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Robust Differentiation of Human Pluripotent Stem Cells into Lymphatic Endothelial Cells Using Transcription Factors

Sanjoy Saha[a, b] Francine Graham[b, c] James Knopp[c, d]
Christopher Patzke[c, d, e] Donny Hanjaya-Putra[a, b, c]

[a]Department of Aerospace and Mechanical Engineering, Bioengineering Graduate Program, University of Notre Dame, Notre Dame, IN, USA; [b]Department of Chemical and Biomolecular Engineering, University of Notre Dame, Notre Dame, IN, USA; [c]Center for Stem Cells and Regenerative Medicine, University of Notre Dame, Notre Dame, IN, USA; [d]Department of Biological Science, University of Notre Dame, Notre Dame, IN, USA; [e]Boler-Parseghian Center for Rare Diseases, University of Notre Dame, Notre Dame, IN, USA

Keywords
Biomedical engineering · Tissue engineering · Stem cell differentiation · Transcription factors · Vascular biology · Lymphatic biology

Abstract
Introduction: Generating new lymphatic vessels has been postulated as an innovative therapeutic strategy for various disease phenotypes, including neurodegenerative diseases, metabolic syndrome, cardiovascular disease, and lymphedema. Yet, compared to the blood vascular system, protocols to differentiate human induced pluripotent stem cells (hiPSCs) into lymphatic endothelial cells (LECs) are still lacking. ***Methods:*** Transcription factors, *ETS2* and *ETV2* are key regulators of embryonic vascular development, including lymphatic specification. While *ETV2* has been shown to efficiently generate blood endothelial cells, little is known about *ETS2* and its role in lymphatic differentiation. Here, we describe a method for rapid and efficient generation of LECs using transcription factors, *ETS2* and *ETV2*. ***Results:*** This approach reproducibly differentiates four diverse hiPSCs into LECs with exceedingly high efficiency. Timely activation of *ETS2* was critical, to enable its interaction with *Prox1*, a master lymphatic regulator. Differentiated LECs express key lymphatic markers, VEGFR3, LYVE-1, and Podoplanin, in comparable levels to mature LECs. The differentiated LECs are able to assemble into stable lymphatic vascular networks in vitro, and secrete key lymphangiocrine, reelin. ***Conclusion:*** Overall, our protocol has broad applications for basic study of lymphatic biology, as well as toward various approaches in lymphatic regeneration and personalized medicine.

Introduction

The lymphatic system, a crucial component of the vascular network, plays an indispensable role in maintaining tissue fluid homeostasis, immune cell trafficking, and lipid absorption [1, 2]. Dysfunction or impairment of lymphatic vessels underlies various pathological conditions spanning neurodegenerative diseases, metabolic syndrome, cardiovascular ailments, and lymphedema [3]. Despite its critical role, therapeutic and disease modeling strategies focused on generating new lymphatic vessels remain relatively underexplored [4, 5].

karger@karger.com
www.karger.com/cto

Correspondence to:
Christopher Patzke, cpatzke@nd.edu
Donny Hanjaya-Putra, dputra1@nd.edu

One promising avenue in addressing this gap lies in harnessing human induced pluripotent stem cells (hiPSCs) for the differentiation and generation of lymphatic endothelial cells (LECs). The development of efficient protocols for generating LECs from hiPSCs is crucial for advancing our understanding of lymphatic biology and for exploring new therapeutic approaches for lymphatic-related diseases. While considerable progress has been made in directing hiPSCs toward blood endothelial cells [6–10], the protocols to effectively differentiate hiPSCs into LECs have been somewhat elusive, hindering advancements in lymphatic-related research and therapeutic interventions. There exists a handful of differentiation protocol to generate LEC from hiPSCs mostly relying on embryonic body (EB) intermediate or the use of murine feeder layers [11–13]. Existing protocols, however, are far from optimal. Limitations stem from the inherent complexity associated with improper cell aggregation and interaction with extraneous cell population [14]. These result in very low yield and poor isolation of final differentiated cells. Moreover, these protocols involve prolonged culture periods and lacks definite functional behavior [15].

Transcription factors, particularly *ETS2* and *ETV2*, have emerged as pivotal regulators governing embryonic vascular development, including the specification of LECs [16]. *ETV2* has shown promise in generating blood endothelial cells efficiently [17–20]. One recent study even shows that *ETV2* is required for lymphangiogenesis and directly regulates VEGFR3/FLT4 expression [21]. Using in vitro differentiated mouse embryonic stem cells, *ETV2* ChIP-Seq analysis revealed specific *ETV2* binding peaks present within VEGFR3 and LYVE-1 promoter/enhancer regions [22]. The VEGFR3 promoter is a likely direct target of *ETV2*, containing an evolutionarily conserved FOX: ETS domain that is bound by *ETV2* and *FOXC2* transcription factors [23]. On the other hand, the expression and colocalization of *ETS2* was identified in the nuclei of LECs [24]. In addition, the work highlights the synergistic enhancement of *ETS2* and *PROX1* in expression of *VEGFR3*. Consistent with the effects on expression profile of *VEGFR3*, *ETS2* induces LEC migration toward VEGF-C. In summary, *ETS2* is reported as a pivotal pro-lymphangiogenic factor in collaboration with PROX1 during lymphangiogenesis. Though a fair number of studies exist to show that *ETV2* and *ETS2* are important regulatory components of LECs, the potential of these factors in the differentiation process toward LECs remains unexplored.

This study aims to address this gap by presenting a novel methodology that reliably and efficiently generates LECs from diverse hiPSC lines using transcription factors – *ETS2* and *ETV2*. The critical temporal activation of *ETV2* and *ETS2* is emphasized in this protocol, as it enables the essential interaction with Prox1, a master regulator pivotal in lymphatic specification [25]. The resulting differentiated LECs express key lymphatic markers such as VEGFR3, LYVE-1, and Podoplanin, exhibiting expression levels comparable to mature LECs. Moreover, the differentiated LECs, following 3 days of culture in hydrogels, demonstrate their capacity to assemble into stable lymphatic vascular networks in vitro. Additionally, these cells exhibit the ability to secrete reelin, a crucial lymphangiocrine [26], further highlighting their functional attributes. Overall, this protocol not only offers significant implications for advancing our fundamental understanding of lymphatic biology but also holds substantial promise for various applications, including approaches in lymphatic regeneration and personalized medicine. The methodology detailed herein lays a robust foundation for further exploration and development of targeted therapies for a spectrum of lymphatic-related disorders and diseases.

Materials and Method

Viral Vector Synthesis and Formulation

Lentiviruses were produced as previously described [27] in HEK293T cells (ATCC) by cotransfection with three helper plasmids (pRSV-REV, pMDLg/pRRE and vesicular stomatitis virus G protein expression vector) with 12 μg of lentiviral vector DNA and 6 μg of each of the helper plasmid DNA per 75 cm^2 culture area) using calcium phosphate transfection. Lentiviral vector DNA plasmids used are pSIN4-EF1a-ETV2-IRES-Puro (61061, Addgene) [28], pLV-tetO-Ets2 (70272, Addgene) [29] and FUW-M2rtTA (20342, Addgene) [30]. Lentiviruses were harvested with the medium 46 h after transfection, aliquoted, and frozen at −80°C. Details of lentiviral constructs are available in Supplementary Table 1 (for all online suppl. material, see https://doi.org/10.1159/000539699) and online supplementary Figure 1. Only virus preparations with >90% infection efficiency as assessed by GFP expression or puromycin resistance were used for experiments (online suppl. Fig. 2).

Culture of hiPSCs

Four different hiPSC lines derived from various tissue origins (online suppl. Table 2) and were obtained from WiCell Research Resources (WiCell, WI). The cells were maintained on growth factor reduced Matrigel (Corning)

in mTeSR™ Plus medium (Stemcell Technologies). The hiPSC colonies were checked regularly and passaged when reached around 70% confluency. Accutase (Innovative Cell Technologies) was used for dislodging the cells and depending on the cell line; ROCK inhibitor (10 μM) was used on the first day. Human iPSCs were routinely examined for pluripotent markers using immunofluorescence staining and flow cytometry analysis for TRA-1-60, TRA-1-81, SSEA4, and OCT4 (online suppl. Table 3). All cell lines were routinely tested for karyotyping and mycoplasma contamination; they expressed normal karyotype and were negative for mycoplasma throughout this study.

Transduction of hiPSCs

The hiPSCs were passaged as usual and resuspended with mTeSR™ Plus (Stemcell Technologies) media and mixed with viral vector solutions. The cells were then seeded on Matrigel coated plate (20,000–30,000 cell/cm^2). The next day the media was changed into STEMdiff™ APEL™2 (Stemcell Technologies) containing 4 μM CHIR99021(Stemcell Technologies). The *ETS2* (co-transduced with rtTA) was activated the next day by addition of doxycycline to the cell culture media and *ETV2* transduced cells were selected through puromycin treatment. This time the media was switched to APEL2 media containing 5 ng/mL VEGF-A and 10 μM SB431542 (Stemcell Technologies). After 48 h the cells were seeded on fibronectin-coated plates and cultured in MV2 media (PromoCell) containing 100 ng/mL VEGF-C. This treatment continued for 3 more days, and the cells were then used for various assays. Growth factor-based endothelial cell differentiation protocol was adopted from [31]. After EC differentiation, the cells were treated with 100 ng/mL VEGF-C similar to the treatment of transduction-based differentiation.

Immunofluorescence Staining

To visualize the lymphatic protein expression, LECs and differentiated LECs were seeded on tissue culture plastic around 60% confluency. Samples were fixed with 4% paraformaldehyde, blocked with 1% BSA, permeabilized with 0.1% Triton- X, and stained for Prox1 and ERG (online suppl. Table 3). Samples were rinsed twice in PBS and counterstained with 300 nM DAPI (Thermo Fisher). All samples were imaged using Nikon AX-R confocal at ×20 magnification.

Flow Cytometry

Differentiated LECs were analyzed for lymphatic markers using flow cytometry (FACS) following standard procedures. Briefly, cells were trypsinized and centrifuged following resuspension in FACS buffer. Suspended cells (1×10^6 cells) were stained with the antibodies (1 μg/mL) for 30 min at room temperature: Anti-LYVE-1 antibody (R&D systems, FAB20892A), Anti-PDPN antibody APC (Biolegend, 337004), as well as their corresponding IgG isotype controls (online suppl. Table 3). For intracellular staining, the cells were fixed and permeabilized with Foxp3/Transcription Factor Staining Buffer Set (Thermo, 00-5523-00) and then incubated with Anti-Prox1 antibody FITC (Novus Biologicals, NBP1-30045AF488) for 30 min. The cells were washed twice and resuspended in FACS buffer for analysis. Then, the cells were analyzed using flow cytometry (BD LSR FortessaX-20), and the metadata were analyzed using FlowJo.

Quantitative Reverse Transcription PCR

To analyze the expression of key lymphatic genes in the differentiated LECs, quantitative reverse transcription PCR was carried out in RNA lysates prepared from cells in culture. Three biological replicates (n = 3) were collected per condition and analyzed with real-time qRT-PCR with triplicate readings as previously described [32]. RNA was reverse transcribed using a high-capacity cDNA reverse transcription kit (Thermo Fisher) according to the manufacturer's protocol. cDNA was then used with the TaqMan Universal PCR Master Mix and Gene Expression Assays for *LYVE-1, Prox1, PDPN, VEGFR3*, and *GAPDH* (online suppl. Table 4). Each sample was prepared in triplicate, and the relative expression was normalized to *GAPDH* and analyzed using the $^{\Delta\Delta}$Ct method.

Enzyme-Linked Immunosorbent Assay

Supernatant samples were collected from cell culture and standardized using Coomassie Protein Assay Kit (ThermoFisher Scientific). Then standardized protocol for ELISA kits (ab100664 – VEGFC Human ELISA Kit, ab284620 – Human Reelin SimpleStep ELISA® Kit) were followed with the samples diluted to correct values. Briefly, standard solutions of target protein were prepared in a two-fold dilution series in assay diluent (online suppl. Fig. 3). Supernatant samples and standards were added to respective wells in triplicate and incubated. Then subsequently detection antibodies, enzyme conjugate, and substrate solution were added and finally the absorbance was measured at 495 nM using a microplate reader.

Tube Formation Assay

Tube formation assay was performed as previously described [33, 34]. Briefly, human differentiated LECs were stained CellTracker™ Green (ThermoFisher) plated at a density of 100,000 cells/cm^2 in one well of a μ-Slide 15 Well

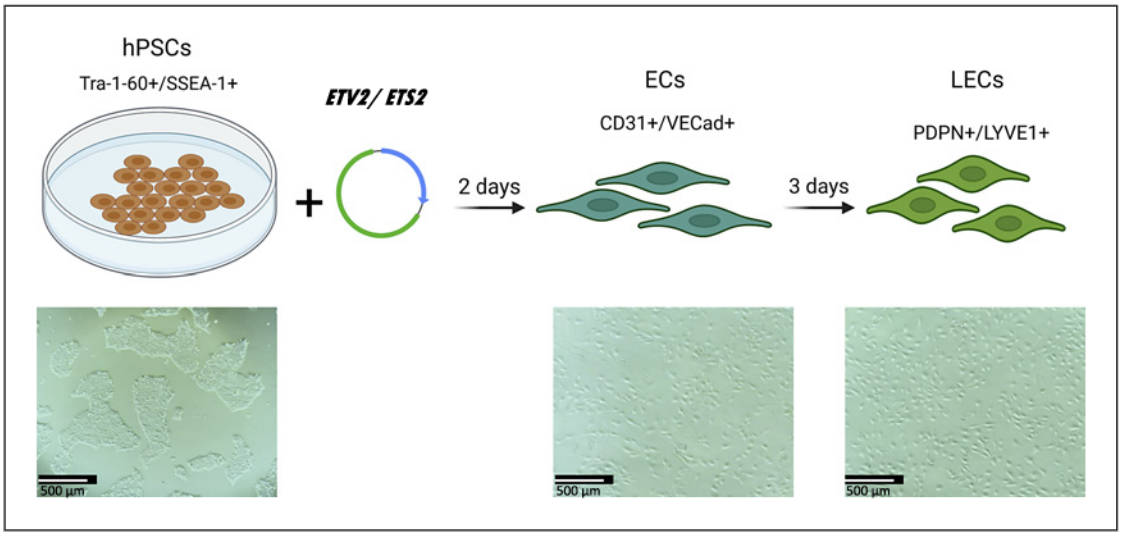

Fig. 1. Schematic of deriving LECs from hiPSCs. The cultured hiPSCs were transduced with viral vector containing specific transcription factor and seeded onto matrigel-coated plate. Next, the transcription factors were activated, and cells were cultured in differentiation media. Then differentiated ECs were seeded onto fibronectin-coated plates and treated with VEGF-C for 3 more days to make them committed to lymphatic endothelial lineage. The second row represents bright-field images of the corresponding cell states.

3D plate (ibidi) on top of solidified Matrigel (10 µL) with differentiation media [34, 35]. After 6 h, cells were imaged using a fluorescence microscope. Numbers of branches were counted by AutoTube script in MATLAB [36].

Statistical Analyses

Unless otherwise stated, data were expressed as means ± SD of the mean. Statistical analyses were performed with GraphPad Prism 10.1.2(324). For each condition, at least three independent experiments were performed with three biological replicates. Statistical comparisons were made using Student's *t* test for paired data, analysis of variance (ANOVA) for multiple comparisons, and with Tukey post hoc analysis for parametric data. Specifically, the Student's *t* test was used to analyze differences between protein expression and gene expression among different transduced groups. Significance levels were set at the following: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

Results

Efficient Transduction of Human Pluripotent Stem Cells into Lymphatic Endothelial Cells

We developed a two-dimensional, feeder-free, and chemically defined protocol that relies on a timely transition of hiPSCs through three distinct stages (Fig. 1). First is the conversion of hiPSCs into intermediate mesodermal progenitor cells (MPCs), which is mediated by the activation of Wnt and Nodal signaling pathways using the glycogen synthase kinase 3 inhibitor CHIR99021. Second, we converted the mesodermal progenitor cells into iECs (transduced endothelial cells). This is done by the transduction and activation of exogenous *ETV2/ETS2*. Third, we treat the cells with vascular endothelial growth factor C (VEGF-C) and transforming growth factor-β (TGF-β) inhibitor. Our stepwise protocol rapidly and uniformly converted hiPSCs into iLECs (transduced LECs). We achieved maximum 88.5% efficiency ($CD144^+$/$Podoplanin^+$) in driving the cells into LEC lineage. In contrast when endogenous *ETV2* was activated via VEGF signaling, the differentiation efficiency was much lower (less than 30%, data not shown). Conversion efficiency of iECs was dependent on amount of *ETV2/ETS2* utilized and thus affecting the EC-LEC conversion as well. We tested 3 different conditions to see how the differentiation efficiency was impacted by *ETV2*, *ETS2* or combination of both. Transduction using *ETV2* produced 69.5% $Prox1^+$ cells (Fig. 2a), while transduction using *ETS2* produced 95.0% $Prox1^+$ cells (Fig. 2b). Transduction using both *ETV2* and *ETS2* produced 81.0% $Prox1^+$ cells (Fig. 2c). Collectively, we found that *ETS2* transduced group had the most efficiency followed by the combination of *ETS2* and *ETV2* group, and lastly *ETV2* alone resulted in the least number of differentiated cells.

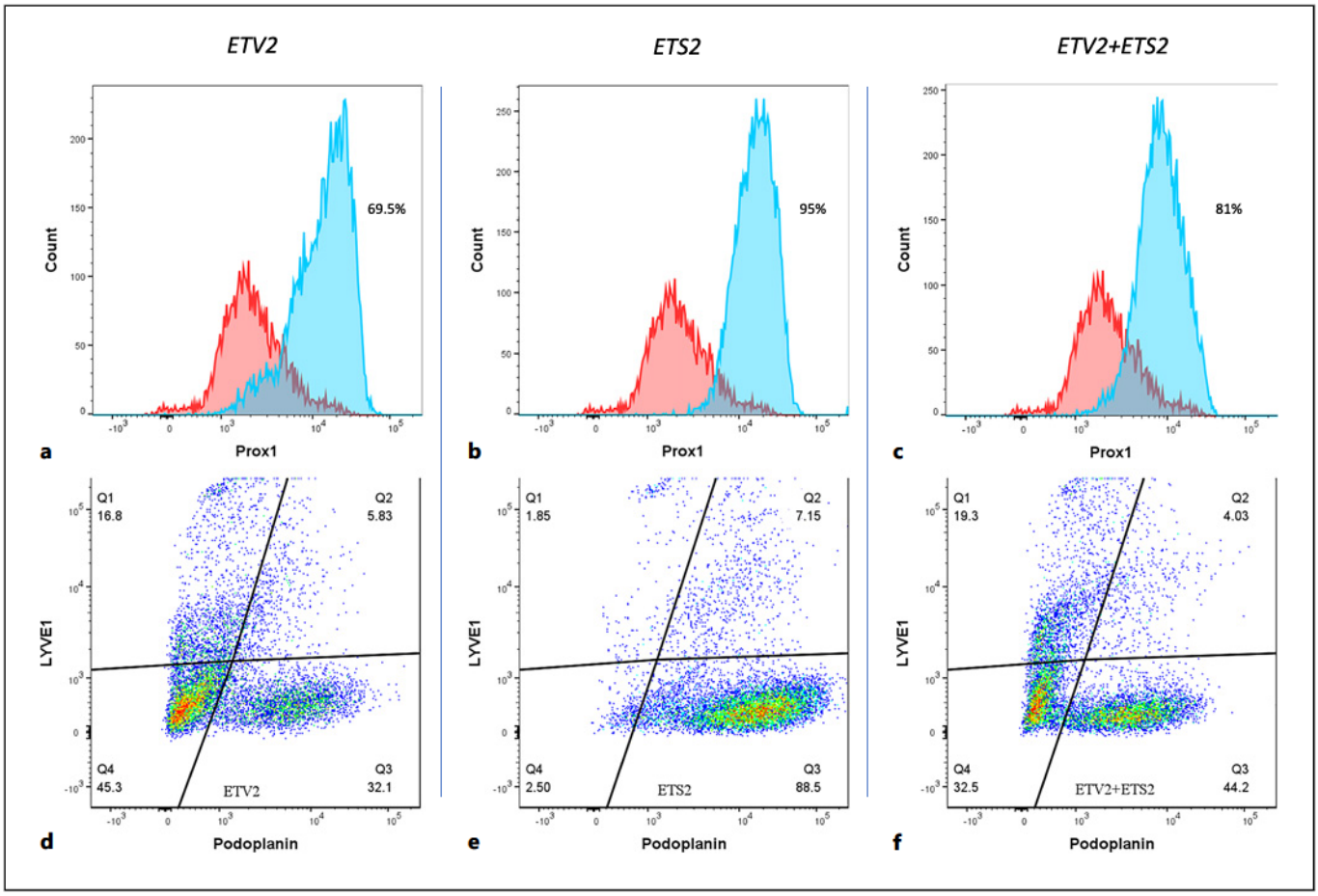

Fig. 2. Characterization of differentiated LEC by flow cytometry. **a**–**c** Prox1 shift compared to Isotype control; **d**–**f** LYVE-1 and Podoplanin expression of three transduced group. h-iLECs were characterized by quantitative protein expression of Podoplanin, LYVE-1, and Prox1. Among three transduced group, *ETS2* showed more Podoplanin expression leading up to over 88%, followed by *ETV2*+*ETS2* group (44.2%) and *ETV2* group. Similarly, Prox1 expression was higher in *ETS2* group as well, rising up to 95% whereas the *ETV2* and combination group showed 69.5% and 81% expression, respectively.

Expression of Key Lymphatic Markers in Differentiated LECs

Next, we further examined the lymphatic endothelial differentiation of the hiPSCs with FACS analysis for key lymphatic markers. Upon differentiation, the resulting iLECs showcased a robust and mature profile of multiple transmembrane key lymphatic markers, including Podoplanin, LYVE-1 and VEGFR3. Importantly, the levels of these markers in the differentiated LECs closely paralleled those observed in mature LECs, indicating a high fidelity of phenotype and maturity in the generated cell population (Fig. 2d–f). Interestingly, we did see differences in the degree of Podoplanin and LYVE-1 expression in the three transduced groups. From the FACS data, it is clearly observed that *ETV2* transduction produced 37.93% Podoplanin$^+$ cells (Fig. 2d) whereas *ETS2* transduction produced 95.65% Podoplanin$^+$ cells (Fig. 2e). The combination of *ETV2* and *ETS2* group produced 48.23% Podoplanin$^+$ cells (Fig. 2f). In terms of LYVE-1 expression, we see the opposite trend. Combination of *ETV2* and *ETS2* produced 23.3% LYVE-1$^+$ cells, *ETV2* produced 22.63% LYVE-1$^+$ cells, and *ETS2* produced 9.0% LYVE-1$^+$ cells (Fig. 2d–f).

To characterize the identity of the cells derived from hiPSCs we investigated whether the differentiated cells expressed lymphatic markers at the protein level with proper localization. For endothelial cells, ERG (ETS-related gene) is expressed in the nuclei of endothelial cells [37], whereas in LECs, PROX-1, a transcription factor, is expressed in the nuclei [25, 38, 39]. As expected, immunostaining results showed that ERG and PROX-1 both were exclusively localized in the nuclei of

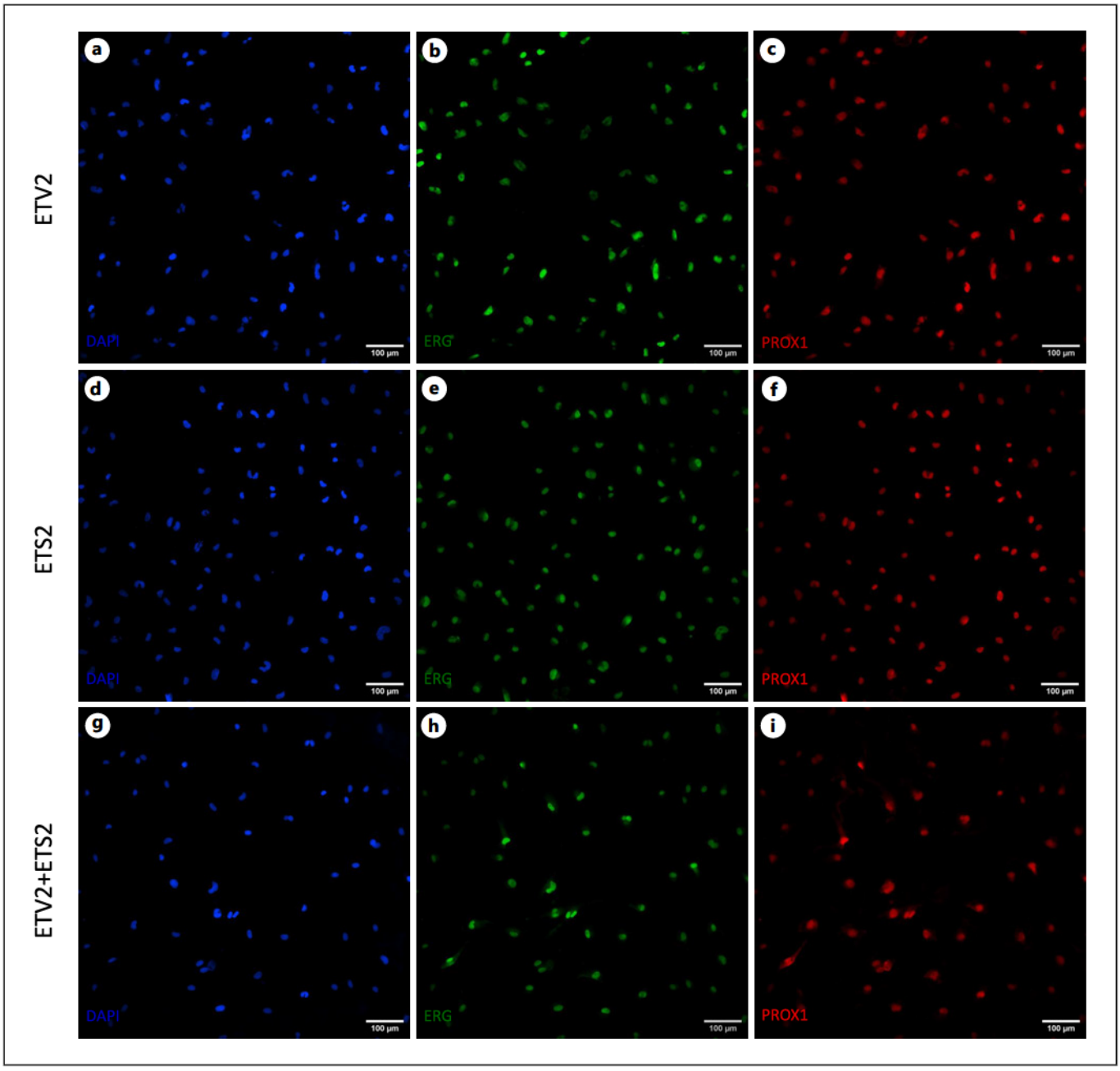

Fig. 3. Immunofluorescent data show presence of key endothelial and lymphatic marker in h-iLECs. **a**–**i** represents *ETV2*, *ETS2*, and *ETV2+ETS2* transduced group respectively. Blue, green, and red are indicator of DAPI, *ETS*-related gene and Prox1 in that order. All three-cohort shows nuclear presence of ERG and PROX1.

the differentiated cells confirming correct localization of the key EC and LEC markers in the differentiated cells (Fig. 3).

Apart from protein level expression, we also looked at the gene expression of the common LEC markers, such as *LYVE-1*, *PDPN*, *Prox1*, and *VEGFR3* (Fig. 4). Compared to growth factor derived LECs, *ETV2* transduced LECs express higher level of lymphatic markers *LYVE-1* (1.92 ± 0.78-fold), *PDPN* (2.84 ± 0.44), *Prox1* (2.06 ± 0.64-fold), and *VEGFR3* (2.20 ± 0.65-fold), *ETV2* and *ETS2* transduced LECs express higher level of lymphatic markers *LYVE-1* (2.14 ± 0.60-fold), *PDPN* (7.75 ± 0.56-fold),

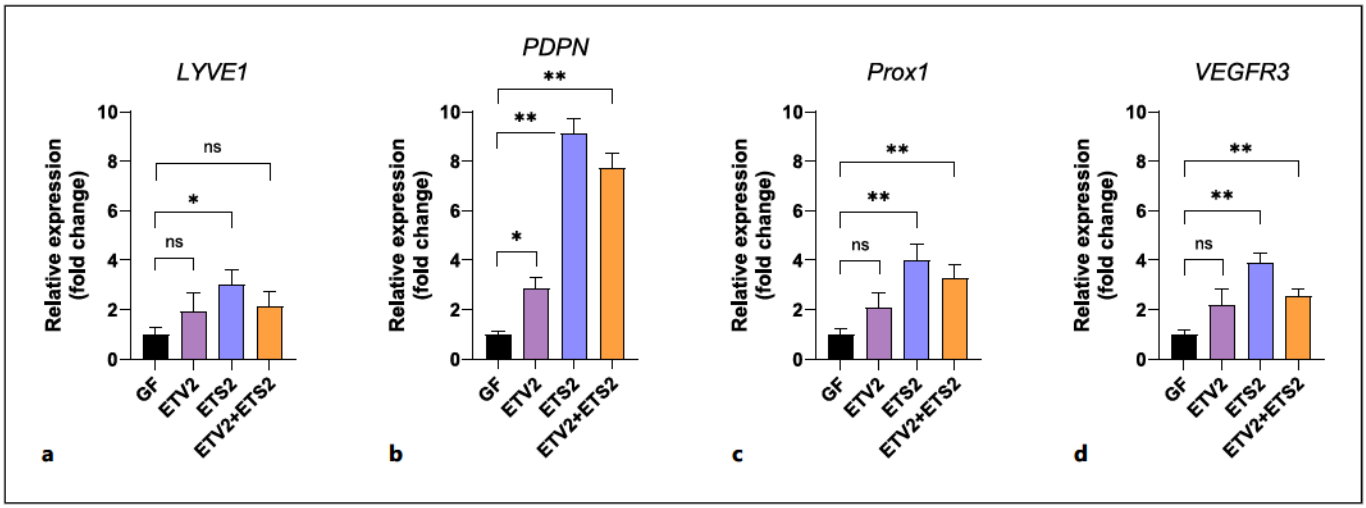

Fig. 4. Genotypic characterization of iLECs. **a**–**d** qRT-PCR data of *LYVE-1, PDPN, Prox1*, and *VEGRR3* of three transduced group, respectively, in each case growth factor derived LECs (endogenous activation of ETV2/ETS2) were used as control. The data show that all three transduced group showed higher expression of the genes of interest compared to control. Especially cohort having *ETS2* showed very significant expression of all the LEC markers. Data represent mean ± standard deviation, $n = 4$ per condition. Significance levels were set at: $^{*}p < 0.5$ and $^{**}p < 0.01$. Three biological replicates ($n = 3$) were collected per condition and analyzed with real-time qRT-PCR.

Prox1 (3.28 ± 0.54-fold), and *VEGFR3* (2.57 ± 0.28-fold), and *ETS2* transduced LECs express the highest level of lymphatic markers *LYVE-1* (3.03 ± 0.78-fold), *PDPN* (9.12 ± 0.61-fold), *Prox1* (4 ± 0.64-fold), and *VEGFR3* (3.90 ± 0.37-fold). Consistent with the differentiation efficiency data, *ETS2* transduced group express the highest lymphatic markers followed by the combination of *ETS2* and *ETV2* group, and lastly the *ETV2* group express relatively higher lymphatic markers compared to the growth factor derived LECs.

Functional Capacity and Phenotypic Maturation

To validate the functionality of the LECs differentiated from hiPSCs under our culture conditions, we performed a series of in vitro studies [40]. First, we tested in vitro activities of iLECs using a tube formation assay (Figure 5a–c, online suppl. Fig. 4). Cells positive for LYVE-1 and Podoplanin were isolated by MACS from differentiating cell population at day 14, labeled with a fluorescent dye, CellTracker™ Green and subjected to tube formation assay. After 12 h of culture, all hiPSC-derived LECs readily formed tube-like structures (Fig. 5a–c). Although all the cells showed some degree of tube formation capability, *ETS2* group formed 33.072 ± 1.268 mm tube length and 109 ± 12.73 branches, followed by *ETS2+ETV2* group that formed 28.271 ± 2.381 mm tube length and 106.5 ± 13.44 branches, and *ETV2* group that formed 24.005 ± 0.235 mm tube length and 66.5 ± 7.78 branches (Fig. 5d, e). A number of branches are not significantly different among the different conditions, but tube length of *ETS2* group was significantly greater than *ETV2* group.

Further, we looked at the secretory properties of our differentiated cells. LECs are known for secreting reelin, one of the major lymphangiocrine factors [26, 41, 42]. In our study, we found that our differentiated LECs secrete reelin comparable to primary LECs in the ranges between 10 and 20 ng/mL (Fig. 5f). We also investigated the level of VEGF-C secreted by iLECs. We confirmed that all the iLECs and primary LECs secreted a very negligible amount of VEGF-C, in the range of pg/mL (Fig. 5g). Overall, the hiPSC-derived LECs showed phenotypic characteristics of mature LECs and faithfully recapitulated functional lymphatic behavior.

Discussion

Promoting the development of new lymphatic vessels has been postulated as an innovative therapeutic strategy for various disease phenotypes [5]. Yet, LECs are difficult to isolate, and they can lose their lymphatic phenotypes during in vitro culture [32]. Therefore, generating LECs from hiPSCs represents an appealing strategy not only for lymphatic regeneration, but also for modeling human diseases in vitro. Thus far, methods to generate LECs from hiPSCs have mainly relied on either EB intermediate

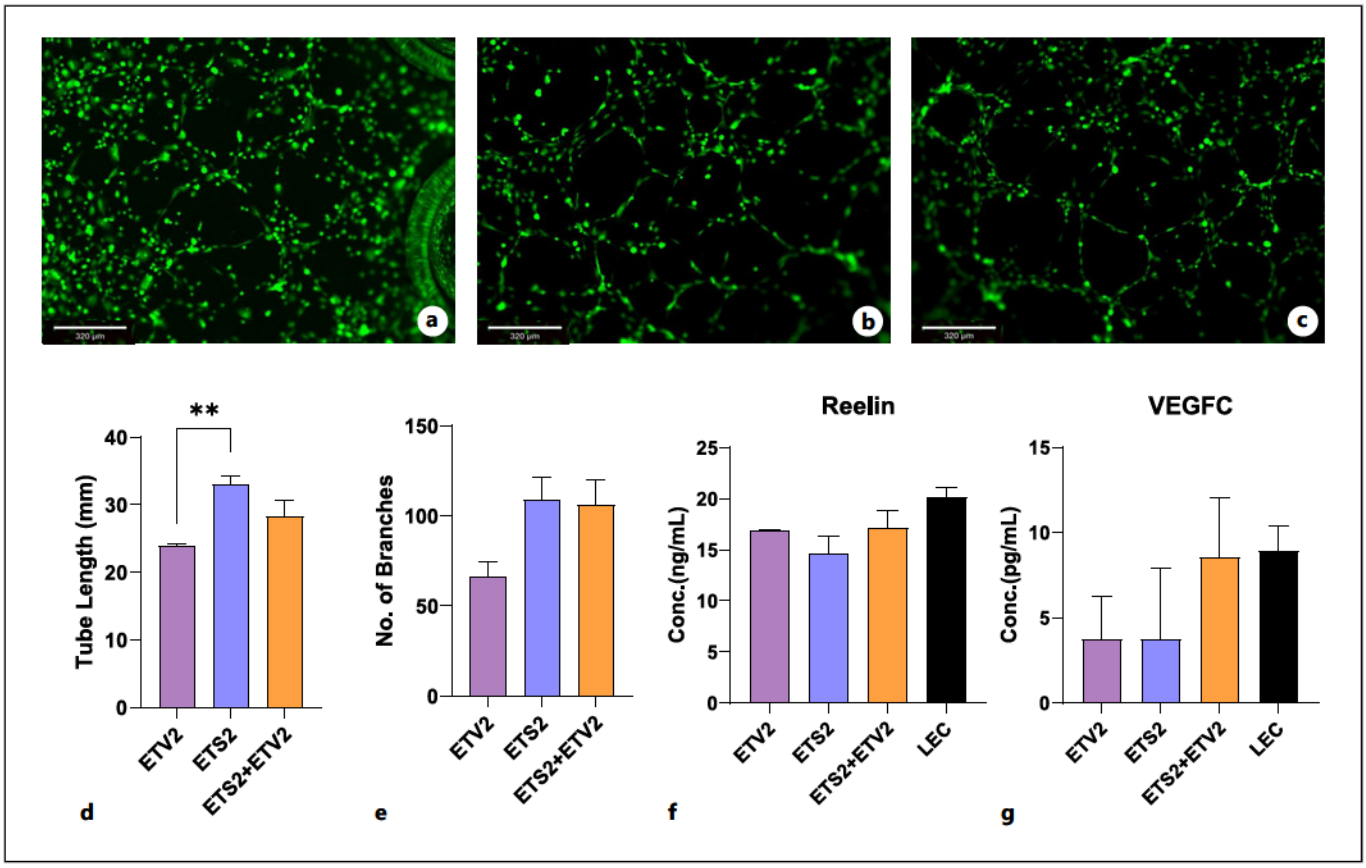

Fig. 5. Functional properties of iLECs. **a**–**c** Capillary-like network formation of *ETV2*, *ETS2*, and *ETV2*+*ETS2* group where Live cells are stained by cell tracker green. **d**, **e** Quantitative analysis of tube formation assay by measuring the tube length and no. of branches respectively. **f**, **g** Quantification of Reelin and VEGFC secreted by the iLECs obtained through ELISA assay. Significance level was set at: $^{**}p < 0.01$.

or the use of murine feeder layers (i.e., OP9) [43, 44]. While generation of EB recapitulate early embryonic development, they require sorting of differentiated cells and therefore produce lower overall yield [15, 43]. On the other hand, co-culture with murine feeder layers introduces xenogeneic components to the final product [4, 5].

Alternatively, direct differentiation can be achieved using lentiviral transduction of *ETV2* and *ETS2*, which are known to be important for blood endothelial cells [45]. In this study, we compared the differentiation efficiency, lymphatic markers, and functionality of differentiated LECs using transduction of *ETV2*, *ETS2*, and combination of *ETV2* and *ETS2*. We discovered that transduction with *ETS2* produced the highest efficiency of Prox1$^+$ cells, which also express high lymphatic markers, such as LYVE-1 and Podoplanin. These results are consistent with previous studies that show *ETS2* interaction with Prox1, the master regulator of lymphatic genes [24, 46]. Transduction with *ETV2* produced lower efficiency of Prox1$^+$, which express lower lymphatic markers. Interestingly, the combination of *ETS2* and *ETV2* produced lower efficiency of Prox1$^+$ cells, which may suggest the opposite effect of *ETS2* and *ETV2*.

LECs are also known to express different lymphatic markers depending on their locations [47]. Lymphatic capillaries highly express LYVE-1, a receptor for hyaluronic acid, which is important for leukocytes trafficking [48]. Lymphatic collecting vessels express Podoplanin, which can bind to platelet receptor CLEC-2 and is important for blood and lymphatic separation [35]. *ETS2* transduced cells highly express Podoplanin, which reflects LECs that reside at the lymphatic collecting vessels [49]. On the other hand, *ETV2* transduced cells highly express LYVE-1, which corresponds to LECs that reside at the lymphatic capillaries. Depending on the final applications, different transcription factors may be more suitable to generate different LECs with varying degree of Podoplanin and LYVE-1 expression.

Regardless of the different lentivirus vectors used, all the transduced cells express key lymphatic markers comparable to primary LECs and are able to form lymphatic networks in vitro. The transduced cells also express reelin in the ranges between 10 and 20 ng/mL, which are comparable to primary LECs. Reelin is a key lymphangiocrine, which has been attributed to cardiac regeneration following heart attack [26]. We also confirmed that the transduced LECs secrete low level of VEGF-C, comparable to primary LECs. While VEGF-C is a key soluble factor to promote lymphangiogenesis [50, 51], it is well known that VEGF-C is not secreted by LECs, rather by macrophages and fibroblasts [51]. All these results suggest that the differentiated LECs exhibit the characteristics and functionalities of LECs.

Overall, the current study demonstrated an efficient method of deriving LECs, which may be useful for modeling human diseases in vitro and basic understanding of human development. For instance, differentiated LECs can be cultured in synthetic hydrogels and microfluidic devices to investigate the molecular mechanism underlying lymphatic disorders in patients with down syndrome and lymphatic malformations [4, 52–55]. Future studies can also generate differentiated LECs that can secrete VEGF-C to provide pseudo-autocrine signaling and further enhance lymphatic regeneration in patients with lymphedema [34, 51]. It is worth noting that while direct differentiation using lentiviral vectors may introduce lentiviral components, future studies can take advantage of modulating *ETV2* and *ETS2* expression with modified mRNA [20]. Furthermore, promising results from the current work warrants future studies to investigate a stepwise and well-defined method to differentiate LECs from hiPSCs, useful for broad applications in basic study of lymphatic biology, as well as toward various approaches in lymphatic regeneration and personalized medicine [5].

Statement of Ethics

This study protocol was reviewed and approved by the University of Notre Dame Institutional Biosafety Committee, Approval No. 21-05-6605. Written informed consent was obtained from donors to participate in the study to generate hiPSC, which are available through WiCell Research Institute.

Conflict of Interest Statement

The authors have no conflicts to disclose.

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Author Contributions

S.S., C.P., and D.H.-P. conceived the ideas, designed the experiments, interpreted the data, and wrote the manuscript. S.S., F.G., and J.K. conducted the experiments and analyzed data. All authors have approved of the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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