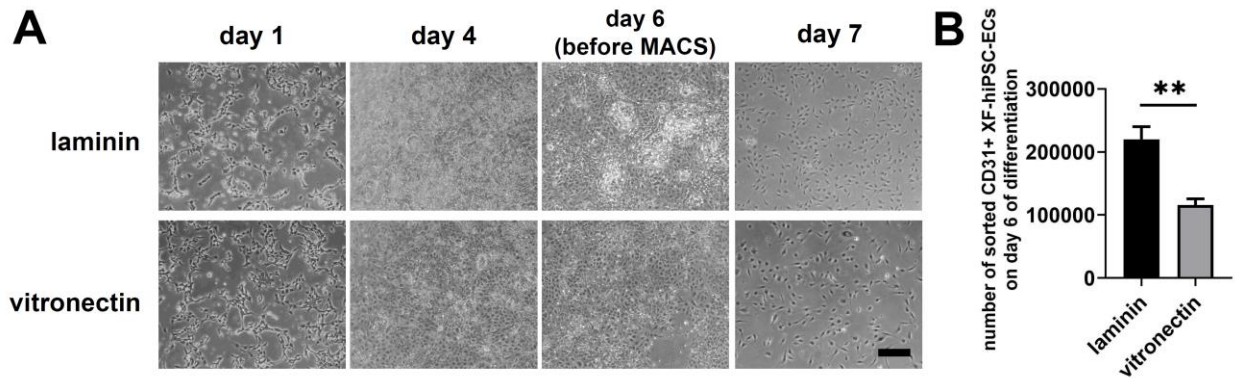


**Figure S1. Xenogeneic-free endothelial differentiation of hiPSCs on human recombinant vitronectin or laminin coated surface.**

**(A)** Bright field images of hiPSCs differentiated toward endothelial lineage on human recombinant vitronectin or laminin coated surface under xenogeneic-free conditions. Images represent differentiated cells on day 1, 4 and 6 and the sorted CD31-positive XF-hiPSC-ECs on day 7 according to xenogeneic-free EC differentiation method (see Materials and Methods) are shown. Scale bar: 200 μm.

**(B)** Number of CD31-positive sorted XF-hiPSC-ECs on day 6 of differentiation on human recombinant vitronectin or laminin coated surface. Note that the XF-hiPSC-ECs were generated from 150,000 XF-hiPSCs seeded on day 0 (Two-tailed unpaired Student's T-test; Mean values and S.E.M indicated by the error bars are shown; n=3; **: p<0.01).

**Figure S2. Flow cytometric analyses of IgG controls (antibody controls for CD31 and VE-cadherin staining) in XF-hiPSC-ECs and XG-hiPSC-ECs and of CD31 and**

**VE-cadherin (cellular controls for XF-hiPSC-ECs) in undifferentiated hiPSCs, XF-HUVECs and XG-HUVECs.**

**(A)** Representative flow cytometric analysis plots of mouse IgC (control for CD31 and VE-cadherin [VECAD]) in XF-hiPSC-ECs and XG-hiPSC-ECs.

**(B)** Quantification of panel (A) (n=3).

**(C)** Representative flow cytometric analyses plot of CD31- or VECAD-positive cells in undifferentiated hiPSCs and HUVECs under xenogeneic-free and xenogeneic culture conditions.

**(D)** Quantification of panel (C) (One-way ANOVA with Tukey's multiple comparisons test; Mean values and S.E.M indicated by the error bars are shown; n=3; ****: p<0.0001).
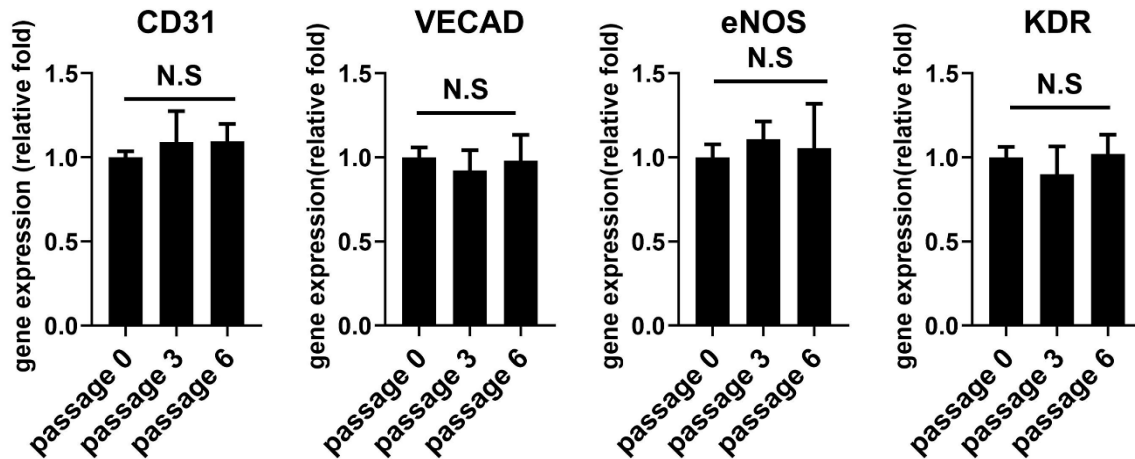
**Figure S3. Relative mRNA transcript amounts of EC markers (CD31, VE-cadherin [VECAD], eNOS and KDR) in XF-hiPSC-ECs at passages 0, 3 and 6.** Values in the y axis represent fold changes relative to human GAPDH expression. Gene expression in each group was normalized to that of XF-hiPSC-ECs at passage 0 (One-way ANOVA with Tukey's multiple comparisons test; Mean values and S.E.M indicated by the error bars are shown; n=3; N.S: not significant).
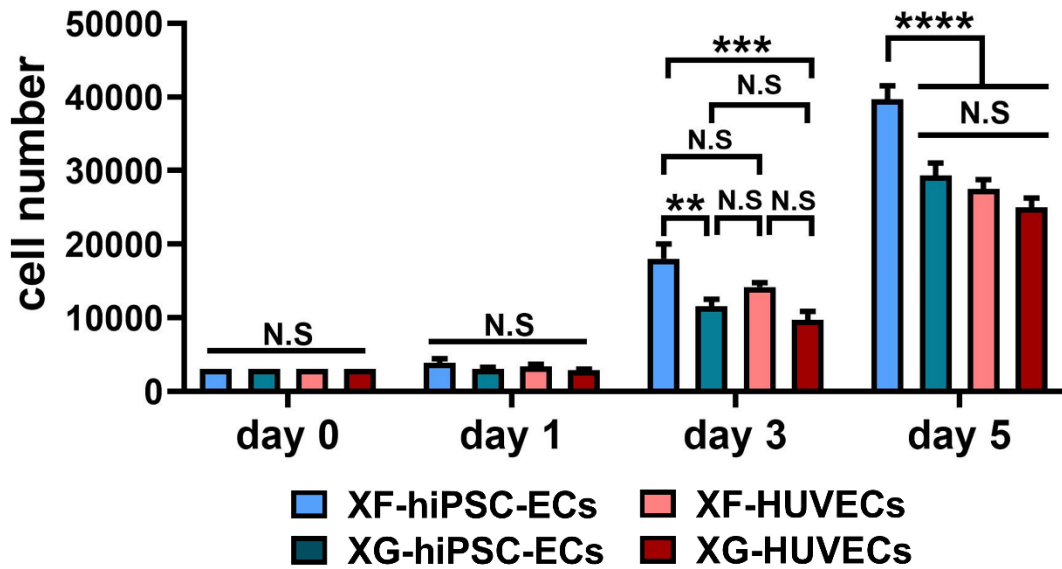
**Figure S4. Proliferation rates of XF- or XG-hiPSC-ECs or HUVECs cultured in the respective expansion medium for 5 days.** XF- or XG-hiPSC-ECs below passage 3 were utilized in this experiment. Two-way ANOVA showed that there was significant interaction between the time point for cell counting and cell type/culture conditions (p<0.0001). Note that XF-hiPSC-ECs appeared to be more proliferative on day 3 and 5 compared with other groups. Mean values and S.E.M indicated by the error bars are shown; n=3; **: p<0.01; ***: p<0.001; ****: p<0.0001; N.S: not significant.
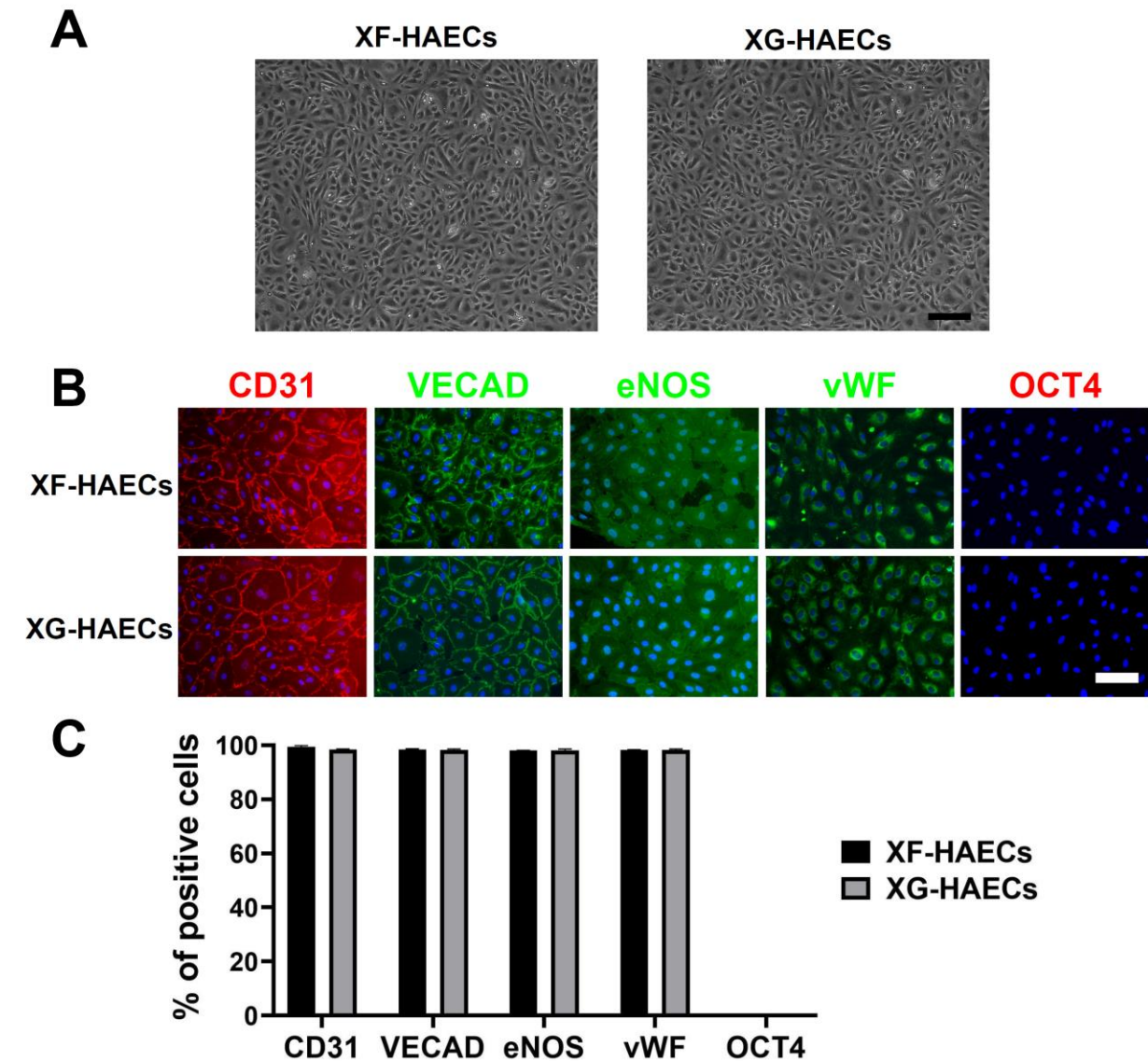
**Figure S5. Marker expression of human aortic endothelial cells (HAECs) under xenogeneic-free or xenogeneic culture conditions (XF-HAECs or XG-HAECs).**

**(A)** Bright field images of XF-HAECs and XG-HAECs. Scale bar: 200 μm.

**(B)** Immunostaining of EC markers (CD31, VECAD, eNOS and vWF) and pluripotency marker OCT4 in HAECs under xenogeneic-free or xenogeneic conditions (XF-HAECs and XG-HAECs). DNA (nuclear) was counterstained by DAPI. Scale bar: 200 μm.

**(C)** Percentages of XF-HAECs and XG-HAECs positive for EC markers (CD31, VECAD, eNOS and vWF) and pluripotency marker OCT4 from immunostaining (B) (Mean values and S.E.M indicated by the error bars are shown; n=3).
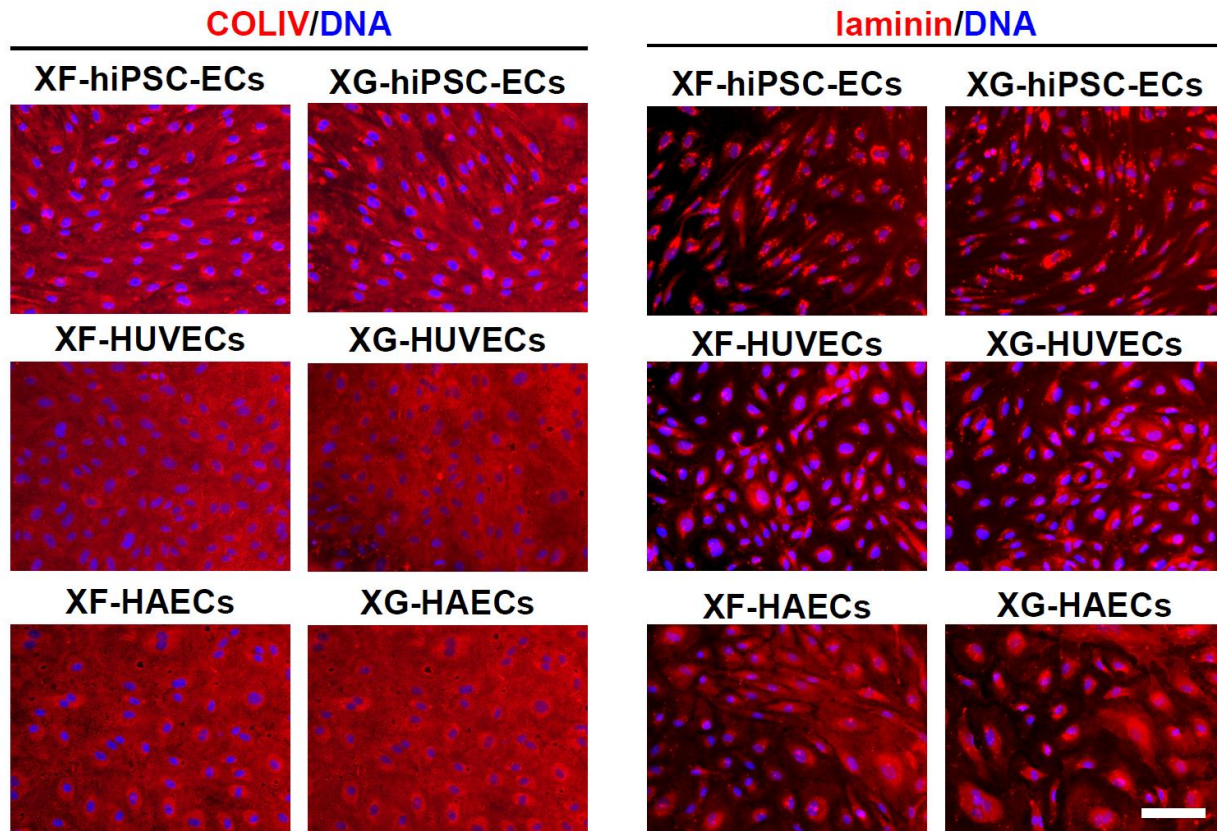
**Figure S6. Expression of basal membrane markers in XF-hiPSC-ECs.**
Immunostaining of basal membrane markers (collagen type IV [COLIV] and laminin) in XF-hiPSC-ECs, XG-hiPSC-ECs, XF-HUVECs, XG-HUVECs, XF-HAECs and XG-HAECs. DNA (nuclear) was counterstained by DAPI. Scale bar: 200 μm.
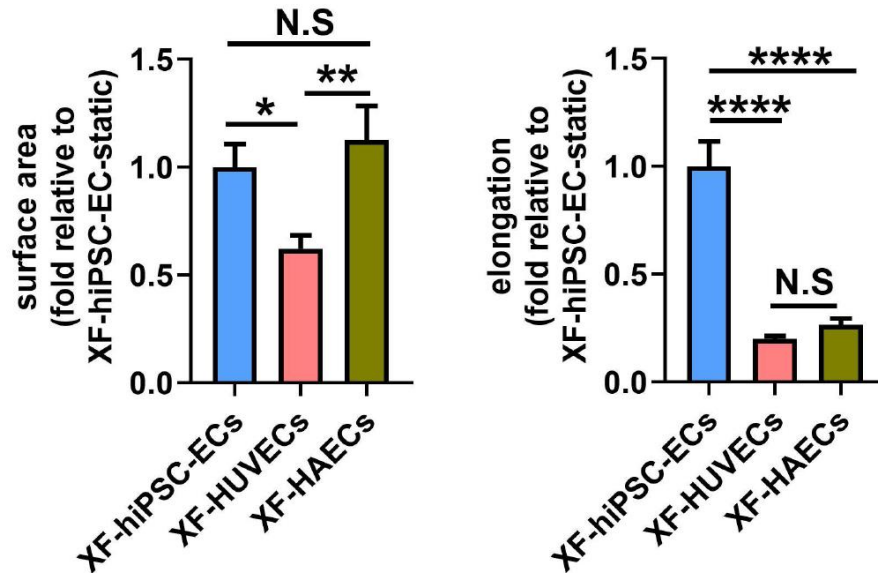
**Figure S7. Cell surface area and level of elongation of XF-hiPSC-ECs.**

(A) Surface areas of XF-hiPSC-ECs, XF-HUVECs and XF-HAECs cultured under static condition relative to that of XF-hiPSC-ECs. Cell surface area was measured using ImageJ software, and at least 20 cells in each image was measured (One-way ANOVA with Tukey's multiple comparisons test; Mean values and S.E.M indicated by the error bars are shown; n=3; *: p<0.05; **: p<0.01; N.S: not significant).

(B) Levels of elongation of XF-hiPSC-ECs, XF-HUVECs and XF-HAECs cultured under static condition relative to that of XF-hiPSC-ECs. Cell elongation was calculated based on the ratio of the long axis and short axis lengths of each cell measured using ImageJ software, and at least 20 cells in each images were measured (One-way ANOVA with Tukey's multiple comparisons test; Mean values and S.E.M indicated by the error bars are shown; n=3; ****: p<0.0001; N.S: not significant).
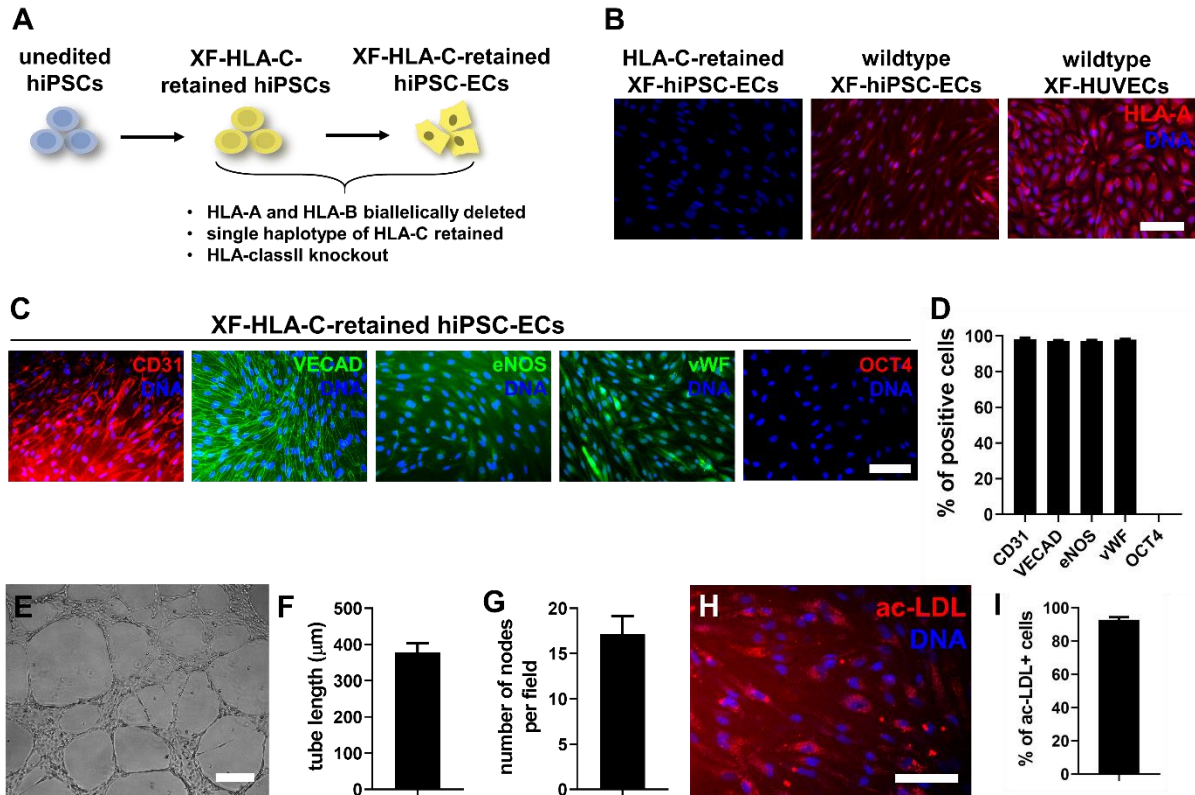
**Figure S8. Characterization of xenogeneic-free ECs derived from hypoimmunogenic, HLA-C-retained hiPSCs (HLA-C-retained XF-hiPSC-ECs).**

**(A)** Schematic illustration of the strategy of generating HLA-C-retained XF-hiPSC-ECs.

**(B)** Immunostaining of human surface antigen marker (HLA-A) in HLA-C-retained XF-hiPSC-ECs. Unedited, wildtype XF-hiPSC-ECs and XF-primary ECs (XF-HUVECs) were stained for HLA-A as positive controls. DNA (nuclear) was counterstained by DAPI. Scale bar: 200 μm.

**(C)** Immunostaining of EC markers (CD31, VECAD, eNOS and vWF) and pluripotency marker OCT4 in HLA-C-retained XF-hiPSC-ECs. DNA (nuclear) was counterstained by DAPI. Scale bar: 200 μm.

**(D)** Percentage of HLA-C-retained XF-hiPSC-ECs positive for EC markers (CD31, VECAD, eNOS and vWF) and pluripotency marker OCT4 from immunostaining (C) (Mean values and S.E.M indicated by the error bars are shown; n=3).

**(E)** Representative phase contrast images of capillary-like networks by HLA-C-retained XF-hiPSC-ECs on Matrigel-coated culture plates 24 hours after cell seeding. Scale bar: 200 µm.

**(F)** Quantification of tube length of cellular networks formed by HLA-C-retained XF-hiPSC-ECs (Mean values and S.E.M indicated by the error bars are shown; n=3).

**(G)** Quantification of nodes per field of cellular networks formed by HLA-C-retained XF-hiPSC-ECs (Mean values and S.E.M indicated by the error bars are shown; n=3).

**(H)** Uptake of fluorescently labelled acetylated low density lipoprotein (ac-LDL) in HLA-C-retained XF-hiPSC-ECs. DNA (nuclear) was counterstained by DAPI. Scale bar: 200µm.

**(I)** Quantification of cells positive for ac-LDL uptake in panel H (Mean values and S.E.M indicated by the error bars are shown; n=3).
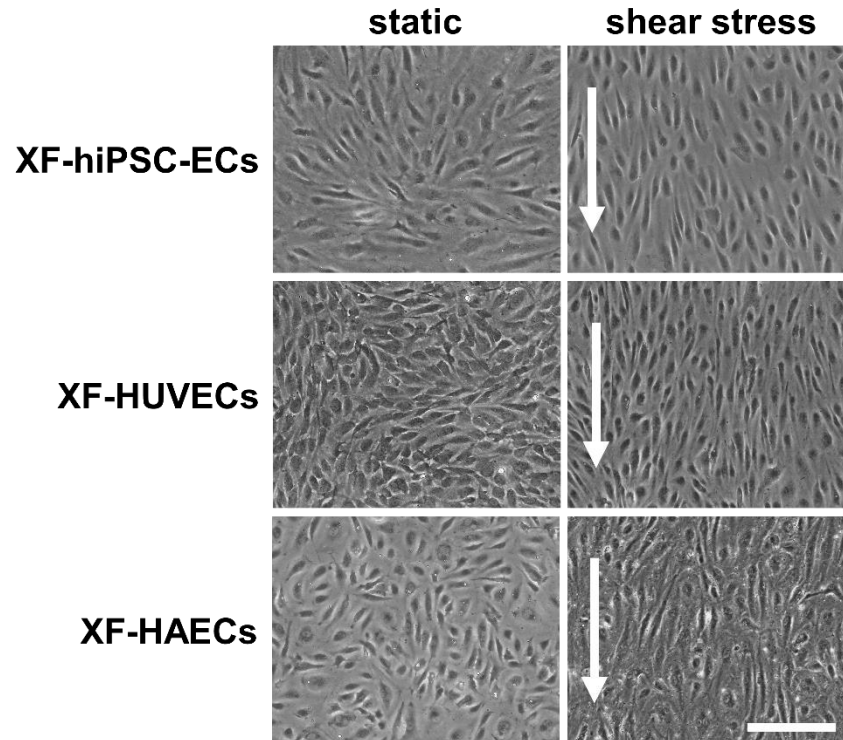
**Figure S9. Bright field images of XF-hiPSC-ECs, XF-HUVECs or XF-HAECs cultured in the presence or absence of shear stress.** 175,000 XF-hiPSC-ECs or XF-primary ECs (XF-HUVECs or XF-HAECs) were seeded onto the human fibronectin-coated surface of µ-Slide I0.4 luer slide chamber and cultured in the xenogeneic-free hiPSC-EC or primary EC expansion medium under static condition for 24 hours, respectively. Cells were then maintained in static culture or exposed to laminar shear stress at 15 dyne/cm$^2$ for two days. Arrows indicate the direction of flow. Scale bar: 200 µm.
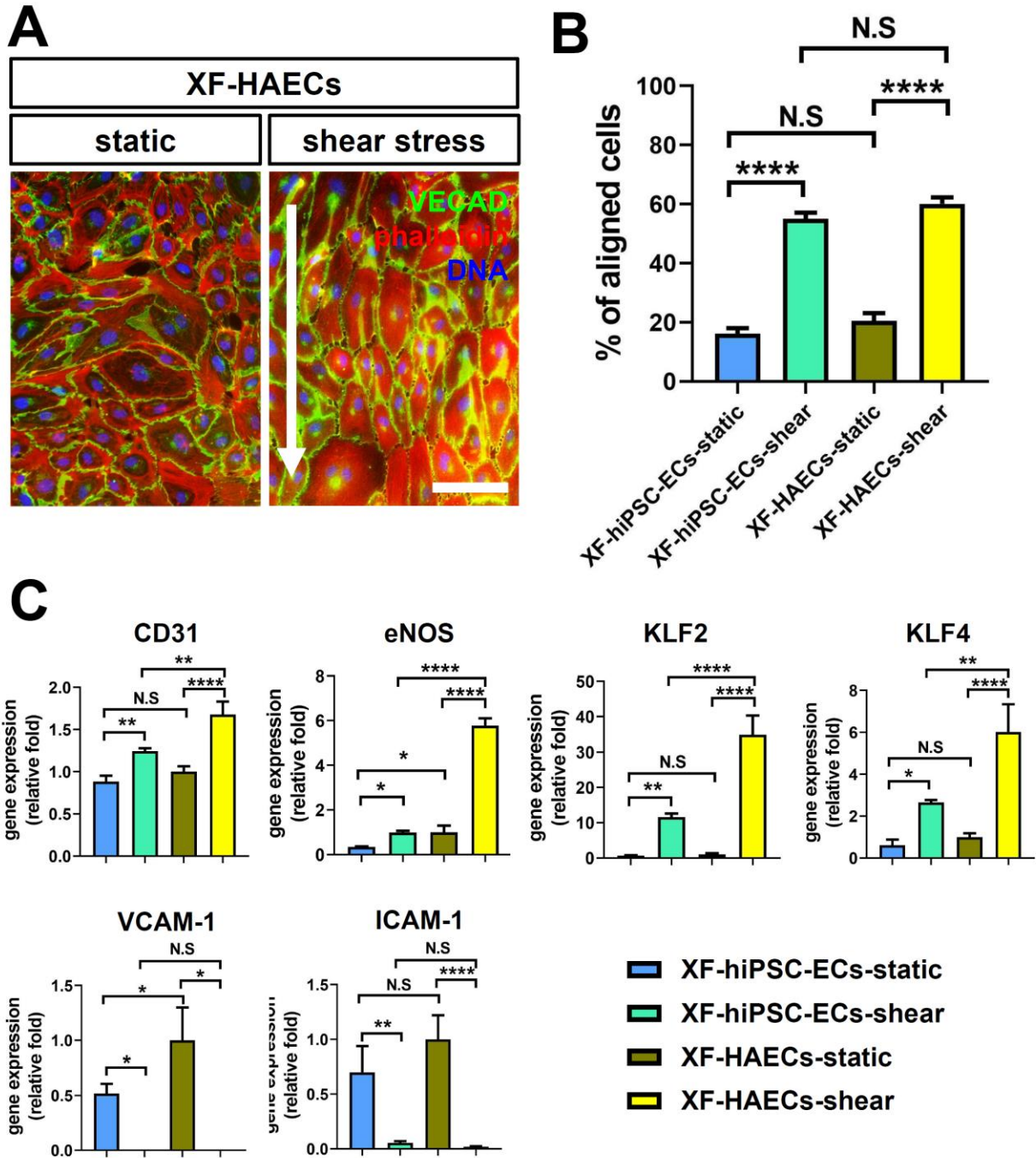
**Figure S10. Response of XF-HAECs to shear stress.**

**(A)** Fluorescence staining of filamentous actin fibers (phalloidin) and VE-cadherin (VECAD) in XF-HAECs cultured in the presence or absence of shear stress. DNA (nuclei) was counterstained with DAPI. Arrows indicate the direction of flow. Scale bar: 200µm.

**(B)** Percentage of cells aligned to the direction of flow in **A**. Cell nuclei with orientation in the direction of flow (±23°) were quantified as aligned. (Two-tailed unpaired Student's T-test; Mean values and S.E.M indicated by the error bars are shown; n=3; ****: $p < 0.0001$).

**(C)** Relative mRNA transcript amounts of EC markers (CD31 and eNOS), anti-thrombotic markers (KLF2 and KLF4) and adhesion molecules (VCAM-1 and ICAM-1) in XF-HAECs cultured in the presence or absence of shear stress. Values in the y-axis represent fold changes relative to human GAPDH expression. Gene expression in each group was normalized to that of XF-HAECs under static culture conditions (One-way ANOVA with Tukey's multiple comparisons test; Mean values and S.E.M indicated by the error bars are shown; n=3; *: $p < 0.05$; **: $p < 0.01$; ****: $p < 0.0001$; N.S: not significant).
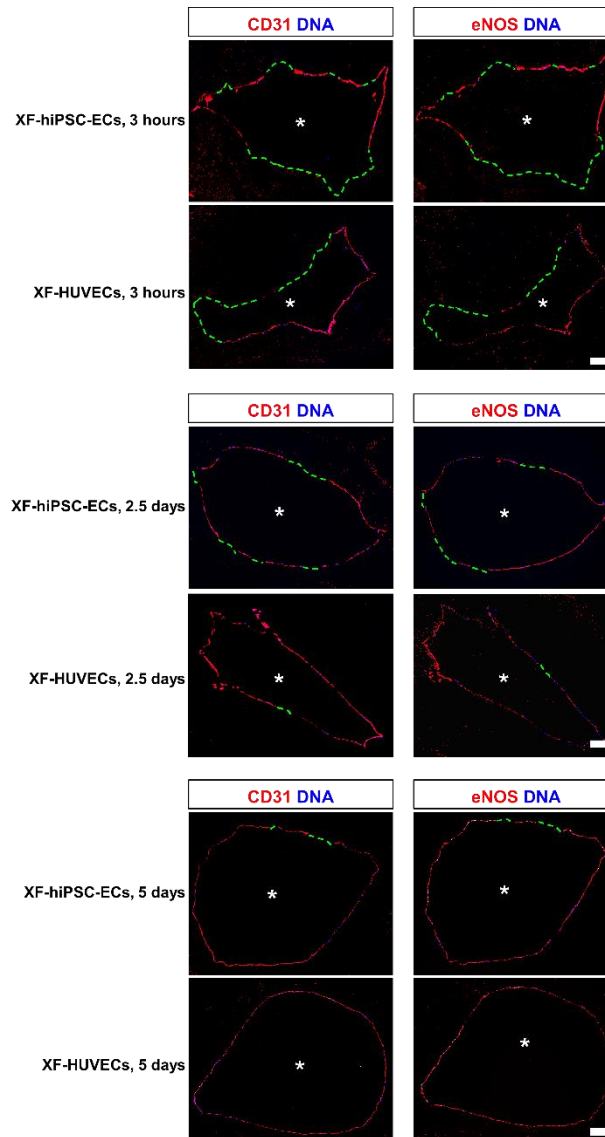
**Figure S11. Immunofluorescence staining of EC markers (CD31 and eNOS) of decellularized human vessels after endothelialization using XF-hiPSC-ECs or XF-HUVECs (original magnification: 4x).** Decellularized human vessels mounted in a dynamic bioreactor system were coated with human fibronectin, seeded with XF-hiPSC-ECs or XF-HUVECs at the density of $5\times10^5/cm^2$ on day 0, and cultured statically for 2 hours. Next, the shear stress was incrementally applied to the lumen of the vessels through generating the intraluminal flow of culture medium. The shear stress was

maintained at 1 dyne/cm$^2$ for 48 hours, progressively increased to the maximum of 15 dynes/cm$^2$ in 36 hours, and eventually maintained at 15 dynes/cm$^2$ for 24 hours. The endothelialized vessels were harvested 3 hours (prior to adding shear stress), 2.5 days, or 5 days post cell seeding, immediately fixed and subjected to immunostaining of endothelial markers CD31 and eNOS. DNA (nuclear) was counterstained by DAPI in immunostaining. Green dashed lines indicated the areas without adhered XF-hiPSC-ECs or XF-HUVECs. Asterisk indicates the vessel lumen. Scale bar: 100 µm.

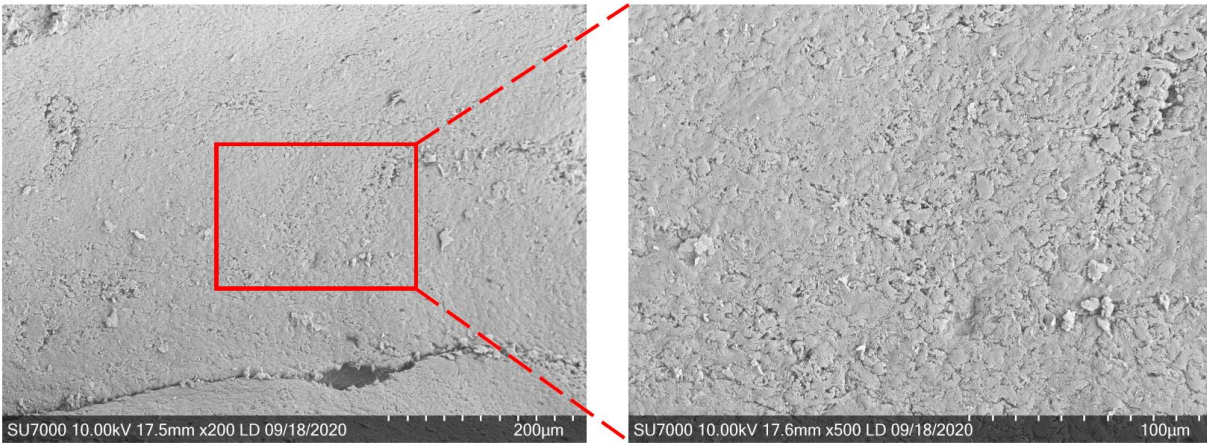**decellularized human vessel without endothelialization**



**Figure S12. Representative images of luminal surface of decellularized human vessel without endothelialization via scanning electron microscopy.** The right panel (magnification 500X) indicates the magnified area in the red frame in the left image (magnification: 200X).

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