



A streamlined method to generate endothelial cells from human pluripotent stem cells via transient doxycycline-inducible ETV2 activation

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Abstract

The development of reliable methods for producing functional endothelial cells (ECs) is crucial for progress in vascular biology and regenerative medicine. In this study, we present a streamlined and efficient methodology for the differentiation of human induced pluripotent stem cells (iPSCs) into induced ECs (iECs) that maintain the ability to undergo vasculogenesis in vitro and in vivo using a doxycycline-inducible system for the transient expression of the ETV2 transcription factor. This approach mitigates the limitations of direct transfection methods, such as mRNA-mediated differentiation, by simplifying the protocol and enhancing reproducibility across different stem cell lines. We detail the generation of iPSCs engineered for doxycycline-induced ETV2 expression and their subsequent differentiation into iECs, achieving over 90% efficiency within four days. Through both in vitro and in vivo assays, the functionality and phenotypic stability of the derived iECs were rigorously validated. Notably, these cells exhibit key endothelial markers and capabilities, including the formation of vascular networks in a microphysiological platform in vitro and in a subcutaneous mouse model. Furthermore, our results reveal a close transcriptional and proteomic alignment between the iECs generated via our method and primary ECs, confirming the biological relevance of the differentiated cells. The high efficiency and effectiveness of our induction methodology pave the way for broader application and accessibility of iPSC-derived ECs in scientific research, offering a valuable tool for investigating endothelial biology and for the development of EC-based therapies.

Keywords Induced pluripotent stem cells (iPSCs) · Endothelial cell differentiation · Doxycycline-inducible · ETV2 · Vasculogenesis · Angiogenesis

Introduction

Endothelial cells (ECs) constitute the inner lining of blood vessels and are essential to the physiological integrity and functionality of virtually all tissues and organs in the body [1]. Their fundamental role extends beyond the mere formation of a barrier to the regulation of key developmental processes, and they are implicated in the pathogenesis of a myriad of diseases [2]. In the field of regenerative medicine, ECs garner particular interest due to their capacity to modulate the activity of various stem cells, thereby playing a critical role in tissue homeostasis and regeneration [3–5]. This dynamic interplay between ECs and stem cells underscores the necessity of harnessing ECs for therapeutic applications. Therefore, the quest for competent ECs is a pursuit within vascular biology and a central endeavor in regenerative

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medicine, aiming to advance treatment options for a broad spectrum of vascular and systemic conditions.

Traditionally, the procurement of human vascular cells predominantly relied on isolating them directly from healthy blood vessels. However, this practice is constrained by limited scalability and applicability [6]. This led researchers to explore postnatal progenitors such as circulating endothelial colony-forming cells (ECFCs) and bone marrow-derived mesenchymal stem cells (MSCs), which serve as precursors to ECs and mural cells, respectively [7, 8]. Despite these advancements, the relatively scarce presence of ECFCs in adult tissues and their diminished functionality in the context of disease pose significant barriers to their clinical utility [9, 10]. The emergence of induced pluripotent stem cell (iPSC) technology has markedly shifted this landscape, offering a seemingly unlimited source of autologous cells [11, 12]. This breakthrough circumvents the limitations associated with traditional cell sources and introduces a novel, non-invasive means of generating patient-specific ECs, heralding a new era in vascular biology research and regenerative medicine applications.

Traditional chemically-induced differentiation protocols emulate vascular development by guiding iPSCs through two key stages: initial differentiation into intermediate mesodermal progenitor cells (MePCs) via Wnt and Nodal pathways, followed by endothelial specification primarily through VEGF signaling [13]. More recent strategies have shifted towards employing transcription factors (TFs) as direct differentiation cues [14, 15]. This approach offers precise control over the differentiation process and the potential for simultaneous differentiation into multiple cell types. ETV2 (ETS variant 2), a TF essential for vascular development [16–20], has emerged as a focal point in this paradigm shift [21]. Its role as a pioneer factor necessitates only transient expression, making it an appealing target for differentiation protocols. For instance, we and others have developed methodologies that leverage the transient activation of ETV2 through modified mRNA technology to direct iPSC differentiation into iECs [22–24]. Others have used viral vectors to induce activation of ETV2 alone and in combination with additional TFs [24–27]. Studies have also included inducible approaches, notably incorporating Tet-ON transgene elements that can be activated with doxycycline [27–31]. Nevertheless, there has yet to be a consensus on the optimal application of ETV2. For example, many studies bypass the mesodermal stage, activating ETV2 directly in the pluripotent cells [24, 26, 27, 31], while others maintain ETV2 activation indefinitely rather than transiently [25]. To date, a definitive method utilizing inducible strategies to exploit ETV2 for generating iECs has not been established.

In this study, we introduce a streamlined methodology for generating iECs from human iPSCs through the activation of ETV2 in a doxycycline-inducible manner. To this end, we generated Dox-inducible ETV2 iPSC lines through a piggyBac transposon system, which enables controlled, transient ETV2 expression, thereby directing MePCs toward the endothelial lineage with exceedingly high efficiency and reproducibility. We anticipate that this methodology will serve as a valuable resource for the field, facilitating the widespread use of iPSC-derived iECs in vascular biology and regenerative medicine research.

Materials and methods

Cell Culture

Human Induced Pluripotent Stem cells (iPSCs)

Seven human iPSC lines were employed. The BJ273 iPSC line was sourced from Boston Children's Hospital's Stem Cell Core. Dox-ETV2-iPSCs (BJ273-C04) and Dox-ETV2-iPSCs-GFP (BJ273-C04-GFP) were engineered from this parental BJ273 line. Six additional iPSC lines, herein termed NYSCF-01 to NYSCF-06, were obtained from the New York Stem Cell Foundation. All iPSCs were cultured in mTeSR Plus medium (STEMCELL Technologies, cat. no. 100–0276) with 5 μ M Y27632 rock inhibitor (Selleckchem, cat. no. S1049) on Matrigel-coated (Corning, cat. no. 354,277) 6-well plates (Gene Clone, cat. no. 25–105). Upon 80–90% confluency, cells were washed with PBS (Thermo Fisher Scientific, cat. no. 10,010,049) and detached using 1x TrypLE Select (TrypLE; Thermo Fisher Scientific, cat. no. 12,563,029), split at a 1:12 ratio, and cryopreserved in FreSR™-S medium (STEMCELL Technologies, cat. no. 05859). Thawing involved resuspension and centrifugation in DMEM medium (DB; Thermo Fisher Scientific, cat. no. 11,995,073).

Human embryonic stem cells (HUES)

The HUES8 embryonic stem cell line, obtained from Harvard University, was maintained under the same culture conditions as the iPSCs.

Human endothelial colony-forming cells (ECFCs)

Human endothelial colony-forming cells (ECFCs) [9] were isolated from umbilical cord blood using previously established methods [32]. These cells were cultured on 1% gelatin-coated p100 dishes (Gene Clone, cat. no. 25–202) in EGM-2 medium (PromoCell, cat. no. C-22,111) without

hydrocortisone, supplemented with 20% FBS (Gene Clone, cat. no. 25–514) and 1x Penicillin-Streptomycin (P/S; Thermo Fisher Scientific, cat. no. 15,140,122). Upon reaching 80–90% confluency, cells were washed with PBS, detached with TrypLE, neutralized with D10 medium (DMEM with 10% FBS and 1x P/S), and split at a ratio between 1:3 and 1:6. Cryopreservation used Recovery™ Cell Culture Freezing Medium (Thermo Fisher Scientific, cat. no. 12-648-010). ECFCs were used between passages 6 and 12.

Human Mesenchymal Stem Cells (MSCs)

Human white adipose tissue-derived MSCs were isolated as previously described [33], cultured on uncoated p100 dishes and maintained in MSCGM medium, comprising Mesenchymal Stem Cell Basal Medium (ATCC, cat. no. PCS-500-030) supplemented with the Mesenchymal Stem Cell Growth Kit (ATCC, cat. no. PCS-500-040) and 1x P/S. For passaging, MSCs were split at ratios from 1:6 to 1:10, following the same protocols as for ECFCs regarding subculturing, centrifugation, and cryopreservation. All experiments were conducted with MSCs at passages 6 to 12.

Human brain microvascular endothelial cells (BMEC)

Human primary BMECs were used as a human primary EC control and were acquired from iXCells Biotechnologies (cat. no. 10HU-051) and cultured on uncoated p100 dishes in Endothelial Cell Growth Medium (iXCells Biotechnologies, cat. no. MD-0010), adopting the same practices for subculturing, centrifugation, and cryopreservation as those applied to ECFCs.

H-1975 epithelial cells

The H-1975 epithelial cell line was sourced from ATCC (cat. no. CRL-5908) and cultured on uncoated T-75 flasks (Corning, cat. no. 430,641), maintained in RPMI-1640 Medium (ATCC, cat. no. 30-2001) supplemented with 10% FBS (ATCC, cat. no. 30-2020). Procedures for subculturing, centrifugation, and cryopreservation mirrored those used for ECFCs.

Human doxycycline-inducible ETV2 endothelial cells (Dox-ETV2 iECs)

Human Dox-ETV2 iECs, generated from Dox-inducible ETV2 iPSC or HUES lines, were cultured on 1% gelatin-coated dishes similar to ECFCs and maintained in EGM-2 medium. EGM-2 was composed of Endothelial Cell Growth Medium 2 (PromoCell, cat. no. C-22,111) without

hydrocortisone, supplemented with 1x P/S. For subculturing, Dox-ETV2 iECs were detached and seeded at a ratio of 1:2, employing the same techniques for washing, detaching, and neutralizing as described for ECFCs. Cryopreservation followed the protocols established for ECFCs. These Dox-ETV2 iECs were utilized in experiments from passages 0 to 3.

Establishment of doxycycline-inducible ETV2 (Dox-ETV2) iPSC and HUES lines

To generate Dox-ETV2 cell lines, we utilized the piggy-Bac (PB) transposon system combined with Super piggy-Bac transposase vectors (SBI, cat. no. PB210PA-1). The PB transposon vector harboring the ETV2 ORF was kindly provided by Dr. George Church at Harvard University. We mixed the PB Dox-ETV2 transposon and transposase vectors at a 5:1 ratio for electroporation into various iPSC and a HUES line, according to a previously established protocol [34]. Specifically, 1 µg of Super transposase and 5 µg of PB transposon were used to transfect 1 million cells using the Neon electroporation system as per the manufacturer's instructions (Invitrogen, cat. no. MPK10096). The electroporation settings were 1150 V for the pulse voltage, 30 ms for the pulse width, and two pulses were applied, employing 3 mL of electrolytic buffer and 120 µL of resuspension buffer R in 100 µL reaction tips. After electroporation, cells were plated on Matrigel-coated 6-well plates (Gene Clone, cat. no. 25–105) and cultured in mTeSR Plus medium with 5 µM Y27632 rock inhibitor added. To select for successfully transfected cells, we added 0.5 µg/mL puromycin (InvivoGen, cat. no. ant-pr-1) to the culture. Positive iPSC clones were isolated manually, and their pluripotency and responsiveness to doxycycline were verified through qPCR and immunostaining assays.

Generation of the Dox-ETV2-iPSC-GFP line

The Dox-ETV2-BJ-C04-GFP iPSC line was established utilizing the Super piggyBac gene editing system. For this purpose, we employed the PB transposon vector pSH231-EF1-GFP-HYGRO (Addgene, cat. no. 115144). The transfection into the Dox-ETV2-BJ-C04 iPSC line followed identical procedures, parameters, and vector quantities as outlined above for generating Dox-ETV2 iPSC and HUES lines. Post-electroporation, cells were cultured under standard iPSC conditions. To select positively transfected cells, 500 µg/mL hygromycin (InvivoGen, cat. no. ant-hg-1) was introduced to the culture medium. Successfully modified clones were manually isolated, with their pluripotency and GFP expression confirmed through qPCR, immunostaining assays, and direct microscopy.

Differentiation of doxycycline-inducible ETV2 endothelial cells (Dox-ETV2 iECs)

Differentiation was conducted over a 4-day protocol, divided into two stages to transition Dox-ETV2-iPSCs into Dox-ETV2 iECs.

Stage 1 (S1): mesodermal progenitor cell differentiation

Initially, Dox-ETV2 iPSCs were seeded in Matrigel-coated 6-well plates. After 12–18 h, at 30–40% confluency, the culture medium was replaced with 2 mL of basal medium enriched with 6 μ M CHIR99021 (Sigma, catalog no. SML1046), termed S1 medium, for 48 h. The basal medium comprised Advanced DMEM/F-12 (Thermo Fisher Scientific, cat. no. 12,634,010), 1x GlutaMAX™ Supplement (Thermo Fisher Scientific, cat. no. 35,050,061), 60 μ g/mL L-Ascorbic acid phosphate (Thermo Fisher Scientific, cat. no. A8960-5G), and 0.4x P/S. This process was initiated at S1-D0 in the S1 medium. After 24 h (S1-D1), the fresh S1 medium was replenished, continuing for another 24 h to complete stage 1 (S1-D2), resulting in mesodermal progenitor cells (MePCs).

Stage 2 (S2): endothelial cell differentiation

MePCs were washed with 1x PBS and dissociated using 1 mL of TrypLE per well for 3 min. The reaction was neutralized with DMEM medium. Typically, this yielded 1–3 million MePCs per 6-well plate. Two million MePCs were then seeded in a p100 Matrigel-coated dish and treated with S2 medium containing the same basal medium as in S1 supplemented with 0.5 μ g/mL Doxycycline Hyclate (Dox; Sigma, cat. no. D9891), 10 ng/mL EGF (ProSpec, catalog no. CYT-798), 50 ng/mL FGF 2 (bFGF; ProSpec, cat. no. CYT-557), and 50 ng/mL VEGF (ProSpec, cat. no. CYT-241). S2 medium was replenished daily. After 48 h of S2 treatment, differentiation into iECs was deemed complete.

Expansion of Dox-ETV2 iECs

Post-differentiation, Dox-ETV2 iECs were treated and cultured similarly to regular human endothelial cells, like ECFCs, as detailed in the [Cell Culture](#) section above.

In vitro vascular network-forming assay

For the in vitro vascular network-forming assay, we used the idenTx 3 Chip and Holder from AIM Biotech Pte. Ltd., following a slightly modified version of the manufacturer's guidelines. We initiated the assay by combining Passage 1 Dox-ETV2 iECs with MSCs at a 2:3 ratio, resulting in a

mixture of 1.2×10^5 cells in 10 μ L. This cell mixture was then embedded in a hydrogel solution, which included thrombin and a specialized medium, and seeded into the chip's cell/gel channel. The hydrogel solution consisted of 6 mg/mL fibrinogen (Sigma, cat. no. F8630) dissolved in 1x PBS and warmed to 37 °C, to which we added thrombin (50 U/mL; Sigma, cat. no. T4648) prepared in 1x PBS. The culture medium used was EGM-2, supplemented with 3% FBS, 50 ng/mL VEGF, 10 ng/mL DAPT (STEMCELL Technologies, cat. no. 72,082), and 10 ng/mL TGF β 1 (ProSpec, cat. no. CYT-716). DAPT (a Notch signaling inhibitor) and TGF β were added to promote vascular branching and perivascular cell maturation, respectively [35, 36]. To prepare the cells for seeding, we first suspended them in a medium-diluted thrombin solution to achieve a concentration of 4 U/mL. This solution was then mixed equally with the fibrinogen solution, resulting in final concentrations of 2 U/mL thrombin and 3 mg/mL fibrinogen. This preparation was directly applied to the chip's gel channel and allowed to polymerize at 37 °C for 30 min. After confirming the hydrogel's polymerization, we added 15 μ L of culture medium to both media channels. To establish a flow gradient critical for network formation, we adjusted the medium volume to 70 μ L on one side and 50 μ L on the other, with daily medium changes to ensure cell viability. Throughout the assay, the chip was maintained at 37 °C in a 5% CO₂ environment. On day 7, we assessed perfusion in the vascular networks using fluorescent microbeads (Supplementary Table S1).

In vivo vascular network formation assay

For our in vivo assay, we utilized athymic nude mice (Foxn1/nu; Envigo) adhering to care guidelines for immune-deficient mice and conducting experiments at Boston Children's Hospital in compliance with protocols approved by the Institutional Animal Care and Use Committee (IACUC). We combined Passage 3 Dox-ETV2 iECs with MSCs in a hydrogel solution for implantation. ECFCs and MSCs served as the control group. Each mouse received a total of 2×10^6 cells, with a 2:3 ratio of Dox-ETV2 iECs (or ECFCs) to MSCs.

The collagen/fibrinogen hydrogel was prepared following a previously described method with some modifications [37]. Briefly, for 1 mL of hydrogel, 30 mg/mL Fibrinogen (Sigma, cat. no. F8630) was dissolved in 0.9% saline solution (VWR, cat. no. BDH7257-2) and warmed to 37 °C; 100 μ L of 10x M199 (Thermo Fisher Scientific, cat. no. 21,180,021), 25 μ L of 1 M HEPES (Thermo Fisher Scientific, cat. no. 15,630,080), 300 μ L of 5 mg/mL Collagen I (R&D Systems, cat. no. 3442-050-01), and 300 μ L of 6 mg/mL Laminin I (R&D Systems, cat. no. 3446-005-01) were mixed, adjusting pH to 7.4 with NaOH (Sigma, cat. no.

S2770-100ML). To this solution, we added 100 μ L FBS, 30 μ L of 1 mg/mL Fibronectin (Sigma, cat. no. FC010), 10 μ L of 100 ng/ μ L EPO (Prospec, cat. no. CYT-201), 10 μ L of 100 ng/ μ L bFGF (ProSpec, cat. no. CYT-557), 100 μ L of Fibrinogen solution, and 20 μ L ddH₂O (Thermo Fisher Scientific, cat. no. 10,977,023), keeping the hydrogel on ice until use.

Anesthetized mice received a subcutaneous injection of 50 μ L of 50 U/mL thrombin (Sigma, cat. no. T4648) followed by 200 μ L of the cell-loaded hydrogel at the same site. After 1 week, the implants were harvested for analysis of vascular formation.

Histological analysis and vessel perfusion quantification

The harvested grafts were fixed in 10% formalin (VWR, cat. no. 89370-094) overnight and subsequently washed thrice with 1x PBS before being transferred to 70% ethanol (Greenfield Global USA Inc., cat. no. 4070) for preservation. Samples were processed for paraffin embedding, sectioning at 5 μ m thickness, and H&E staining to facilitate histological examination (iHisto Inc.; Salem, MA, USA). Multiple slides from each sample were stained and imaged using a MoticEasyScan Infinity 60 microscope (Motic) to ensure consistent quality and representation of tissue architecture. To assess functional vascular perfusion within the explanted grafts, we identified perfused vessels as lumens visibly filled with erythrocytes in the H&E-stained sections. We quantified the density of these erythrocyte-filled vessels (vessels/mm²) to measure the functional vessel density within each graft. This analysis was performed using ImageJ2 (2.9.0/1.53t) and QuPath (0.4.2) software.

Immunofluorescence staining

Human iPSCs and iECs were cultured and fixed upon reaching 80–90% confluency using 4% paraformaldehyde (PFA; Electron Microscopy Sciences, cat. no. 15,714 S) for 15 min. Permeabilization was achieved with 0.5% Triton X-100 (Bio-Rad, cat. no. 161-0407), followed by blocking and antibody incubation in 1.5% bovine serum albumin (BSA; Sigma, cat. no. A7906-100G) buffer. Primary antibodies targeting OCT4, NANOG, SOX2, ETV2 for iPSCs, and CD31, VE-Cadherin, VWF for iECs were incubated either for 1 h at room temperature or overnight at 4 °C. Secondary antibodies were then applied, followed by DAPI counterstaining. Slides were mounted with ProLong™ Diamond Antifade Mountant (Thermo Fisher Scientific, cat. no. P36965). Antibody specifics are listed in Supplementary Table S1.

For immunofluorescence staining in the in vitro chip assays, cells were also fixed with 4% PFA, permeabilized with 0.1% Triton X-100, and blocked with 1.5% BSA. Antibody incubations were performed similarly to iPSCs and iECs, followed by DAPI counterstaining. Chips were sealed and stored at 4 °C until imaging.

For in vivo characterization of vascular structures, sections were first deparaffinized with xylene and sequentially rehydrated through graded ethanol solutions. After rehydration, sections were subjected to antigen retrieval in a Tris-EDTA buffer, then blocked with 5% BSA to prevent non-specific binding. Overnight incubation with primary antibodies specific to human endothelial markers was conducted at 4 °C, followed by the application of secondary antibodies. DAPI was used for nuclear counterstaining. Sections were then mounted using ProLong™ Diamond Antifade Mountant for fluorescence imaging. Detailed antibody information is provided in Supplementary Table S1.

Flow cytometry analysis

Cells were dissociated into single-cell suspensions using 1x TrypLE, incubated at 37 °C with 5% CO₂ for 3 min. The enzymatic reaction was neutralized using DB or D10 medium, followed by centrifugation at room temperature at 4,000 rpm for 3 min using an Eppendorf Centrifuge 5425. Subsequently, cells were washed with FACS buffer (1x PBS, 0.5% BSA, and 0.2 mM EDTA). For staining, cells were incubated with specific fluorochrome-conjugated antibodies for 20 min at 4 °C, washed to remove unbound antibodies, and resuspended in FACS buffer. Flow cytometry was performed using a BD Accuri™ C6 Plus flow cytometer (BD Biosciences), and data analysis was carried out using FlowJo 10.8.1 software (BD Biosciences). The cells were analyzed immediately post-preparation without fixation. Detailed antibody information is available in Supplementary Table S2.

Quantitative RT-PCR

For quantifying mRNA levels, we used the Power SYBR™ Green Cells-to-CT™ Kit (Thermo Fisher Scientific, cat. no. 4,402,954) for total RNA extraction and subsequent cDNA synthesis, following the manufacturer's protocol. Quantitative PCR was performed using the Power SYBR™ Green PCR Master Mix (Thermo Fisher Scientific, cat. no. 4,368,706) on a QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific, cat. no. A28567). We normalized gene expression data to the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer sequences for the qPCR assays are listed in Supplementary Table S3.

RNA-seq analysis

We analyzed three biological replicates for iPSCs (BJ273), ECFCs, modRNA-ETV2 iECs, and Dox-ETV2 iECs (96 h). Total RNA extraction, library preparation, and sequencing were performed at Azenta Life Sciences (South Plainfield, NJ, USA). Total RNA was extracted using the Qiasymphony RNA kit (Qiagen, cat. no. 931,636). Sequencing libraries were prepared with the NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB, cat. no. E7770S), including mRNA enrichment, cDNA synthesis, and PCR enrichment. Library quality and quantification were assessed using Agilent TapeStation and Qubit 2.0 Fluorometer, respectively. Sequencing was performed on an Illumina HiSeq 2500 platform in a 2 × 150 paired-end mode.

Sequencing data were processed using Illumina's bcl2fastq 2.17 software for file conversion and de-multiplexing. Raw reads were first quality controlled (FastQC, version 0.11.7) and trimmed (Trim_Galore, version 0.4.4). Then trimmed reads were mapped to the human hg38 (UCSC Genome Browser) reference genome, and gene expression (FPKM and count) was calculated with RSEM (version 1.2.28). Pairwise comparison differentially expressed (DE) genes were called by DESeq2 (version 1.38.1, threshold used: fold change > 2, $P < 0.05$). Intersected combined DE genes were subjected to heatmap and principal component analysis (PCA) plotting (R version 4.2.1). RNA-seq data used in this study are deposited in the Gene Expression Omnibus (GEO) under accession number [GSE263058].

Proteomic analysis

Proteomic analysis was performed at the Multiplexed Proteomics Core, Harvard Medical School. For cell preparation, upon reaching 90% confluence, cells were washed with PBS and harvested using a rubber scraper. The cells were then centrifuged at 500 g, the supernatant discarded, and cell pellets stored at -80 °C. Proteomes were extracted from the cell pellets with a buffer containing 200 mM EPPS pH 8.5, 8 M urea, and protease inhibitors. Following lysis, samples were reduced with 5 mM TCEP and alkylated using 10 mM iodoacetamide for 20 min in the dark. Excess iodoacetamide was quenched with 10 mM DTT. Proteomes (100 µg) were precipitated, re-solubilized in 200 mM EPPS, and digested first with Lys-C (1:50) overnight, then with trypsin (1:100) for 6 h at 37 °C. Anhydrous acetonitrile was added to reach 33% concentration. Peptides (50 µg from each sample) were labeled with TMTPro reagents (Thermo Fisher Scientific) for 2 h, quenched with 0.5% hydroxylamine, and acidified with formic acid. Labeled peptides were pooled and desalted with Sep-Pak (Waters). Proteomic data were collected on an Orbitrap Eclipse mass spectrometer (Thermo

Fisher Scientific) with FAIMS, coupled to a Proxeon EASY-nLC 1200 LC pump.

Microscopy

Images were captured using a Zeiss Axio Observer Z1 inverted microscope equipped with an AxioCam MRc5 camera and ApoTome 2. Both 20X and 40X objective lenses were used for fluorescent imaging, while 5X or 10X objective lenses were employed for phase contrast images. For the in vitro vascular network model, images were further analyzed with a Zeiss LSM880 Laser Scanning Confocal microscope. Image analysis was conducted using ZEN 3.6 (blue edition), ZEN 2.3 SP1, and ImageJ2 (2.9.0/1.53t) software.

Statistical analysis

Data were presented as means ± standard error of the mean (s.e.m.). When comparing two groups, mean values were assessed using unpaired two-tailed Student's t-tests. For comparisons involving multiple groups, analysis of variance (one-way ANOVA) followed by Bonferroni correction was utilized. No criteria for exclusion were applied to any of the analyses. Statistical calculations were performed using Prism v.10 software (GraphPad Software Inc.). Statistical significance was considered at $P < 0.05$.

Results

Engineering human iPSCs with dox-inducible ETV2

In pursuit of an efficient protocol for the differentiation of iPSCs into iECs, we previously demonstrated the necessity of a mesodermal intermediary, as well as the transient induction of ETV2 [22]. This laid the groundwork for a more refined approach that could circumvent the technical challenges associated with direct gene delivery methods such as electroporation or lipofection. Drawing on these insights, we engineered iPSCs to express ETV2 in a doxycycline-inducible manner, thereby enabling precise temporal control over ETV2 activation and subsequent endothelial differentiation.

Utilizing the piggyBac system, we constructed an ~8-kbp transposon vector that harbors the *ETV2* gene (specifically, ETV2, transcript variant 1) under the control of a doxycycline-responsive Tet-On element, driven by an EF-1α promoter, a promoter recognized for its robustness in maintaining high levels of transgene expression [38] (Fig. 1A). Transfection of the iPSCs with the piggyBac construct, in conjunction with a super piggyBac transposase vector, was

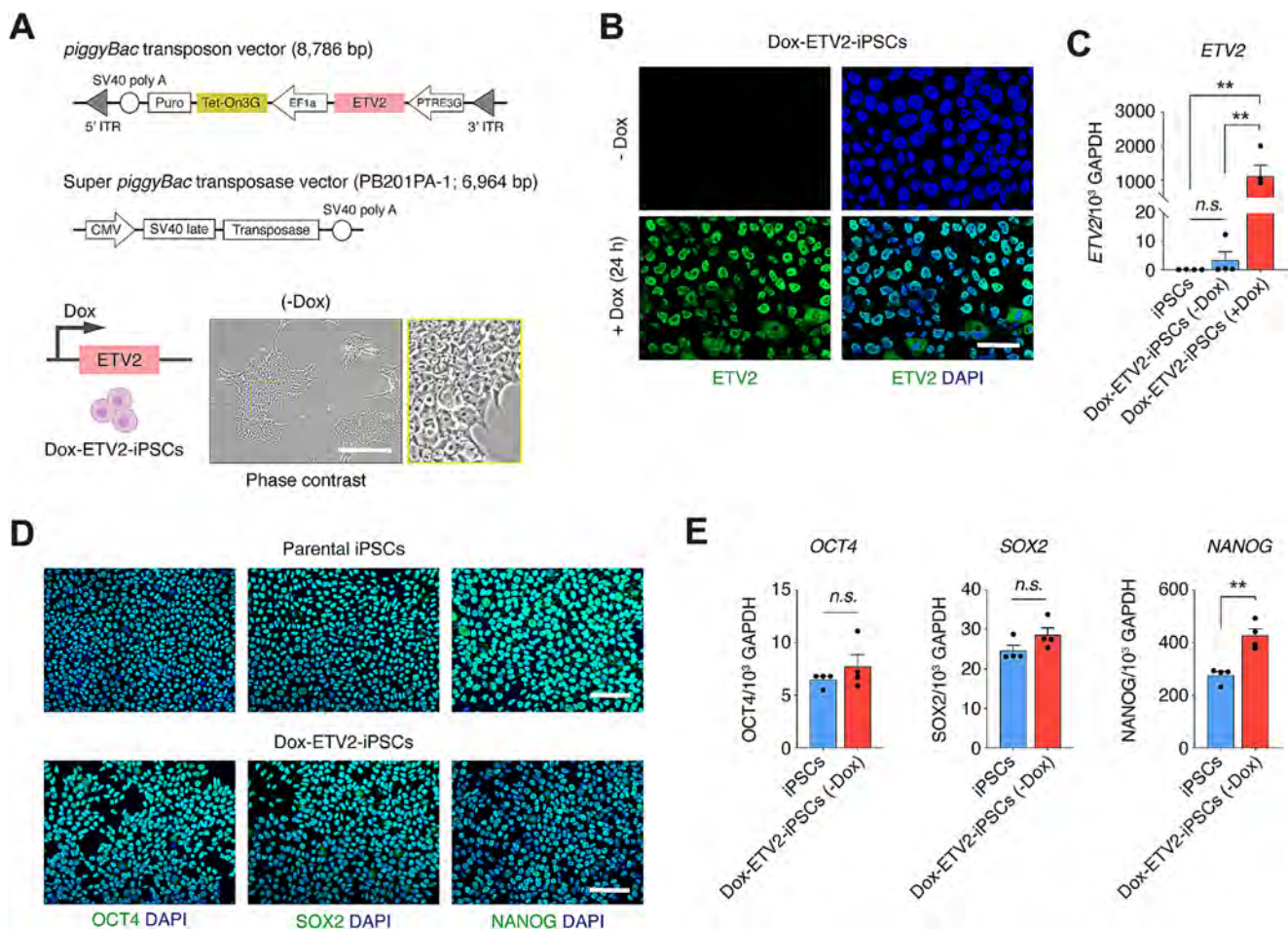


Fig. 1 Engineering Dox-inducible ETV2 human iPSCs and validation of pluripotency. **(A)** Schematic of the piggyBac transposon and transposase vectors for doxycycline-inducible ETV2 expression; phase-contrast images display iPSC morphology before doxycycline induction. **(B)** Immunofluorescence demonstrates ETV2 expression 24 h post-doxycycline treated versus non-treated controls. Cell nuclei are counterstained with DAPI. **(C)** Quantitative PCR shows significant upregulation of ETV2 mRNA with doxycycline (24 h) and negligible leakiness without it. *n.s.* is not statistically significant; $^{**}P \leq 0.01$

followed by selection using puromycin to isolate successfully engineered cells. These Dox-ETV2 iPSC lines retained morphological characteristics consistent with pluripotent stem cells and were cultured under the same standard conditions used for the unmodified parental iPSCs (Fig. 1A).

To validate the inducibility of our system, we exposed Dox-ETV2 iPSCs to doxycycline and confirmed a robust upregulation of ETV2 expression at both the mRNA and protein levels within 24 h (Fig. 1B, C). Importantly, we assessed the potential for leaky expression of ETV2 in the absence of doxycycline and observed negligible levels, confirming the tight regulation of our system (Fig. 1C).

In parallel, we verified the preservation of pluripotency in Dox-ETV2 iPSCs without doxycycline, evidenced by the robust expression of the pluripotency markers OCT4,

($n=4$; one-way ANOVA with Bonferroni's post-test). **(D)** Immunofluorescence for OCT4, SOX2, and NANOG confirms pluripotency in both the Dox-ETV2 engineered and the parental iPSCs in the absence of doxycycline. **(E)** Quantitative PCR confirms the expression of pluripotency markers in the engineered Dox-ETV2 iPSCs in the absence of doxycycline. All PCR data is normalized to GAPDH. *n.s.* is not statistically significant; $^{**}P \leq 0.01$ ($n=4$; paired two-tailed t-test). Data are mean $\pm$ s.e.m.; n are biological replicates (C, E). Scale bars: 250 μ m (A), 50 μ m (B), 100 μ m (D)

SOX2, and NANOG, both at the mRNA and protein levels (Fig. 1D, E). These findings not only demonstrated the successful engineering of iPSCs with a Dox-inducible ETV2 but also confirmed the functional integrity of the iPSCs post-engineering, setting the stage for subsequent endothelial differentiation studies.

Inducible protocol to generate iECs: efficiency and reproducibility

To study the differentiation of the Dox-ETV2 iPSCs, we implemented a feeder-free, two-dimensional, and chemically defined protocol. This protocol is designed to ensure the orderly transition of iPSCs through two critical developmental stages, spanning a period of 48 h each. Initially, iPSCs

are cultivated on Matrigel-coated dishes to induce their differentiation into mesodermal intermediates (MePCs), a process driven by the glycogen synthase kinase 3 (GSK-3) inhibitor CHIR99021, which catalyzes Wnt signaling activation [39] (Fig. 2A). After this, the cells are transferred to fresh Matrigel-coated dishes to embark on the second stage, transitioning from MePCs to iECs. This crucial conversion is achieved by activating the ETV2 transgene via doxycycline in the presence of angiogenic factors EGF, bFGF, and VEGF (Fig. 2A). The iECs that emerge from this process are then expanded on gelatin-coated dishes using standard EGM-2 endothelial growth medium.

Our streamlined protocol, termed S1-Dox-ETV2, has proven highly effective, converting MePCs into iECs with remarkable efficiency. Within just 48 h of doxycycline

exposure, approximately 95% of cells were characterized as VE-Cadherin+/CD31+ as per flow cytometry analysis (Fig. 2B). This high efficiency points to the capacity of ETV2 to drive endothelial differentiation while sidestepping non-endothelial pathways. Moreover, we observed that iECs maintained their endothelial phenotype over multiple passages in culture up to passage 3, as evidenced by sustained VE-Cadherin and CD31 expression (Fig. 2C).

Given the variability of iPSC differentiation efficiency noted in conventional chemically induced protocols, we set out to test the reproducibility of our S1-Dox-ETV2 method across multiple clonal Dox-ETV2 iPSC lines (BJ273 and NYSCF-01 to NYSCF-06 lines). These lines were derived from a variety of cellular sources, reinforcing the robustness and broad applicability of our methodology. Additionally,

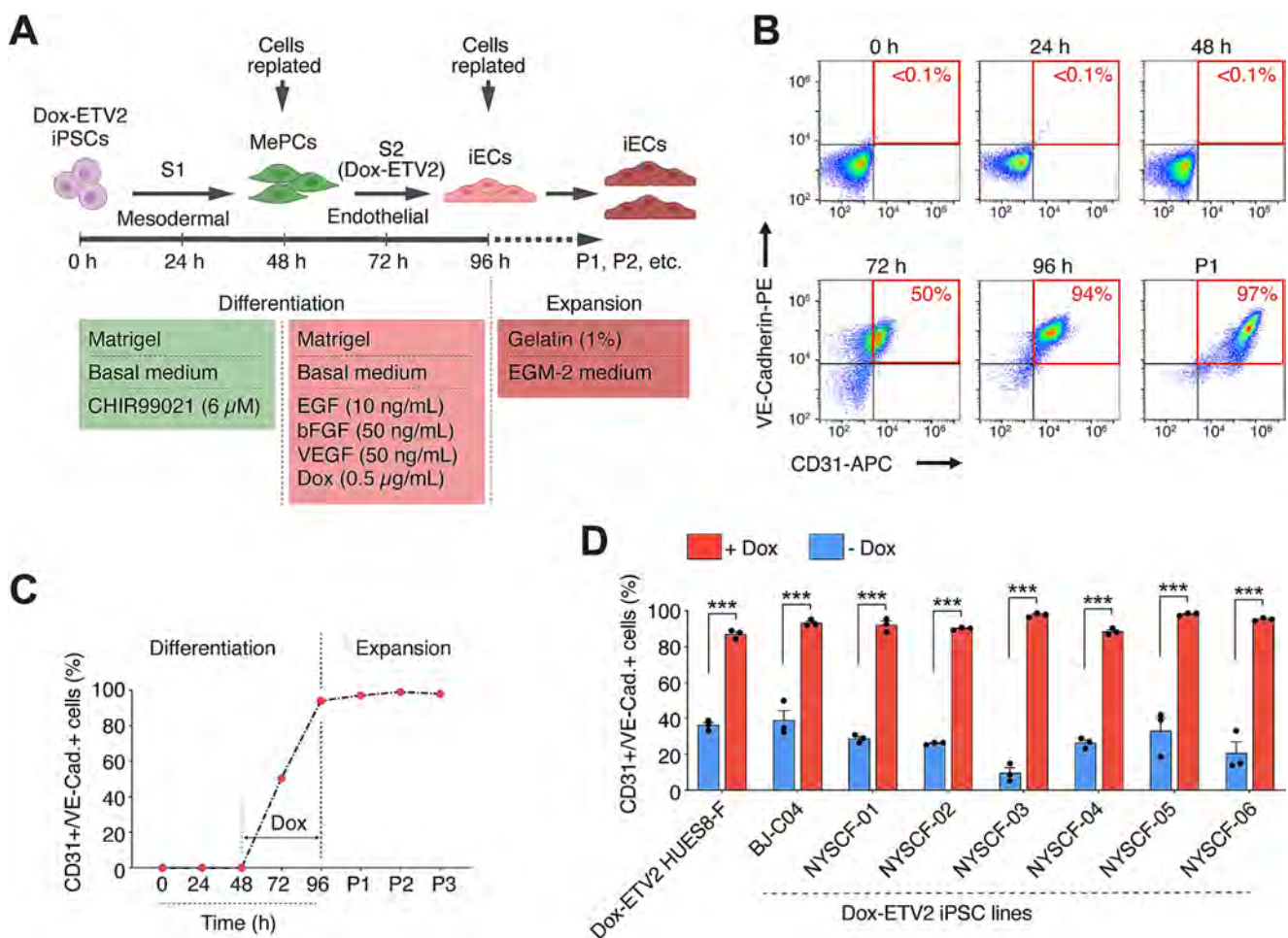


Fig. 2 Inducible differentiation of iPSCs to iECs with high efficiency and reproducibility. **(A)** Schematic of the two-stage differentiation protocol (S1-Dox-ETV2) from iPSCs to mesodermal progenitor cells (MePCs) and subsequent iECs, with transitions driven by GSK-3 inhibitor CHIR99021 and doxycycline-induced ETV2 expression, respectively. **(B)** Flow cytometry analysis displaying the rapid induction of VE-Cadherin+/CD31+ iECs after 48 h of doxycycline treatment, achieving approximately 95% differentiation efficiency at 96 h. **(C)** Maintenance of endothelial phenotype over multiple passages (P1-

P3) is shown by the sustained expression of VE-Cadherin and CD31. **(D)** Reproducibility of the S1-Dox-ETV2 protocol across various iPSC lines, including an engineered embryonic stem cell line (HUES8), indicated by consistent induction of VE-Cadherin+/CD31+ iECs upon ETV2 activation with doxycycline (red bars), compared to the lower efficiency and higher variability of iEC induction in the absence of doxycycline (blue bars). Data are mean $\pm$ s.e.m.; n are biological replicates. *** $P \leq 0.001$ ($n = 3$; paired two-tailed t-test)

we genetically engineered an established embryonic stem cell line (HUES8) to produce a Dox-inducible ETV2 variant (Dox-ETV2 HUES8-F), thus broadening the protocol's utility to encompass embryonic stem cells (Fig. 2D).

A total of seven Dox-ETV2 iPSC clones were evaluated using our streamlined S1-Dox-ETV2 differentiation protocol. Control VEGF-mediated differentiation in the absence of doxycycline showed variability and lower efficiency (10–40%) of iEC induction, with conversion rates fluctuating significantly across clones (Fig. 2D, blue bars). However, the induction of exogenous ETV2 via doxycycline uniformly enhanced differentiation efficiency across all clones, yielding between 90 and 98% VE-Cadherin+/CD31+ iECs at 96 h post-induction, regardless of their origin (Fig. 2D, red bars). This stark contrast highlights our method's capacity for consistent, high-fidelity differentiation. These outcomes align with our prior findings, where exogenous ETV2 provision via modified mRNA established a similar high-efficiency benchmark [22].

Phenotypic validation of Dox-ETV2 iECs

Following differentiation, we examined the phenotype of the Dox-ETV2 iECs. At 96 h post-differentiation (P0) and after the first passage (P1), the cells exhibited the characteristic cobblestone-like morphology typical of ECs in culture. Notably, cell alignment and organization appeared more refined at P1, following subculture onto gelatin-coated plates and subsequent culture in endothelial growth medium (Fig. 3A). Protein expression analysis at P1 by immunofluorescent staining revealed robust CD31 and VE-cadherin localization at cell-cell junctions, along with von Willebrand factor (vWF) in punctate cytoplasmic patterns (Fig. 3B). These observations are consistent with the endothelial phenotype and align with previous characterizations of iECs derived through a modified mRNA ETV2 protocol (modRNA-ETV2) [22].

To compare the transcriptomic profiles of iECs generated with the Dox-inducible and modRNA ETV2 differentiation protocols, we performed bulk RNA sequencing (RNA-seq) in triplicates, with human ECFCs and undifferentiated parental iPSCs serving as controls. Differential gene expression was extensive across all groups; however, hierarchical clustering revealed close transcriptional proximity among the Dox-ETV2 and modRNA-ETV2 iEC groups. iECs clustered more closely with ECFCs than with iPSCs, indicating a successful transcriptional transition towards an endothelial phenotype (Fig. 3C, D). These relationships were substantiated by principal component analysis (Fig. 3C) and pairwise correlations (Fig. 3D).

Further validation of key endothelial genes via qPCR confirmed expected expression patterns (Fig. 3E). *ETV2*

expression, highly upregulated during differentiation at P0, was silenced by P1 upon Dox withdrawal, matching the non-expression of *ETV2* in control ECFCs (Fig. 3E). This cessation of ETV2 expression at P1 is consistent with the gene's transient role during endothelial development. Moreover, at P1, our Dox-ETV2 iECs expressed endothelial markers such as *PECAM-1* (CD31), *CDH5* (VE-Cadherin), *VWF*, *KDR* (VEGFR2), and *NOS3* (eNOS), with a notable increase in expression levels compared to P0, indicative of maturation following passage and culture under endothelial-specific conditions (Fig. 3E). The expression levels of these endothelial markers generally surpassed those in control ECFCs, with the exception of *NOS3* and *VWF* (Fig. 3E). The low *VWF* expression indicate some degree of immaturity in the iPSC-derived iECs, which is consistent with other differentiation protocols as reported previously [22]. Lastly, the pluripotency marker *OCT4* was significantly downregulated in iECs, with more than six-fold reduction compared to iPSCs, reaching levels comparable to those in control ECFCs (Fig. 3E). Collectively, these analyses validated the endothelial phenotype of the differentiated Dox-ETV2 iECs and corroborated their maturation and divergence from a pluripotent state.

To complement our transcriptomic data, we conducted proteomic analyses to interrogate the protein expression of various EC markers in the differentiated Dox-ETV2 iECs at P0. For this proteomic assessment, primary human brain microvascular ECs (BMECs) and the epithelial cell line H-1975 were employed as positive and negative controls, respectively. Our proteomic results indicate a consistent expression of EC markers at the protein level in iECs (Fig. 3F and G). Notably, aside from vWF, which was expressed significantly lower in iECs—paralleling our mRNA level observations (Fig. 3E)—other EC markers, including CD31 and VE-cadherin, were highly expressed, matching the levels observed in BMECs. These findings substantiate the endothelial identity of the iECs, further corroborated by the absence of epithelial markers, which confirms the selectivity of our differentiation protocol against epithelial lineage development.

Functional assay: vascular network formation on-a-chip

Beyond phenotypic validation, functional assessment is crucial for confirming the endothelial identity of differentiated cells. Previous studies, including our own, have raised concerns that iECs generated by direct ETV2 induction in iPSCs—without transitioning through an intermediate mesodermal stage—may exhibit an endothelial-like phenotype, yet possess impaired functionality [22]. Therefore, rigorous functional testing is imperative to ensure that the

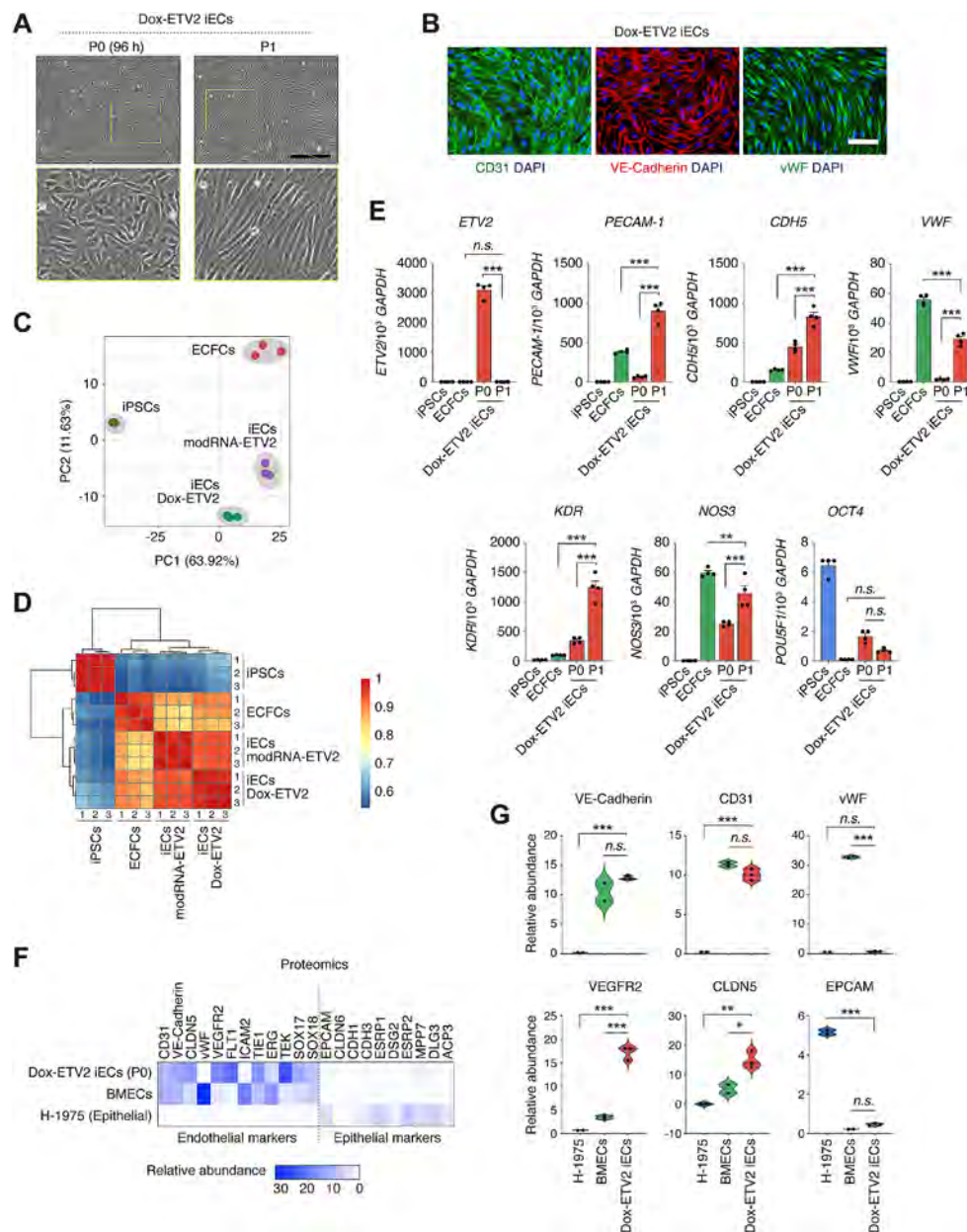


Fig. 3 Phenotypic validation of Dox-ETV2-induced iECs. **(A)** Morphological assessment of Dox-ETV2 iECs at post-differentiation (P0) and after the first passage (P1), displaying typical EC cobblestone-like morphology with enhanced alignment and organization at P1. **(B)** Immunofluorescent staining of Dox-ETV2 iECs at P1 for endothelial markers CD31, VE-cadherin, and vWF, confirming the endothelial phenotype. Cell nuclei are counterstained with DAPI. **(C–D)** Bulk RNA-seq analysis showing transcriptional profiles of iECs, with **(C)** principal component analysis and **(D)** hierarchical clustering indicating close transcriptional similarity between Dox-ETV2 iECs and modRNA-ETV2 iECs, and their closer relation to ECFCs compared to iPSCs ($n=3$). **(E)** Quantitative PCR validation of endo-

thelial-specific gene expression, demonstrating the transient upregulation of ETV2 during differentiation, maturation of EC markers at P1, and the downregulation of pluripotency marker OCT4 in iECs. All PCR data is normalized to GAPDH ($n=4$). **(F)** Proteomics heatmap comparison reveals relative protein abundance levels of endothelial markers in Dox-ETV2 iECs (P0) ($n=3$). Primary human BMECs and the epithelial cell line H-1975 served as positive and negative controls ($n=2$), respectively. **(G)** Violin plots depict quantified proteomic analysis of endothelial markers. Data are mean $\pm$ s.e.m.; n are biological replicates. *n.s.* is not statistically significant; $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ (one-way ANOVA with Bonferroni's post-test). Scale bars: 250 μ m (**A**), 100 μ m (**B**)

differentiated cells truly embody the defining characteristics and capabilities of ECs. This validation of common EC attributes should not preclude the identification of additional tissue-specific properties and heterogeneity that might be

present when following the various differentiation protocols [40–42].

A diverse suite of assays is available to probe the multifaceted functional attributes of ECs, encompassing angiogenic

activity, regulation of leukocyte adhesion, alignment in response to shear stress, and the synthesis of nitric oxide [43]. Indeed, many of these assays were used in our previous work with modRNA-ETV2 iECs [22]. Among these, a critical assay that encapsulates EC function is the ability to form a network of perfused vessels. Thus, we assessed this capability in our Dox-ETV2 iECs through both in vitro and in vivo models.

For the in vitro analysis of vascular network formation, we employed a microphysiological system often utilized in vascular biology. We adopted a microfluidic ‘on-a-chip’ model that enables manipulation of the microenvironment,

including mechanical and biochemical factors, that shape the microvascular network assembly through vasculogenesis [44, 45]. Using this perfusable microfluidic platform, we could monitor and modulate variables such as paracrine signaling, cell seeding density, and the matrix’s mechanical properties (Fig. 4A). We incorporated Dox-ETV2 iECs expressing GFP into the device alongside human MSCs at a 2:3 ratio, embedded within fibrin gels in the central channel of the chips. Nutrient provision was facilitated by the culture medium supplied to the flanking channels, enriched with VEGF, TGFβ1, and the Notch pathway inhibitor DAPT.

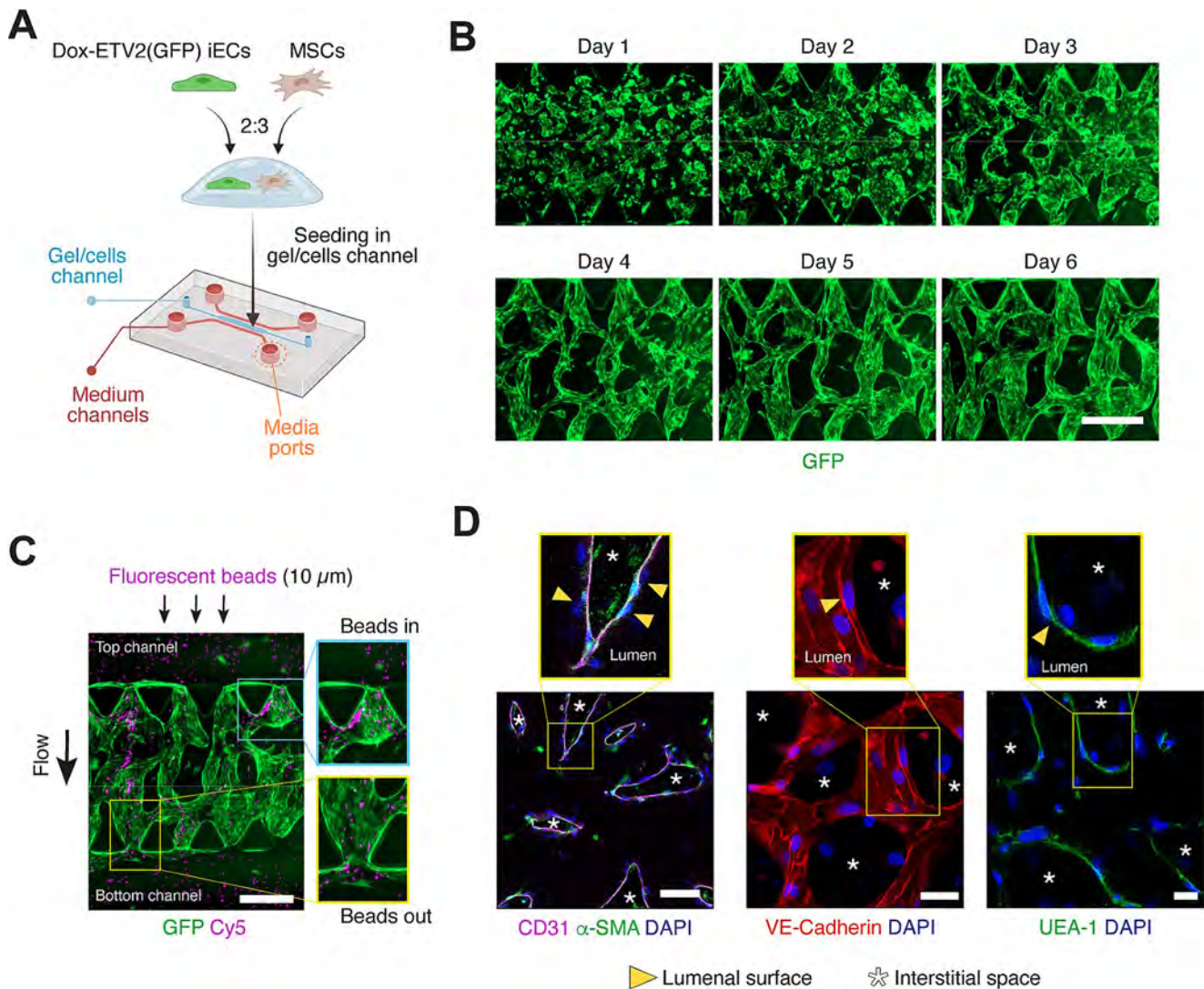


Fig. 4 In vitro vascular network formation using a microfluidic platform. **(A)** Schematic illustrating the setup of the microfluidic ‘on-a-chip’ system for vascular network assembly, highlighting the co-culture of GFP-labeled Dox-ETV2 iECs with MSCs in a fibrin gel matrix. **(B)** Time-lapse GFP fluorescence imaging depicts the progression of iEC network formation from day 1 to day 6. **(C)** Confirmation of vascular perfusion on day 7 is shown by the successful transit of fluorescent microspheres through the luminal structures within the

iEC network. **(D)** Immunofluorescence characterization of the vascular networks, with UEA-1 + iECs forming the lumens and expressing CD31 and VE-Cadherin, and perivascular cells expressing α-SMA in the surrounding interstitial spaces. Cell nuclei are counterstained with DAPI. Scale bars: 500 μm (B, C), and 100 μm, 40 μm, and 50 μm for left, middle, and right panels (D), respectively. Related results in Supplementary Video S1

GFP fluorescence imaging allowed us to visualize the spontaneous network formation by iECs, with initial structures observable at 24 h and increasingly complex networks by day 6 (Fig. 4B). The interaction between MSCs and iECs was indispensable for averting network regression and ensuring morphological stability beyond day 4. By day 7, the engineered vessels featured patent lumens capable of supporting the flow of fluorescent microspheres, indicative of perfusion, when a pressure gradient was applied across the media channels (Fig. 4C, Supplemental Video S1). Immunofluorescence further verified that the luminal iECs expressed canonical endothelial markers, CD31 and VE-Cadherin, as well as their affinity for UEA-1 lectin binding, delineating an intact endothelial lining (Fig. 4D). Concurrently, CD31-negative MSCs populated the interstitial spaces, with subsets expressing the perivascular marker α -smooth muscle actin (α -SMA) positioned on the abluminal side, adjacent to the lumens (Fig. 4D).

Collectively, this in vitro approach substantiates that the Dox-ETV2 iECs, in conjunction with perivascular cells, are capable of orchestrating the formation of perfusable vascular networks. This proficiency serves as a benchmark for their functional maturation and validates their potential for applications in vascular tissue engineering and regenerative medicine.

Functional assay: vascular network formation in vivo

Notwithstanding the advantages of microfluidic systems, evaluating EC functionality in vivo represents a more definitive test, extending beyond the capacity to assemble perfusable networks in vitro. The crucial test is to evaluate iECs' capacity to engraft and form functional vascular networks that integrate with the host's circulation. To this end, we used our subcutaneous xenograft model in immunodeficient (nude) mice [8]. Dox-ETV2 iECs were combined with human MSCs and implanted subcutaneously into nude mice for 7 days (Fig. 5A). Control grafts comprising ECFCs mixed with MSCs were included for comparative analysis. Initial macroscopic examination of the explanted grafts revealed a red hue, indicative of perfusion (Fig. 5A). Hematoxylin and eosin (H&E) staining of the graft sections confirmed the presence of extensive networks of microvessels that carried red blood cells, suggesting functional integration with the host's vasculature (Fig. 5B). Quantitative analysis of microvessel density showed a more extensive vessel formation in grafts with ECFCs (~ 178 vessels/mm²) compared to those with iECs (~ 92 vessels/mm²). The average microvessel density found in grafts with ECFCs was statistically higher than in iPSC-derived iECs, which could reflect their more mature phenotype [9]. Nevertheless, both

ECFCs and iECs were proficient in forming perfused vessels in vivo (Fig. 5C). Human-specific CD31 expression corroborated that the vessels were indeed lined with iECs (Fig. 5D).

Subsequent analysis of mural cell recruitment, an essential process for vessel maturation and stability, demonstrated that the majority of the human-derived vessels within the Dox-ETV2 iEC grafts were invested by perivascular cells expressing α -SMA (Fig. 5D). This pattern of perivascular cell coverage was also consistently observed in control grafts containing ECFCs (Fig. 5D).

In summary, our streamlined methodology yields iECs with proper phenotypic and functional competence. This includes their capacity to assemble into stable, perfused vascular networks, a crucial attribute for ECs, demonstrated both in vitro and in vivo.

Discussion

In this study, we introduce a streamlined method for inducing the differentiation of human iPSCs into iECs, utilizing a doxycycline-inducible system for the transient activation of ETV2. This methodology offers significant enhancements over existing strategies for generating ECs from iPSCs through the expression of TFs. Here is a summary of the key advantages this method provides:

1. *Streamlined and efficient differentiation*: Our method consistently achieves over 90% efficiency in just four days, streamlining the process and ensuring high yield.
2. *Reproducibility across different cell lines*: Our approach demonstrates robust reproducibility across a variety of iPSC and embryonic stem cell lines. This overcomes the variability often encountered with chemically induced differentiation methods, ensuring consistent outcomes.
3. *Reduction in protocol technical complexity*: Our method simplifies the differentiation process by eliminating the need for repeated transfections, a significant improvement over other strategies used to transiently activate ETV2, including our previous modified mRNA technique.
4. *Controlled transgene expression*: The doxycycline-inducible system allows for precise temporal control over the expression of ETV2, enabling a transient induction that more closely mirrors developmental endothelial differentiation. This represents a key improvement over methods that induce ETV2 expression either in a non-transient manner or prematurely, which may compromise EC functionality.
5. *Phenotypic stability and functional integrity*: The iECs generated through our protocol exhibit enduring

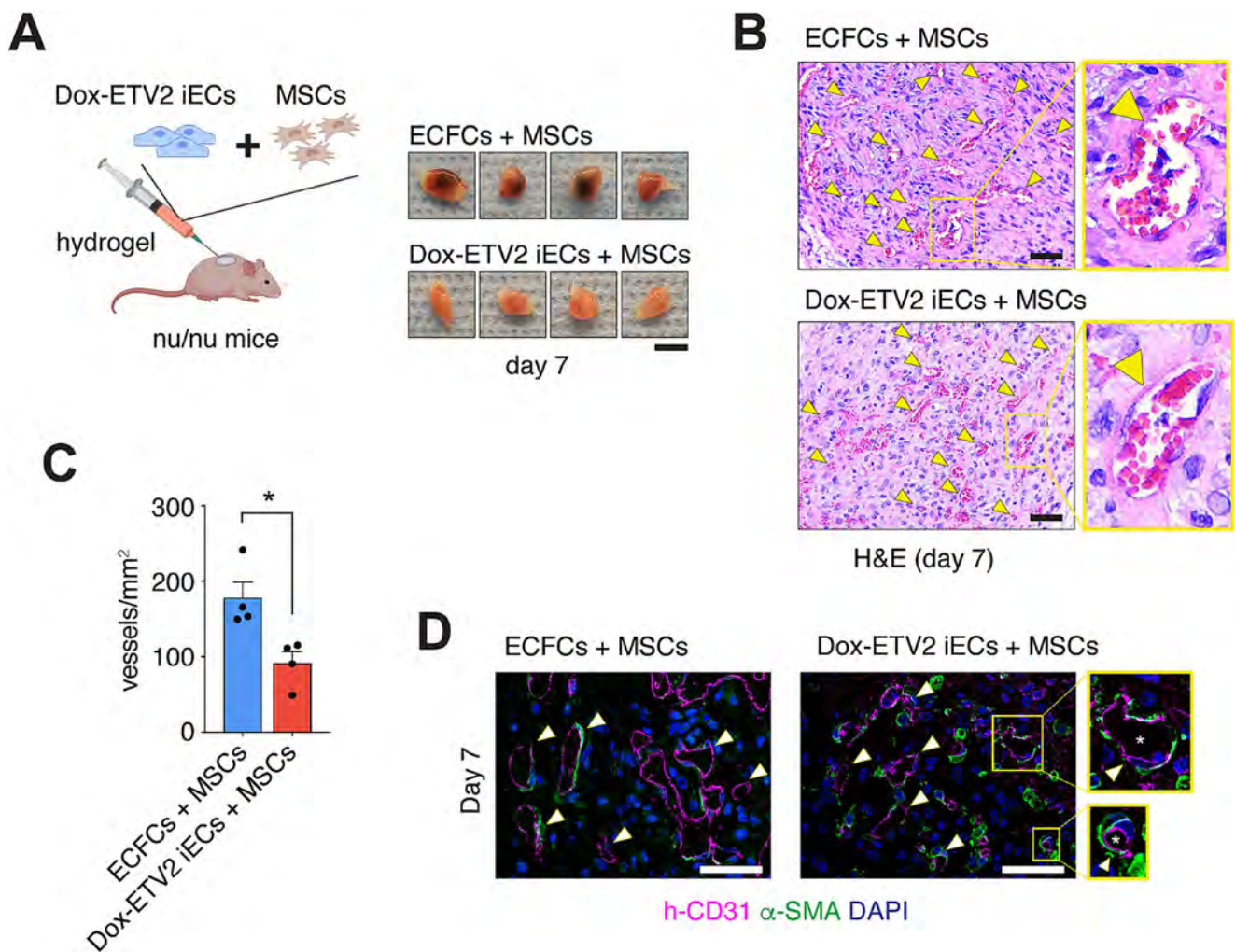


Fig. 5 In vivo formation of functional vascular networks by Dox-ETV2 iECs. **(A)** Schematic illustrating the subcutaneous xenograft model in nude mice used to assess the vascular network-forming capability of Dox-ETV2 iECs in conjunction with MSCs. Macroscopic images of the explanted grafts on day 7. **(B)** Hematoxylin and eosin (H&E) staining of the explanted grafts revealing perfused microvessels containing red blood cells (yellow arrowheads) on day 7 post-implantation, suggesting functional anastomosis with the host vas-

culature. **(C)** Quantitative analysis of the microvessel density within the grafts shows significant vessel formation by both Dox-ETV2 iECs and ECFCs. Data are mean $\pm$ s.e.m.; n are biological replicates. $*P \leq 0.05$ ($n=4$; paired two-tailed t-test). **(D)** Immunofluorescence confirmation of human-specific CD31 expression by iECs lining the vessels, and α -SMA expression by perivascular cells surrounding the vessels, indicative of successful engraftment and vessel maturation. Cell nuclei are counterstained with DAPI. Scale bars: 4 mm (A), 50 μ m (B, D)

endothelial characteristics and the capability to form vascular networks both in vitro and in vivo. These critical features, which underscore the functional and phenotypic fidelity of our iECs, are often not adequately demonstrated in other protocols.

Collectively, these advancements, which are elaborated in more detail and with more context below, position this doxycycline-inducible ETV2 method as a significant contribution to the field, offering a more accessible and reproducible strategy for generating iECs.

Our methodology capitalized on the use of ETV2, a pioneer TF [20]. The approach builds upon previous findings where exogenous ETV2 was leveraged to substantially

enhance differentiation efficiency [15, 22, 24]. A primary advantage of our method is the implementation of an inducible system, which eliminates the need for recurrent and cumbersome transfection procedures (e.g., electroporation, lipofection), thereby streamlining the protocol and enhancing its utility. For example, this method distinguishes itself from our previously reported modRNA-based technique [22], which, while effective, required direct transfection of modified mRNA encoding ETV2. Our current approach simplifies the differentiation process, significantly reducing the technical complexity and potential for variability inherent in direct mRNA transfections. Also, the rapid progression from iPSCs to iECs, achieved in just four days, represents a significant reduction in the cellular reprogramming timeline.

Another important feature of this protocol is its reproducibility, which is critical, particularly in the field of regenerative medicine, where protocols frequently require optimization across different iPSC lines or clones. Our results demonstrate a high degree of consistency in the production of iECs expressing canonical endothelial markers across multiple iPSC lines as well as an embryonic stem cell line, confirming the robustness of this approach. This uniformity in differentiation efficiency illustrates the advantage of using TFs and is consistent with findings from our prior study employing the modified mRNA technique to provide exogenous ETV2 [22]. Furthermore, the differentiated cells exhibit a stable endothelial phenotype without regression across multiple passages, although further studies extending to longer passage numbers are warranted to provide more robust evidence for long-term phenotypic stability. This reproducibility, coupled with the robustness and expedited timeline, underscores the utility of the protocol.

Our methodology calls for the use of the piggyBac transposon system for the insertion of the Dox-inducible ETV2 transgene into iPSCs. The piggyBac system has been previously recognized for its efficacy in gene insertion and its ability to facilitate controlled overexpression of exogenous TFs [14, 30]. One significant advantage of the piggyBac transposon system is the absence of size constraints on the transgene, which contrasts with the limitations observed with lentiviral vectors. However, considering the modest size of the ETV2 gene, alternative methods, such as lentiviral transduction, have also been proven feasible for insertion [24–27]. In addition, the piggyBac system enables the integration of a larger number of gene copies into the genome. However, while, in certain contexts, this might enhance gene function [34], our previous work with modified mRNA suggests that the number of ETV2 copies does not markedly influence differentiation outcomes [22].

The choice of an inducible over a constitutively active system for ETV2 expression is not arbitrary, and several studies have previously attempted inducible strategies [27–31]. A constitutive system precludes the maintenance of iPSC pluripotency due to the persistent activity of ETV2. On the contrary, an inducible system allows for the temporal control of ETV2 expression, aligning it with the differentiation stage where its activity is required. Also, the integrated inducible approach described here circumvents the inefficiencies and impracticalities associated with repeated viral transductions and cell line selections for each differentiation cycle that would be required if constitutive lentiviral systems were to be used.

Other strategies might propose the co-activation of additional TFs alongside ETV2, such as FLI1 and ERG [15, 25]. However, studies have shown that the induction of ETV2 alone appears sufficient to upregulate downstream

endothelial-specific TFs [14, 22, 30]. In any case, future investigations could explore whether overexpressing ETV2 with other TFs confers any phenotypic or functional advantages to the derived iECs. Nonetheless, our present findings confirm the efficacy and practicality of the ETV2-centric, piggyBac-mediated, inducible approach in generating iECs from iPSCs.

Our protocol outlines a precise and efficient method for directing the initial conversion of iPSCs into mesodermal progenitors, a critical step achieved within 48 h using the GSK-3 inhibitor CHIR99021 to activate Wnt signaling. However, the field lacks a unanimous approach for inducing mesoderm, with some studies employing additional factors like BMP-4 and activin A to stimulate Nodal signaling as well as longer differentiation periods [46–48]. These alternative strategies, varying in their concentration and combination of growth factors, might influence the characteristics of the downstream vascular cells produced. In any case, integrating different mesodermal induction techniques into our protocol is feasible, as these modifications would occur upstream of ETV2 activation. While we anticipate that such alterations might not significantly impact the capacity of ETV2 to drive the differentiation of mesodermal intermediates into iECs, comprehensive evaluations would be necessary to determine whether these modifications affect the phenotypic and functional attributes of the resultant iECs.

Another important consideration is the timing of ETV2 activation. Several recent methodologies have suggested the possibility of activating ETV2 directly in iPSCs, thereby circumventing the mesodermal induction phase [24, 26, 27, 30, 31]. Although this approach appears to streamline the differentiation process further, our previous findings indicate that such direct activation might yield iECs with compromised functionality, particularly in their capability to form perfused vascular networks in vivo [22]. As a result, while exploring variations in the mesodermal induction step or bypassing it altogether may seem appealing for simplification, a careful functional assessment of the cells obtained through these alternative routes is recommended to ensure their competency and applicability in regenerative medicine.

In our study, we have also delineated methods to evaluate the vascular network formation capabilities of the resultant iECs both in vitro and in vivo. While these assays provide crucial insights into endothelial function, they should be recognized as part of a broader suite of functional evaluations rather than definitive or exhaustive tests. These assays—forming perfused vascular networks in vitro using a microphysiological on-a-chip model and in vivo through a subcutaneous xenograft model in immunodeficient mice—represent just a fraction of the potential methodologies to examine iEC functionality. Additional functional attributes,

such as angiogenic response, leukocyte adhesion regulation, sprouting, lumen formation, alignment under shear stress, and nitric oxide production, can also be assessed through various other assays detailed elsewhere [43].

Our *in vitro* demonstration of iECs' ability to form perfused networks aligns with findings from other studies utilizing similar microfluidic platforms [45, 49]. However, it is important to note that the conditions for assembling these platforms vary significantly across studies, emphasizing the need to tailor the assay conditions to the specific characteristics of the ECs being tested.

For a more definitive evaluation of iEC functionality, the *in vivo* context presents a critical test of their capacity to engraft, integrate into host circulation, and form functional vascular networks. Indeed, our use of a subcutaneous xenograft model in nude mice in this study provided valuable evidence of the practical applicability of Dox-ETV2 iECs in regenerative medicine settings. This model, by demonstrating the engraftment and perfusion capabilities of the iECs, offers a direct assessment of their potential for contributing to vascular networks in a living organism [43, 50]. In any case, these functional assays, while essential for characterizing the endothelial phenotype, should be considered starting points for a comprehensive evaluation of iEC functionality. Researchers are encouraged to expand upon these foundational assays to explore further biological and translational applications of Dox-ETV2 iECs, tailoring their investigative approach to the specific questions and contexts of their studies.

Our streamlined methodology, while presenting numerous advantages, also has potential limitations that merit consideration. A primary concern is the genomic footprint left by the integration of the ETV2 transgene via the piggyBac transposon system. While the piggyBac's ability to incorporate large transgenes offers flexibility, the resultant genomic footprint raises potential concerns about insertional mutagenesis and genomic stability, particularly in contexts where genomic integrity is crucial. The piggyBac system produces random insertions of the transgene at 'TTAA' sites, which could inadvertently influence the expression of proximal genes or disrupt critical genomic regions [51]. This phenomenon is not exclusive to the piggyBac system – lentivirus and retrovirus have an even higher frequency of exon integration [51]. Although for most research applications, this may not present a significant issue, the implications for clinical translation and therapeutic use demand careful consideration. For instance, investigators could routinely screen for undesirable insertions on every single clone, which is cumbersome and not very practical. To circumvent these concerns, alternative strategies such as CRISPR/Cas9-mediated insertion into 'safe harbor' genomic sites like the AAVS1 locus could be employed to achieve more

precise control over transgene integration [28]. Additionally, for applications requiring a transient expression without a permanent genomic footprint, methodologies relying on temporary transfection with modified mRNA might be preferred despite being less streamlined than our current approach [22–24].

A second limitation is related to our methodology's reliance on ETV2 as the sole driver of endothelial differentiation, which overlooks the potential synergistic benefits of co-expressing other TFs that might enhance differentiation efficiency or EC functionality [15, 25]. This represents an area for further exploration, offering the potential to improve the quality of iECs derived from this process.

Lastly, the risk of transgene silencing over time poses a potential challenge to the long-term stability of ETV2 expression and the enduring differentiation capacity of the engineered iPSCs [52]. Although the piggyBac system's capacity for multiple insertions may minimize this risk [53], regular monitoring of ETV2 inducibility in engineered cell lines is advisable to maintain consistent differentiation outcomes.

In conclusion, our work introduces a streamlined method for the efficient and reproducible differentiation of human iPSCs into iECs, leveraging the transient, doxycycline-inducible activation of the ETV2 TF. The adoption of this TF-inducible approach underscores a broader trend in stem cell research towards more controlled and precise differentiation processes. By developing and sharing research on this methodology and the related Dox-ETV2 iPSC lines, we aim to enhance their utility in advancing vascular biology research and exploring regenerative medicine applications. As the utility of these inducible systems gains recognition, we anticipate their increased incorporation into future vascular biology studies, offering a valuable resource for investigators seeking to reliably generate human iECs.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10456-024-09937-5>.

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Author contributions A.C.L., J.W., J.G. and J.M.M.-M. conceived and designed the project. A.C.L., J.W., K.W., Y.Z., L.G., U.L., X.L., and R.-Z.L. performed the experimental work. Y.Z. performed bioinformatical analyses. All authors discussed and analyzed the data and edited the results. A.C.L., and J.M.M.-M. wrote the manuscript with input from D.E.I and all other authors.

Data availability RNA-seq data used in this study are deposited in the Gene Expression Omnibus (GEO) under accession number [GSE263058].

Declarations

Competing interests The authors declare no competing interests.

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