

**COMPREHENSIVE ANALYSIS OF HEK293 CELLS REVEALS A LEC-LIKE
PHENOTYPE**

by

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ABSTRACT

The history of cell culture dates back to 1907 when Ross Harrison discovered that neuronal cells could be cultured in vitro. Two types of cells used within cell culture include primary and established cell lines. Among established cells HEK 293 and 293T cells are among the most widely used in the technique of cell culture. Lymphatic endothelial cells (LECs) are often represented as primary cells, which have been isolated from tissues. Previous studies have characterized 293 and 293T cells as neuronal in origin although phenotypic analyses have not been reported. Similarly, studies dealing with the binding characteristics of HIV have revealed the expression of podoplanin, a typical LEC marker, by 293T cells. Additionally, our lab has observed the expression of podoplanin by 293 cells through flow cytometry analysis. These observations of podoplanin expression suggest that the phenotype of 293 and 293T cells needs to be further explored and that they might resemble LECs. This study outlines the comprehensive mRNA and protein analysis of 293 and 293T cells in the context of LECs using standard and real-time RT-PCR and flow cytometry analysis. Furthermore, this study evaluates the influence of culture conditions on LEC marker expression by 293 and 293T cells in an attempt to find optimal LEC growth conditions for these cell lines. Evaluation of mRNA expression levels revealed that 293 and 293T cells express multiple LEC markers including Prox-1, Lyve-1, PDPN, and VEGFR-3. Studies investigating the manipulation of culture conditions revealed that

293 and 293T cells do not significantly change in LEC marker expression. Taken together, these studies suggest that 293 and 293T cells phenotypically resemble LECs and should be defined as LEC-like. Defining 293 and 293T cells as LECs is highly relevant to the field of public health as it provides a new model by which to study LEC function in several different contexts including vaccine interaction and cancer metastasis.

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1.0 INTRODUCTION

1.1 EARLY HISTORY OF CELL CULTURE

Though the human body functions as a system comprised of tissues, the isolation of specific cells from tissues has provided the ability to understand specific cellular functions. Cell culture is one method by which researchers can investigate the properties of cells. The first cell culture experiment was performed in 1907 when Ross Harrison documented nerve growth [1]. Since 1907, cell culture has provided insight into the study of specific cellular function and understanding of the body as a whole. Cells grown in culture can be classified as either “primary” or “established”. The distinct difference between primary and established cells is the modification of established cells to continually divide [2]. Through the years, the study of cell culture has been developed to include methods to manipulate and differentiate cells using supplemented cell culture media, such as growth factors [3]. Similarly, the field of cell culture has continued to develop and more recently scientists have been modifying culture conditions from a two-dimensional to a three-dimensional level. The advantage of three-dimensional growth is that it better replicates the interaction between neighboring cells and between cells and their host environment. Depending on the area of research, different cell types might be preferred. For example, when studying cellular response to vaccine treatment the results would

more likely mimic host response if primary cells were used. However, many cells within the body are not easily maintained and thus, must be modeled using established cell lines.

1.1.1 Primary cells

Primary cells are typically isolated from tissue samples that are taken from an individual or animal and have a limited life span. Tissue samples often contain a variety of cell types and must be sorted after isolation based on cell size, granularity, or specific cellular markers [3]. Once cells are isolated from tissue, they are passaged in culture vessels containing supplemented media to allow further differentiation. Unfortunately, some cell lines are difficult to culture and therefore have a limit to their research potential due to lack of known cellular markers, limit on passage number, or longevity of cellular marker expression.

1.1.2 Established cells

Similar to primary cells, established cells are often isolated from tissue for the purpose of cell culture. Once in culture, the cells are manipulated by transformation of tumor or viral genes that allow for continuous cellular division [2]. A drawback to the use of established cells is the unknown change in phenotype after manipulation. However, this does not limit their use in research, for example; established cell lines can still be used in various areas of research including cystic fibrosis and cancer research [4]. A common established cell line used in numerous areas of research is the human embryonic kidney (HEK) cell line first isolated by Franklin Graham in the 1970s [5].

1.2 HUMAN EMBRYONIC KIDNEY CELLS

1.2.1 The History of HEK293 cells

The history of human embryonic kidney (HEK) cells dates back to the 1970s when the method of transducing cells with DNA was first devised. Among the first cells to be infected with human virus, Adenovirus type 5, were human embryonic kidney cells [5]. Post infection these cells were referred to as HEK293 or 293 cells and have since been used for many types of research within the scientific community. Not even 20 years after the origin of 293 cells came the origin of 293T cells, the result of transducing 293 cells with a segment of simian virus 40 containing the large-T antigen [6]. The addition of the SV40 large T antigen into the 293 genome provided cells with the ability to replicate vectors containing the SV40 origin of replication [7]. 293 and 293T cells have been used in various areas of study including expression of recombinant proteins and vaccine development for Hepatitis E virus [8, 9]. Despite their frequent use, 293 and 293T cells have not been well characterized. A limited number of studies have been performed in an attempt to determine the origin of 293 cells, resulting in the classification of 293 and 293T as neuronal or epithelial in origin [5, 10]. A thorough characterization of this cell line could provide insight into interpreting previously published data and might open up additional avenues for use of 293 cells.

1.2.2 Previous Characterization and Use of the HEK293 Cell Line

Though 293 and 293T cells have not been thoroughly characterized, they have been used in many areas of research. Research using 293 cells has revealed a previously unknown insulin

receptor substrate, IRS-4, which undergoes phosphorylation when in the presence of insulin [11]. Similarly, virology studies have shown novel cytopathic effects in 293 cells infected with Hantavirus, a virus that does not normally produce cytopathic effects in mammalian cells [12]. Previous research has shown the expression of several related neuronal proteins by 293 cells. Shaw *et al.* investigated 293 cells for the expression of neurofilament, a typical neuronal marker, and the impact transformation with human adenovirus had on 293 cells [10]. The results revealed that 293 cells expressed both the major neurofilament protein and neurofilament light chain protein, atypical expression for an isolated kidney cell. They offered two interpretations of their findings: that adenovirus has a rare ability to turn on neurofilament genes not normally expressed, or that 293 cells were neuronal in origin [10]. Furthermore, they suggested that 293 cells do not resemble normal kidney cells and should not be used in kidney related research [10]. This study investigates the phenotype of 293 and 293T cells in an attempt to better understand their use and suggest that they might be lymphatic endothelial cells.

1.3 THE LYMPHATIC SYSTEM

One function of the human lymphatic system is to maintain fluid balance within the body. The major cell type within the lymphatic endothelium is lymphatic endothelial cells (LECs), which make up the subcapsular, cortical, and medullary sinuses of the lymph node [13]. Lymphatic vasculature is found in most vascularized tissue within the body, with the brain being one exception [14]. The lymphatic vascular system was first discovered in the 17th century but remained understudied for many years due to a lack of known cellular markers [15]. However, an early established function of the lymphatic endothelium is to serve as a conduit to traffic DCs

from tissues to the draining lymph nodes during infection [14]. The functions of the lymphatic system have been more recently uncovered due to the discovery of lymphatic endothelial cell markers Prox-1, PDPN, Lyve-1, and VEGFR-3 which when used in conjunction, aid in pure isolation of LECs [15].

1.3.1 Lymphatic Endothelial Cell Function

The role lymphatic endothelial cells play within the immune system continues to be discovered. LECs have a unique physical structure where neighboring cells do not overlap and are attached to the membrane through fine elastic fibers [16]. This physical property is most likely due to the function of the cells to maintain fluid balance between the lymphatic and blood vascular system [16, 17]. Known functions of LECs include production of chemokines, such as CCL21, to attract mature dendritic cells expressing CCR7 to the draining lymph node [18]. More recently, reports have suggested that LECs might play a more significant role in detection and presentation of antigen through the expression of antiviral factors and toll like receptors [19, 20]. Similarly, LECs have been shown to express D6, a receptor that is able to remove chemokines such as CCL21, from the environment. This function helps LECs regulate trafficking of DCs during local inflammation [21].

1.3.2 Lymphatic Endothelial Cell Marker Expression

Lymphatic endothelial cells express multiple markers that allow for a more definitive characterization and differentiation. Five commonly used markers to distinguish LECs include Prox-1, PDPN, VEGFR-3, CCL21 and Lyve-1. The recent discovery of these markers has

allowed for a better understanding of LECs and the lymphatic vasculature as a whole [14]. LEC phenotype, like many other cell lines, is defined by the expression of multiple cellular markers. This is particularly true for LECs because some markers expressed by LECs are also expressed by other cell types [16]. For example, Lyve-1 is expressed in LECs and is often used to separate primary LECs from blood endothelial cells (BECs), but can also be found in liver and spleen sinusoidal tissue [16]. Similarly, VEGFR-3 is frequently used in conjunction with another LEC marker to distinguish LECs, but can be expressed by BECs albeit to lower levels [22]. Though LECs maintain their phenotype when cultured for a minimal number of passages, the method of isolation (use of Lyve-1 or VEGFR-3 and PDPN) might determine the cellular marker expression profile of the cultured cells post isolation [16]. These five markers play distinct roles in LEC phenotype and since each of its discovery have been used to isolate and characterize LECs.

1.3.2.1 Prox-1

Prospero-related homeobox gene-1 (Prox-1) is a nuclear protein that functions as a transcriptional regulator for LEC marker expression [23, 24]. At approximately day nine of developing mouse embryos, a specific subset of blood endothelial cells begins expressing Prox-1. These Prox-1 expressing cells are among the first to emerge from the cardinal vein and begin the lymphatic vasculature and to further differentiate into LECs [25]. Studies have shown Prox-1 knock-out mice are deficient in lymphatic vasculature and that IL-3 might regulate the presence of Prox-1 on LECs [23, 26]. Prox-1 expression also functions as the major factor determining differentiation between BECs and LECs, with expression conferring an LEC phenotype [13].

1.3.2.2 Podoplanin

Podoplanin (PDPN) is a cell surface transmembrane protein that is expressed on a variety of cell types from LECs to kidney cells. PDPN expression is regulated depending on the cellular environment. For example, LECs upregulate PDPN through expression of LEC marker, Prox-1 [24]. However, there must be other mechanisms for regulation of PDPN other than Prox-1 due to the fact that fibroblasts express PDPN, but not Prox-1 [24]. Similar to the unknown regulation is the relatively unknown function of PDPN. It was recently demonstrated that on LECs PDPN functions in the trafficking of DCs to the lymph node and plays a role in wound healing and migration of cells [24]. PDPN has also been shown to bind both CLEC-2, normally expressed on DCs, and CCL21, a chemokine expressed by LECs both of which help direct DCs to the lymph node [24].

1.3.2.3 VEGFR-3

Another cellular marker expressed by LECs is vascular endothelial growth factor three (VEGFR-3). In addition to Prox-1, VEGFR-3 expression on LECs causes them to sprout from the cardinal vein and differentiate into LECs [13]. Furthermore, mice deficient in VEGFR-3 lack many of the lymphatic vessels and often die around day 15 [13]. Previous research has shown that though Prox-1 is needed for LEC differentiation and formation of vasculature, signaling via VEGF-C, the ligand for VEGFR-3, is needed for lymphatic sprouting [13]. The signaling cascade driven by VEGFR-3 is still being uncovered, but to date it is understood that VEGFR-3 induces the PI3-K pathway and eventually the Akt and mitogen-activated protein (MAP) kinase pathway [13]. VEGFR-3 has five tyrosyl phosphorylation sites along the carboxyl terminal tail, one of which specifically binds Shc and GRB2, which activate the RAS signaling pathway [27].

1.3.2.4 Lyve-1

Lymphatic endothelial hyaluronan (HA) receptor, Lyve-1, was first discovered by searching for HA receptors similar to CD44, which is the HA receptor expressed on BECs [28]. Lyve-1 and CD44 share approximately 43% similarity, with the majority of homology comprising the HA binding region [29]. Studies also confirmed that Lyve-1 was expressed by endothelial cells through staining of human umbilical vein endothelial cells (HUVECs) and by co-expression of other LEC markers [30]. Lyve-1 has been shown to bind HA although the functional significance of binding to HA within the lymphatics is still being investigated [28]. Two possible hypotheses exist for LEC expression of Lyve-1. First, Lyve-1 might act as a transporter for HA, which would then be processed by LECs. Alternatively it might play a structural role in which it helps with the passage of cells through the endothelium [28].

1.3.2.5 CCL21

CCL21 is a chemokine that is typically produced by LEC to aid in the movement of DCs to the draining lymph nodes. The CCL21 receptor, CCR7 is expressed by DCs that have come into contact with antigen and been successfully activated [31]. In the presence of inflammation, LECs have been reported to up-regulate CCL21 production [21]. Other reports suggest that DC migration patterns are determined by type of inflammatory stimulation, but overall movement is primarily dependent on LEC production of CCL21 [31]. Along with up-regulation during inflammation, CCL21 production might be determined in part by expression of another cellular marker, CD137 [32]. CD137 is known for its activation properties among T cells and NK cells and is a member of the TNF- α superfamily, which in turn, can signal through the NF- κ B pathway [32]. Reports have shown that LECs co-express Lyve-1 or PDPN with CD137 post

inflammation and that expression of CD137 on LECs causes an increase in CCL21 production [32].

1.4 DETERMINANTS OF CELL CHARACTERIZATION

Within basic research, cell culture remains one of the most widely used methods to model *in vivo* responses with one of the main challenges being reproducibility between cellular responses *in vitro* and cellular responses *in vivo*. Choosing an appropriate cell line to model the human system is one way in which investigators can maximize reproducibility. Ideally, the chosen primary cell or cell line would be fully characterized and well explored. However, many cell lines have not been fully characterized and this limitation could affect our understanding of the results when unsuitable cells are used. For instance, HeLa cells were recently observed to differ from normal human cells on multiple levels after DNA and RNA deep sequencing [33]. In fact, it is reported that misidentified cell lines are becoming increasingly public and that actions should be pursued to eliminate misleading results [34].

According to the FDA for the production of biological products for licensure, cell lines should be characterized based on several criteria including, but not limited to, the history of the cell line, including species of isolation and culture methods, characteristics of the cell line including expression of specific markers, and how much the cell line resembles other cultures of the same cell line [35]. Though slightly different in application, these standards should also be taken into consideration in the field of basic research. Defining the expression of specific markers is of particular importance when evaluating cells or cell lines that do not have a strongly

defined function, making evaluation of mRNA and protein expression a significant part of cell line characterization.

1.5 SUMMARY

Cell culture plays an important role within the field of research and development from being involved in initial experiments on vaccine potency to discovery of specific cellular function within the human body. Primary cells and established cell lines are the two cell states used in cell culture differing in their potential to continuously divide. HEK293, also known as 293 cells, are an example of an established line that is able to undergo continuous division due to transformation by human adenovirus 5 DNA [5]. In contrast, primary cells, which do not have the potential for continuous division, are isolated from host tissues. LECs are an example of primary cell populations that are typically isolated from tissues using markers such as Lyve-1, PDPN, Prox-1, and VEGFR-3. LECs differ from blood endothelial cells (BECs) through expression of other specific markers and through their function, trafficking DCs and lymphocytes to the lymph node and maintaining fluid homeostasis between the blood and lymphatic systems [16]. Investigations into the phenotype of 293 cells have been minimal and previous results have revealed the expression of neurofilament proteins, leading others to propose they are neuronal [10]. Despite this highly limited phenotypic characterization of 293 cells, they remain one of the most widely used cell types for cell culture.

2.0 STATEMENT OF THE PROBLEM

Until the discovery of lymphatic endothelial cell markers, isolation and study of LECs was difficult [14]. LECs still remain a somewhat understudied cell population due to the recent identification of specific LEC markers: Prox-1, VEGFR-3, Lyve-1, and PDPN. Similarly, 293 and 293T cells have not been thoroughly studied on their surface markers and do not have a defined phenotype. However, recent studies by Chaipan *et al.* using 293T cells to investigate HIV binding strategies revealed that an HIV attachment factor, CLEC-2, could bind and incorporate into virus particles the cellular receptor PDPN, a LEC marker expressed by 293T cells [36]. The expression of PDPN by 293T cells, though not the primary focus of their study, provided an initial suggestion that HEK293 and 293T cells might have some connection to cells of the lymphatic lineage. Similarly, our lab has observed the expression of PDPN on 293 cells intended for use as a negative control for flow cytometric staining of LECs (Berendam, unpublished data). The revelation that 293 cells express an LEC marker, PDPN, raised the question as to whether they might express multiple LEC markers, and to what extent they resemble LECs. If 293 cells do express LEC markers and arguably can be re-characterized as LEC-like, they might be used as model LECs. **The objective of these studies was to investigate the extent to which 293 and 293T cells resemble LECs and to explore their LEC-likeness through alternative culture conditions.**

2.1 SPECIFIC AIM 1: DETERMINE THE PHENOTYPIC RESEMBLANCE BETWEEN HEK293, HEK293T CELLS AND LECs

LECs play an integral role in the lymphatic endothelium and are characterized by their expression of cellular markers including: PDPN, Prox-1, Lyve-1, VEGFR-3, and CCL21. Each of the cellular markers provides structural and functional properties and their presence and levels of expression are one determinant of LEC-likeness. Cellular marker expression by 293 and 293T has not been thoroughly studied and is necessary to determine the phenotype of these cells. The observed expression of PDPN by 293 and 293T cells reveals a possible new phenotype based on LEC marker expression. 293 and 293T cells must exhibit expression of multiple LEC markers to be considered LEC-like. Furthermore, characterization of 293 and 293T cells, as LEC-like is dependent on the level of expression of LEC markers relative to a known control. Standard RT-PCR was performed to determine the expression of some LEC markers by 293 and 293T cells. Real-time RT-PCR was used to quantify expression of a large set of additional markers, both LEC markers and LEC-associated markers, relative to a known endogenous control, beta-glucuronidase (GUSB). To determine if the protein expression of 293T cells was similar to LECs flow cytometry staining was performed for PDPN and Lyve-1. Real-time RT-PCR expression levels revealed that 293 and 293T cells express multiple LEC markers to similar levels as human dermal LECs (HDLECs). Similarly, flow cytometry staining revealed that 293T cells exhibit protein expression concordant with observed mRNA levels.

2.2 SPECIFIC AIM II: DETERMINE THE LEC-LIKE POTENTIAL OF HEK293 AND 293T CELLS USING CULTURE MANIPULATION

Cells grown in cell culture can be influenced by supplemented factors within the growth medium [3, 37]. Primary LECs are often cultured in the presence of VEGF-C, one of the two ligands of VEGFR-3 [16, 38]. Since the presence of VEGFR-3 has been associated with LEC attachment and sprouting from the cardinal vein, VEGF-C has been proposed as being essential for the growth of LECs in cell culture conditions [38]. Similarly, manipulation of culture conditions by transforming cells or growing in three-dimensional environments can induce changes in cellular marker expression and differentiation [39, 40]. As previously discussed, another factor that determines the LEC phenotype is the transcriptional regulator, Prox-1 and forced expression of this protein might result in up-regulation of LEC marker mRNA expression levels [23, 24]. Three-dimensional culture of cells has become a recent model that better reflects the in vivo environment [41]. It represents the in vivo environment by providing more realistic cell-cell contact and cell-environment contact [40]. One factor influencing the study of 293 and 293T cells grown in three-dimensional culture is that previous studies have reported that 293 cells have the potential to de-differentiate when grown in low attachment environments [40]. This de-differentiation might provide an ability to re-differentiate 293 cells further into LECs, which eventually could serve as an LEC cell line. Additionally, since 293 and 293T cells are LEC-like, they might up-regulate LEC markers when grown in three-dimensional culture, similar to human umbilical vein endothelial cells (HUVECs) previously grown in three-dimensional culture using fibrin matrices [42].

To further investigate 293 and 293T LEC-like potential and resemblance, cells were grown in a variety of manipulated culture conditions including: VEGF-C treatment, transfection

with a plasmid expressing Prox-1, and seeding cells into three-dimensional culture conditions. Results revealed that 293 and 293T cells do not up-regulate cellular marker expression similar to LECs, as previously shown, in response to VEGF-C [37]. Transfection studies reveal that over expression of Prox-1 in 293 cells might significantly impact Prox-1 and Lyve-1 mRNA expression but in 293T cells does not significantly change LEC marker mRNA expression. Results from three-dimensional culture studies also revealed little to no change in differentiation and up-regulation of LEC markers by 293 and 293T cells using either a hanging drop method or low attachment method [39, 40].

3.0 MATERIALS AND METHODS

3.1 CELL CULTURE CONDITIONS

HEK293 (ATCC CRL-1573) and HEK293T (ATCC CRL-11268, Michele Calos, Stanford Registry no. S97-079) cells were maintained in DMEM (Fisher, cat no. BW12-614F) supplemented with 5% fetal bovine serum (Hyclone), 1X penicillin/streptomycin (Invitrogen, 15140163) at a final concentration of 2000U/500mL media, and 1X L-glutamine (Life Technologies) with a final concentration of 2mM. Table 1 shows culture conditions for each of the cell lines used. Cells were split every 2-3 days or when 70% confluency was reached by treating with 0.025% for 293T cells or 0.05% for more adherent cells, 293, Tzm-bl, and Caco-2, trypsin (Fisher). Cells were then resuspended in media containing FBS to stop trypsin action and aliquoted into 15 mL conical tubes. Tubes were pelleted for 5 minutes at 1200 rpm and then resuspended in fresh media and seeded into T25 or T75 culture flasks. Established cell lines were grown to a maximum of approximately 30 passages.

Table 1. Cell Lines and Culture Media.

Cell Line	Culture Medium	Received From
Human dermal LECs	EGM-2MV	In house stock
HEK293	DMEM (5%FBS, 1X L-glut, 1X Pen/Strep)	In house stock
HEK293T	DMEM (5%FBS, 1X L-glut, 1X Pen/Strep)	In house stock
Caco-2	DMEM (10%FBS, 1X L-glut, 1X Pen/Strep)	Dr. Tanya Wang
A549	RPMI 1640 (10% FBS, 1X L-glut, 1X Pen/Strep)	Dr. Tianyi Wang
786-O	RPMI 1640 (10% FBS, 1X L-glut, 1X Pen/Strep)	Dr. Tianyi Wang
Tzm-bl	DMEM (5%FBS, 1X L-glut, 1X Pen/Strep)	In house stock
Thp-1	RPMI 1640 (10% FBS, 1X L-glut, 1X Pen/Strep)	In house stock
CEM	RPMI 1640 (10% FBS, 1X L-glut, 1X Pen/Strep)	In house stock
CEM 174	RPMI 1640 (10% FBS, 1X L-glut, 1X Pen/Strep)	In house stock
Jurkat	RPMI 1640 (10% FBS, 1X L-glut, 1X Pen/Strep)	In house stock
U937	DMEM (5%FBS, 1X L-glut, 1X Pen/Strep)	In house stock

3.2 ISOLATION OF RNA

To dissociate cells, 900 μ L of Trizol reagent (Invitrogen) was added to cell monolayer or pellet and incubated for five minutes at room temperature. Cell lysates were then treated with chloroform, isopropanol, and ethanol for purification. Cell pellets were also treated to remove contaminating genomic DNA with DNase I (Ambion) and then eluted using RNeasy mini kit

(Qiagen). RLT and RPE buffers were prepared for use by adding 10 μ L of beta-mercaptoethanol to every 1mL RLT buffer used and adding 44mL 96%-100% ETOH to buffer RPE (RNeasy mini kit protocol). RNA was washed by mixing 250 μ L 96%-100% ETOH and 350 μ L RLT buffer and eluted using columns included in RNeasy mini kit. Columns were washed two times with buffer RPE and RNA was eluted in a final volume of 20-30 μ L nuclease free water. Final RNA concentration was measured using a NanoPhotometer (Implen, version 2.1).

3.3 cDNA SYNTHESIS FOR STANDARD RT-PCR

cDNA for standard RT-PCR was prepared by mixing 2 μ g of total RNA with 2 μ L Oligo dT (from Promega RT kit) and adjusted to 5 μ L with the addition of NFW. Samples were placed into Peltier Thermal Cycler model PTC-200 from MJ Research at 70°C for 5 minutes and then immediately placed on ice for 5 minutes. RT mixture was set up according to Table 2 for one sample. A volume of 5 μ L of prepared RNA was added to 15 μ L of RT mixture. Reactions were placed in the Peltier Thermal Cycler from MJ Research and run according to the following conditions:

25°C for 5 minutes
43°C for 1 hour
70°C for 15 minutes

Table 2. cDNA Synthesis Reaction Setup for Standard RT-PCR.

10X RT Buffer (Promega RT kit)	2 μ L
25mM MgCl ₂	4.4 μ L
10mM dNTPs	1 μ L
NFW	6.1 μ L
Rnase Inhibitor	0.5 μ L
Reverse Transcriptase (replace with NFW for NRT)	1 μ L

3.4 STANDARD RT-PCR

Standard RT-PCR was performed using previously designed primers or newly designed primers as listed in Table 3. Primers were designed by aligning human sequences obtained from the GenBank sequence repository for the target marker using the Vector NTI program. Table 4 outlines the volume and mass of reagents combined for one reaction. To prepare cDNA, 2.0 μ g of RNA was added to each reaction mixture. To perform standard RT-PCR, 2.5 μ L of cDNA was added to each reaction tube. 3-5 μ L of PCR product was run using 0.5X TBE buffer on a 1% agarose gel against a 1Kb plus ladder (Invitrogen). PCR conditions were as follows using a Peltier Thermal Cycler model PTC-200 from MJ Research.

94°C for 3 minutes
94° C for 30 seconds
68°C for 30 seconds
72°C for 2 minutes
72°C for 10 minutes
4°C for forever

} 35 cycles

Table 3. Primer Sequences for Standard RT-PCR.

Gene of Interest	Designed By	Primer Sequences
PDPN	Amarendra Pegu	F: 5' CAG CTC AGA ATC TTG CTG CTC GGC 3' R: 5' GAC TGA GAC ACG GGA CAG GGA CTC GAG 3'
VEGFR-3	Amarendra Pegu	F: 5' GAC AAG GTG TAC ACC ACG CAG AGT GA 3' R: 5' AAG CCG CTT TCT TGT CTA TGC CTG CT 3'
Prox-1	Amarendra Pegu	F: 5' AGT GCG GCG ATC TTC AAG ATA TGT 3' R: 5' CAT GAA GCA GCT CTT GTA GGC AGT T 3'
Lyve-1	Amarendra Pegu	F: 5' CTT GCA GCT ATG GCT GGG TTG GAG A 3' R: 5' ACG GTA GTT TTG CTT GGA CTC TTG G 3'
CD31	Nicole Phillips	F: 5' TGT GCT GCA ATG TGC TGT GA 3' R: 5' TCA AGG GAG CCT TCC GTT CT 3'

Table 4. Standard RT-PCR Master Mix Setup.

5X GoTaq Gene Buffer (Fisher Scientific)	5 μ L
2mM dNTPs	2.5 μ L
25 μ M Forward and Reverse primers	1 μ L
GoTaq DNA Polymerase	0.2 μ L
Nuclease Free Water	12.8 μ L

3.5 cDNA SYNTHESIS FOR REAL-TIME RT-PCR

cDNA was prepared for real-time RT-PCR using 5 μ L of 80ng/ μ L purified RNA, for a total of 400ng RNA per reaction tube. No reverse transcription (NRT) tubes served as a negative control by lacking RT SSII (Invitrogen). Both RT and NRT reaction mixtures are included in

Table 5. Amplification was performed in Peltier Thermal Cycler model PTC-200 from MJ Research using the following conditions:

25°C for 10 minutes
 48°C for 30 minutes
 95°C for 5 minutes
 4°C for forever

Table 5. cDNA Master Mix Setup for Real-Time RT-PCR.

Reagent	RT	NRT
Nuclease Free Water	56.875 μ L	56.875 μ L
10X PCR Buffer (ABI)	10 μ L	10 μ L
25mM MgCl ₂ (ABI)	22 μ L	22 μ L
dNTPs (25mM each)	2 μ L	2 μ L
Random Hexamers (100 μ M)	2.5 μ L	2.5 μ L
RNAsin (40 units/ μ L)	1 μ L	1 μ L
RT SSII (200 units/ μ L)	0.625 μ L	0.625 μ L (NFW)

3.6 REAL-TIME RT-PCR

Real-time RT-PCR was performed using commercially available Taqman primer and probe sets (Table 6) or by SYBR green detection assay (WT1 and Synaptopodin) (Table 7). Previously designed primers for macaque sequences were aligned with human WT1 and Synaptopodin from NCBI to determine if primer regions were in consensus [43]. Primers were modified due to single nucleotide differences between human and macaque sequences and purchased through IDT website for SYBR Green detection (Life Technologies) (Table 7).

Table 6. Real-time Taqman Assays Purchased from Life Technologies.

Gene of Interest	Catalogue Number
PDPN	Hs00366766
Prox-1	Hs00160463
VEGFR-3 (FLt-4)	Hs00176607
Lyve-1	Hs00272659
CCL21	Hs00171076
CCL20	Hs00171125
CD31 (PECAM-1)	Hs01065279
CD206	Hs00267207
CD209	Hs01588349
CD40	Rh02621776
CD26	Hs00175210
IL-17	Rh02621732
IL-17R α	Rh02841231
IL-3	Rh02621715
IL-3R α	Hs00608141
NEFL-L	Hs01034882
Apol L2	Hs01853832
Nestin	Hs04187831
Nanog	Hs04260366
Rpl13a	Hs03043884
*GUSB	Hs99999908

Table 7. Primer Sequences for SYBR Green Assays.

Gene of Interest	Primer Sequence
Wilm's Tumor-1	F: 5' CTT CAG AGG CAT TCA GGA TGT G 3' R: 5' TCT CAG ATG CCG ACC GTA CA 3'
Synaptopodin	F: 5' GAC GTT GGG CCG GAG CAC TAG 3' R: 5' CTG GAC GCC ACG GGA AT 3'

3.7 FLOW CYTOMETRY STAINING

Cells were removed from culture dish using 0.025% trypsin for five minutes at RT and trypsin action was stopped using media containing FBS and spun down at 1200rpm for 5 minutes at RT. Cells were counted using a haemocytometer and resuspended to a total number of cells based on cell density. DNase I (Roche) was added to the total number of cells (200,000 cells per sample) to obtain a final concentration of 0.1mg/mL and were incubated for 30 minutes at 37°C, 5% CO₂. Cells were washed with 1XPBS, resuspended, and stained using Live-Dead Aqua Stain (Life Technologies) for 30 minutes at RT covered from light using aluminum foil. Cells were resuspended in 20µL of azide-free blocking buffer (Innovex) and stained using anti-human podoplanin-PE (Angibio, cat no. 11-009PE), anti-human lyve-1-APC (R&D Systems, cat no. FAB20892A), Rat IgG2a-PE (BD Biosciences cat no. 555844) as a matched isotype control to anti-human podoplanin-PE, or Mouse IgG1-APC (BD Biosciences cat no. 555751) as a matched isotype control to anti-human lyve-1-APC. Cells were stained for one hour at 4°C then washed with 1X PBS and resuspended in blocking buffer/paraformaldehyde for a final concentration of 0.25% PFA and acquired using BD Fortessa (access kindly granted by Dr. Rinaldo and the MACS) using the PE yellow/green channel for detection of cells stained positively for anti-human podoplanin and APC channel for detection of cells stained positively for anti-human lyve-1.

3.8 VEGF-C TREATMENT OF 293 AND 293T CELLS

293 and 293T cells were seeded at a density of 8,000 cells/cm² in a T25 flask with complete DMEM and incubated overnight at 37°C, 5% CO₂ to allow cell attachment. At 24 hours post-plating, medium was supplemented with 0.2µg/mL VEGF-C (Acro-biosystems) at 37°C, 5% CO₂, for 48 hours. Cells were then washed twice with 1X PBS (without calcium and magnesium) and prepared for RNA extraction (above) using 900µL Trizol reagent. RNA was purified including DNase I treatment (Ambion) and RNeasy column purification (Qiagen) and analyzed using real-time RT-PCR (Taqman primer/probe set for Apolipoprotein L2).

3.9 3-D HANGING DROP MODEL FOR CELL CULTURE

Spheroids were formed using the hanging drop model by plating 500 cells in either 25µL or 50µL onto non-adherent square petri dishes (Greiner Bio-One) in a 20% methylcellulose (Sigma-Aldrich cat no. M0512) supplemented DMEM medium, as previously described [39]. Methylcellulose media was made by measuring 0.6g methylcellulose and placing into a bottle able to be autoclaved with a sterile stir bar and autoclaving. Warm 50mL complete media to 60 °C and after autoclaving methylcellulose, add 25mL warmed media under sterile conditions to bottle and place on stir plate (medium speed) for 20 minutes at RT. Add 25mL warmed media to jar under sterile conditions and placed on stir plate overnight at 4 °C. Aliquot 25mL media into two 50mL conical tubes and spin in centrifuge for 3 hours, 3500g, at 4 °C [39]. Cells were incubated upside down overnight at 37°C and 5% CO₂. Spheroids grown for seven days were formed and maintained in culture with sterile water in the bottom of the plate to maintain

hydration of methylcellulose drops. Spheroids were washed from square petri dishes using 1X PBS without calcium and magnesium to harvest spheroids. Spheroids were allowed to settle for 30 minutes by gravity sedimentation in a 50mL conical tube and then all liquid was removed above the spheroids. For extraction of RNA, 900 μ L of Trizol was then added to the spheroids and pipette up and down and allowed to sit for 5 minutes then stored at -80°C until time of extraction. To form a spheroid plug, 50 μ L of 4% paraformaldehyde (in 1X PBS) was added to the spheroids and incubated on ice for 15 minutes and then at RT for 15 minutes. Paraformaldehyde/PBS was carefully removed and replaced with 50 μ L of a 0.33% agarose/PBS solution stored at 37°C and placed on ice for 15 minutes to solidify. After the plug had solidified it was placed in 10% sucrose/PBS followed by 20% sucrose/PBS each for 4 hours and then frozen using methylbutane stored on dry ice for 45 minutes to reach a temperature of -65°C. Frozen plugs were stored at -80°C and allowed to thaw at -20°C for 2 hours prior to cryosectioning. Spheroid plugs were cut into 10 μ m cryosections and placed on glass slides (SuperFrost Plus, Fisher). Glass slides containing sections were immediately placed on a 60°C warm plate for 20 minutes before being stored at -80°C. Slides were allowed to sit at room temperature and then placed in paraformaldehyde for 5 minutes then stained for hematoxylin and eosin by being dipped in hematoxylin for 2 minutes followed by a single dip in eosin. Slides were washed twice with Millipore water and then washed with 70%, 80%, and 95% ETOH for 5 minutes each followed by xylenes for 5 minutes before being cover slipped using Permount (Fisher Scientific).

3.10 3-D LOW ATTACHMENT METHOD

Cells were cultured in low-attachment conditions as previously described [40]. Low-attachment dishes were prepared by dissolving 13.4g of DMEM high glucose powder (GIBCO) in 3.7g of sodium bicarbonate solution (Life Technologies) then filtered in sterile conditions using a 0.22 micron filter. Prior to use, media was pre-warmed at 37°C. Agarose solution was made by mixing 1.0g standard agarose (Invitrogen) into 100mL deionized water and boiling in microwave between 30 seconds and 1 minute to dissolve. Lastly, equal parts of agarose solution and warmed DMEM were used to coat cell culture dish to a thickness of approximately 4mm. For this protocol, G-10 Agarose (Biowest) was replaced with standard agarose. 293T or 293 cells were seeded at a density of 3×10^6 onto 60mm petri dishes and incubated at 5% CO₂, 37°C for six or seven days, respectively. Spheroids were collected and spun down at 1200rpm for 5 minutes at room temperature then gently re-suspended in PBS and centrifuged again to remove any excess agarose within the media. Spheroids were analyzed for change in Nanog, Nestin, or LEC marker mRNA expression using real-time RT-PCR (Table 6).

3.11 OVEREXPRESSION OF PROX-1 VIA TRANSFECTION

Transfections were completed by plating 500,000 or 1 million cells in 12-well or 6-well, respectively, on to tissue culture treated plates. Plates were coated with a 1:5 dilution of poly-L-lysine to ensure attachment of cells to culture dish. Poly-L-lysine solution was made by reconstituting 3.0mg of poly-L lysine in 30mL sterile water under sterile conditions. The solution was then filtered using a 0.22 micron filter and working stock was created by diluting 1:5 in

nuclease free water. Plates were coated with 1:5 dilution by adding enough poly-L lysine that it covers the well. The solution was allowed to sit for 10 minutes with swirling of the plate every few minutes to ensure coverage. The mixture was then pulled off and washed with 1X PBS and set to dry for 2 hours before plating cells. Cells were seeded at a density of 500,000 cells in 1 mL or 1×10^6 cells in 2mL onto 12-well or 6-well plates, respectively, and allowed to incubate at 37°C overnight or until cells reach 70-80% confluency before beginning the transfection. Complete medium was changed to a volume of 0.5mL 30-60 minutes prior to addition of the Polyjet (Signa Gen)/DNA reagent mixture. For a 12-well plate, DNA was diluted to 0.403µg and suspended in 20.16µL of serum-free medium for each well. Similarly, 1.21µL Polyjet reagent was mixed into 20.16µL for each well to form a DNA to Polyjet ratio of 1:3. Diluted Polyjet reagent was added to diluted DNA and incubated for 15 minutes at room temperature to allow the DNA and Polyjet to form a complex. DNA/Polyjet mixture was then added dropwise to each well and incubated for 12 hours. Transfection efficiency was checked by visualizing GFP transfected wells using a FITC cube at approximately at 48 hours post-transfection for harvesting of cells. RNA was isolated from lysates as described above and analyzed for changes in LEC marker expression. Plasmids used included empty vector pcDNA3.1, pcDNA3.1rhesus-Prox-1, and pcDNA3.1human-Prox-1, the latter expressing the rhesus macaque and human Prox-1 cDNAs, respectively (generated by Stella Berendam).

3.12 STATISTICAL ANALYSIS

Statistical analysis for repeated studies (VEGF-C treatment, Prox-1 transfections, and three-dimensional culture of spheroids) was performed using GraphPad Prism version 6. A

student's paired t-test was performed to determine significance of data at a 0.05 level. Spearman correlation analysis was performed using the $2^{-\Delta Ct}$ values for the multiple genes and cell lines and performed using GraphPad Prism version 6.

4.0 RESULTS

To date, there is no cell line that can appropriately model LECs. Given that 293 cells have been previously shown to express PDPN, a LEC marker, analysis of their phenotype might reveal their potential to be defined as LEC-like and provide a model cell line for LECs. To explore further LEC marker expression by 293 and 293T cells standard and real-time RT-PCR were performed. Flow cytometry staining was used to validate protein expression by 293T cells and several attempts were made to deepen the LEC-like phenotype of 293 and 293T cells through manipulation of culture conditions using treatment with VEGF-C, transfection with a plasmid containing Prox-1, and placement into three dimensional culture. The results revealed a strong LEC-like phenotype for 293 and 293T cells and suggested that these cells should be re-defined as LEC-like.

4.1 STANDARD RT-PCR REVEALS HEK293 AND 293T CELLS EXPRESS MULTIPLE LEC MARKERS

To confirm expression of PDPN and to evaluate expression of other LEC markers by 293 and 293T cells, cDNA was prepared using RNA from cell lysates. Human dermal lymphatic endothelial cells (HDLEC) served as a positive control population with no reverse transcriptase (NRT) tubes serving as negative controls. Primers were previously designed or newly designed

using published human sequences for each specific marker, based on sequences in GenBank, choosing an area of approximately 50% G-C content and avoiding areas that will fold back and self-align. Primers were evaluated and purchased using the IDT website.

Standard RT-PCR using PDPN-specific primers revealed that 293 and 293T cells express PDPN mRNA as do HDLEC, with NRT controls being clean (Figure 1). These results are concordant with previously observed data within our lab and from previous study using 293 and 293T cells (Berendam, unpublished data) [36]. To validate further the uniqueness of expression of PDPN by 293 and 293T cells using standard RT-PCR, three additional cell lines were obtained. The three human cell lines included were: Caco-2, intestinal epithelial cells; A549, airway epithelial cells; and 786-O, kidney epithelial cells. Standard RT-PCR results showed that PDPN expression was not unique to 293 and 293T cells when compared to three other epithelial lines, but expression appears to be higher among 293 and 293T cells than other epithelial cell lines (Figure 2). To investigate mRNA expression of other LEC markers, standard RT-PCR was also performed using specific primers for VEGFR-3, Lyve-1, and Prox-1, on six cell types (Figures 3-5). CD31, also known as PECAM-1, a typical endothelial cell marker, was also investigated for mRNA expression among the six cell lines, using primers designed for standard RT-PCR (Figure 6). Lastly, to evaluate the presence of an endogenous expression of beta-glucuronidase (GUSB), standard RT-PCR was performed on the six cell lines previously analyzed (Figure 7). Six additional cell lines were included in standard RT-PCR analysis for GUSB that will be included in real-time RT-PCR analysis (Figure 12).

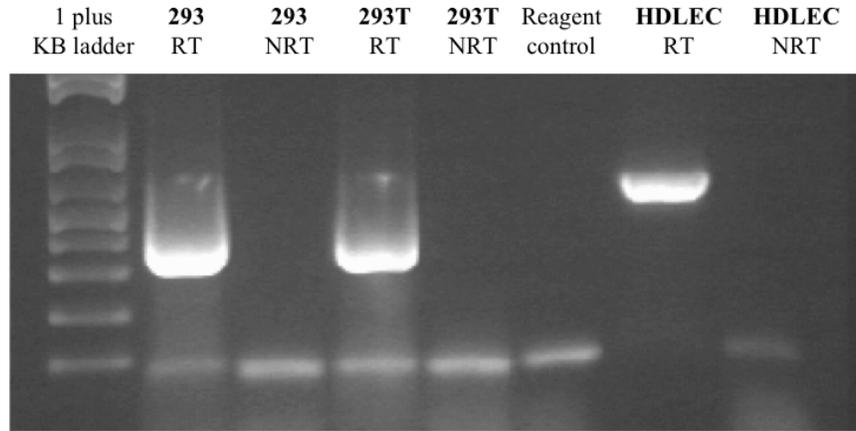


Figure 1. PDPN mRNA expression by 293 and 293T cells Revealed Through Standard RT-PCR.

Results confirm PDPN mRNA expression by 293 and 293T cells using human dermal lymphatic endothelial cells (HDLEC) as positive control. RT indicates samples where reverse transcriptase was added and NRT refer to control samples in which reverse transcriptase was not added. Shifted bands might be due to a splice variation within the PDPN mRNA sequence. Expected band size = 560bp

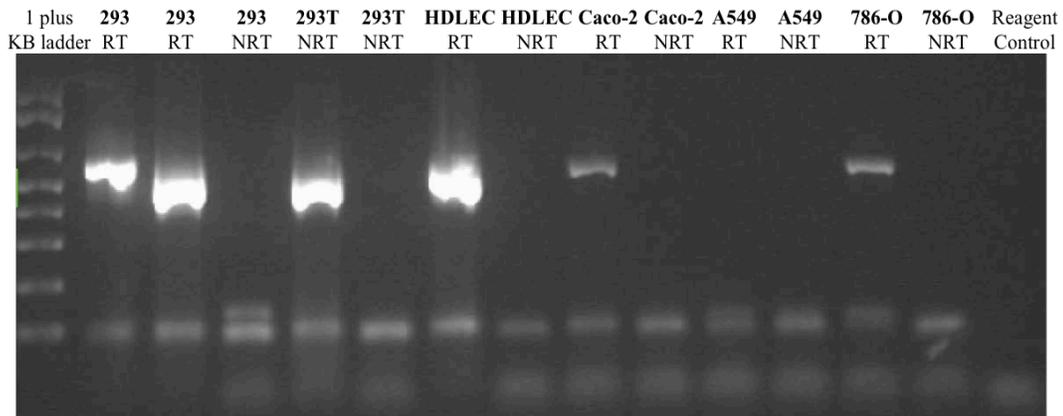


Figure 2. PDPN mRNA Expression by Six Cell Lines Revealed Through Standard RT-PCR.

Results confirm PDPN mRNA expression by 293 and 293T cells using human dermal lymphatic endothelial cells (HDLEC) as positive control. RT indicates samples where reverse transcriptase was added and NRT refer to control samples in which reverse transcriptase was not added. Caco-2, A549, and 786-O are epithelial cells included to determine uniqueness of PDPN mRNA expression. Shifted bands might be due to a splice variation within the PDPN mRNA sequence. Expected band size = 560bp

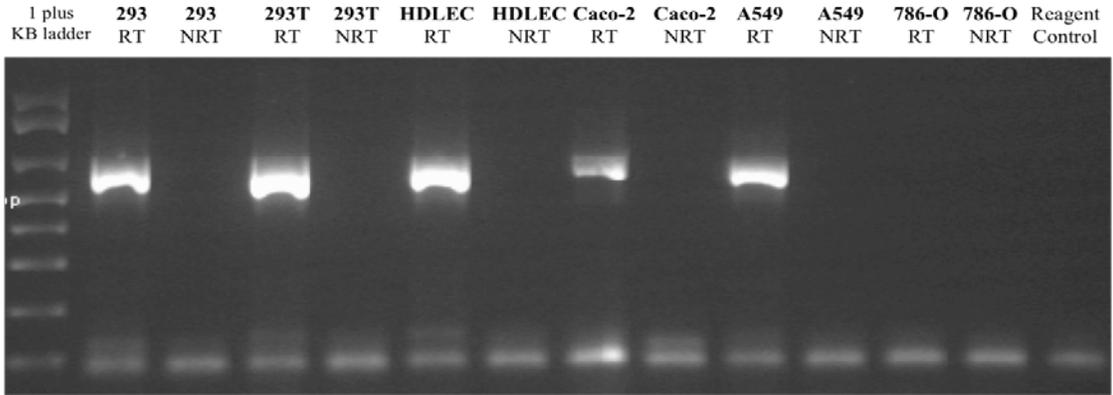


Figure 3. VEGFR-3 mRNA Expression by Six Cell Lines Revealed Through Standard RT-PCR.
 Results confirm VEGFR-3 mRNA expression by 293 and 293T cells using human dermal lymphatic endothelial cells (HDLEC) as positive control. RT indicates samples where reverse transcriptase was added and NRT refer to control samples in which reverse transcriptase was not added. Caco-2, A549, and 786-O are epithelial cells included to determine uniqueness of VEGFR-3 mRNA expression. Expected band size = 550bp

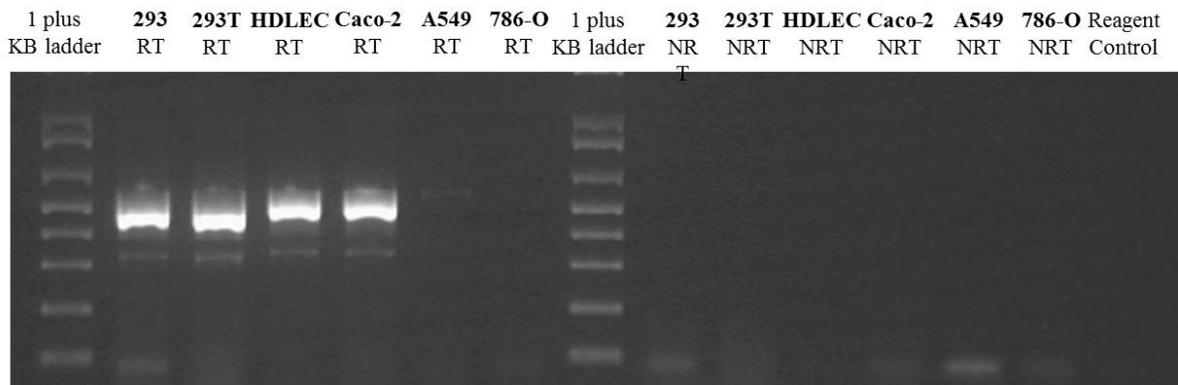


Figure 4. Prox-1 mRNA Expression by Six Cell Lines Revealed Through Standard RT-PCR.
 Results confirm Prox-1 mRNA expression by 293 and 293T cells using human dermal lymphatic endothelial cells (HDLEC) as positive control. RT indicates samples where reverse transcriptase was added and NRT refer to control samples in which reverse transcriptase was not added. Caco-2, A549, and 786-O are epithelial cells included to determine uniqueness of Prox-1 mRNA expression. Expected band size = 550bp

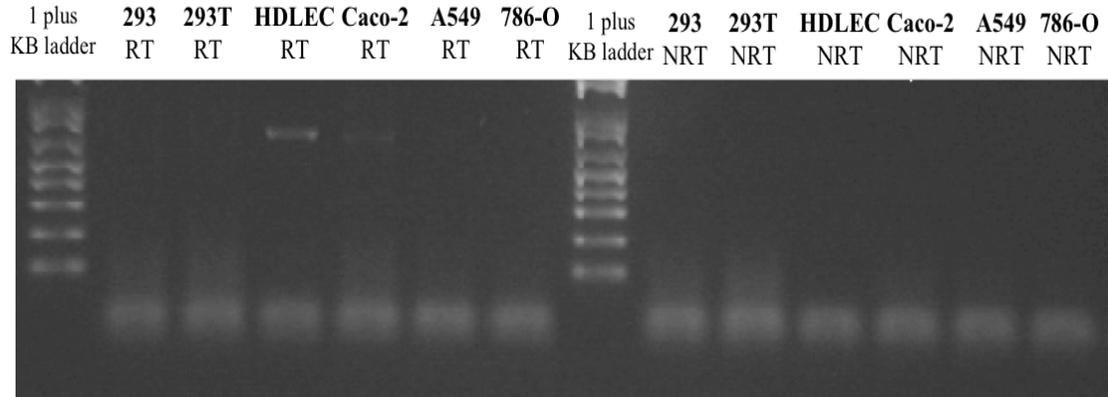


Figure 5. Lyve-1 mRNA Expression by Six Cell Lines Revealed Through Standard RT-PCR.

Results confirm Lyve-1 mRNA expression by 293 and 293T cells using human dermal lymphatic endothelial cells (HDLEC) as positive control. RT indicates samples where reverse transcriptase was added and NRT refer to control samples in which reverse transcriptase was not added. Caco-2, A549, and 786-O are epithelial cells included to determine uniqueness of Lyve-1 mRNA expression. Expected band size = 530bp

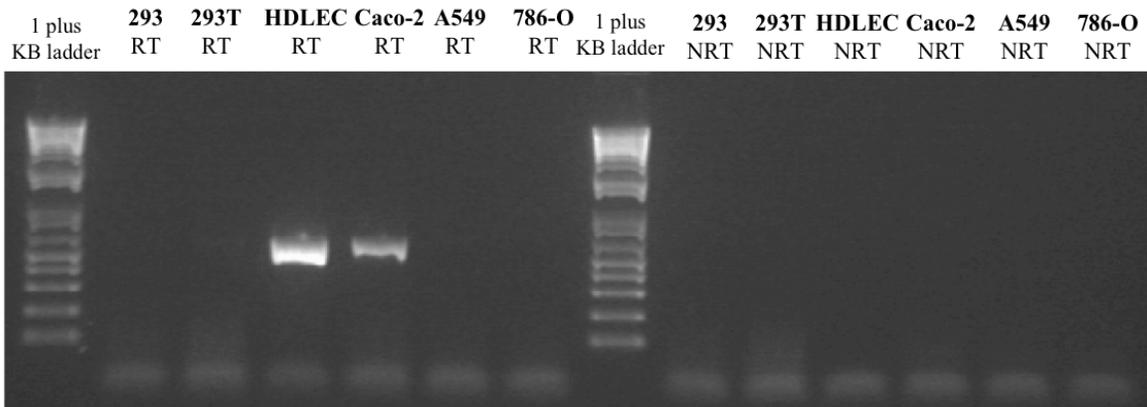


Figure 6. CD31 mRNA Expression by Six Cell Lines Revealed Through Standard RT-PCR.

Results confirm CD31 mRNA expression by 293 and 293T cells using human dermal lymphatic endothelial cells (HDLEC) as positive control. RT indicates samples where reverse transcriptase was added and NRT refer to control samples in which reverse transcriptase was not added. Caco-2, A549, and 786-O are epithelial cells included to determine uniqueness of CD31 mRNA expression. Expected band size = 650 bp

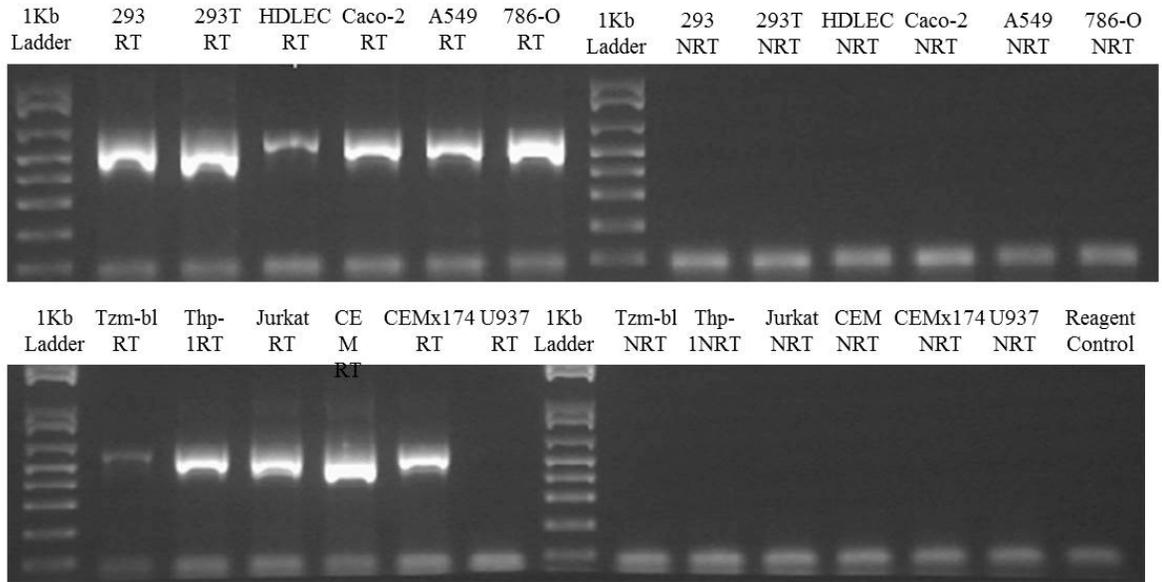


Figure 7. GUSB mRNA Expression by Six Cell Lines Revealed Through Standard RT-PCR.

Results confirm GUSB mRNA expression by 293 and 293T cells using human dermal lymphatic endothelial cells (HDLEC) as positive control. RT indicates samples where reverse transcriptase was added and NRT refer to control samples in which reverse transcriptase was not added. Caco-2, A549, and 786-O are epithelial cells included to determine uniqueness of GUSB mRNA expression. Expected band size = 576 bp

Overall, standard RT-PCR confirmed previous findings that 293 and 293T cells express PDPN, a known LEC marker (Berendam, unpublished data) [36]. Standard RT-PCR also revealed that 293 and 293T cells express two other LEC markers, VEGFR-3 and Prox-1. However, this expression was not observed using primers designed for Lyve-1 or CD31, a common endothelial cell marker. Lastly, standard RT-PCR showed that mRNA expression of LEC markers is not unique to 293 or 293T cells because the included epithelial cell lines, Caco-2, A549, and 786-O, also expressed several of the LEC markers. Though standard RT-PCR also revealed relatively equal levels of GUSB expression, quantitative measurement of mRNA expression levels would more accurately show the comparative level of expression and allow for a more thorough interpretation of overall LEC marker expression.

4.2 REAL-TIME RT-PCR REVEALED THE COMPARATIVE LEVELS OF LEC MARKER EXPRESSION BETWEEN 293, 293T CELLS, HDLECS, AND OTHER CELL LINES

Standard RT-PCR confirmed LEC marker mRNA expression by 293 and 293T cells but further analysis using real-time RT-PCR was performed to measure relative expression levels. Secondly, to better understand the mRNA expression levels in 293 and 293T cells, six additional cell lines were added to the study including epithelial cells, lymphocytes, and pre-monocytic cells. The cell lines, tissue origin, host disease state, and cell type as described by American Type Culture Collection (ATCC) are listed in Table 8. In conjunction with incorporation of multiple cell types, it was necessary to evaluate the appropriateness of different endogenous controls, beta-glucuronidase (GUSB) and Rpl13a, for use in comparing the different cell types. Figure 8 shows the amplification plots and mean, median, range, and standard deviation values calculated using Ct values from the cell populations for GUSB and Rpl13a. These analyses revealed that GUSB had a smaller range and standard deviation than Rpl13a, with a difference of 0.2 in standard deviation and 0.88 in range (Figure 8b). This difference indicates that GUSB is a more suitable endogenous control for use across multiple cell lines within this study.

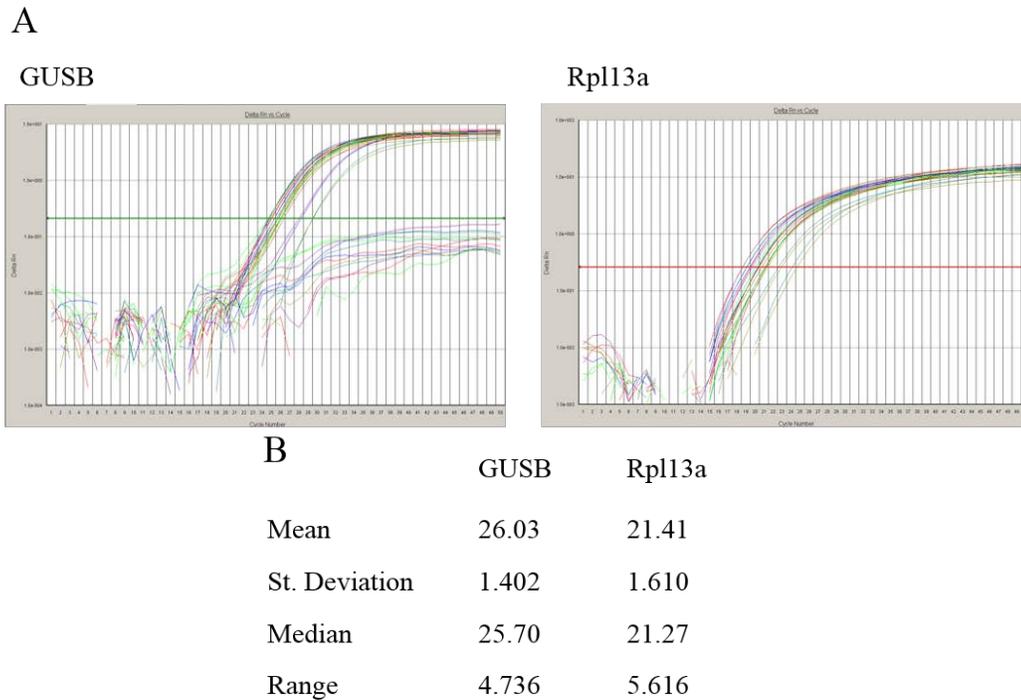


Figure 8. Statistical Analysis of Endogenous Controls, GUSB and Rpl13a.

Statistical analysis from mRNA expression of endogenous controls using Ct values of 12 different cell lines. A) Amplification plots for both GUSB and Rpl13a visibly show a difference in the range of Ct values between GUSB and Rpl13a. B) Statistical analysis revealed that GUSB had a smaller standard deviation and range than Rpl13a, making it a more appropriate endogenous control.

Real-time RT-PCR was performed on RNAs from 293 and 293T cells for LEC marker expression using purchased Taqman primers and probes as listed in Table 6. Along with investigating LEC marker mRNA expression, many other cellular markers were included, including CD26, Wilm's tumor 1 (WT1), and Synaptopodin (Synpo). Podocytes, which are a kidney cell type, also express the LEC marker podoplanin, and so to determine if 293 and 293T cells resemble kidney podocytes, WT1 and Synpo were included in this initial analysis. Primers for WT1 and Synpo were adapted for human sequences from previously published macaque primers and the sequences are listed in Table 7 [43]. CD26, also known as dipeptidylpeptidase IV (DPPIV), is an enzymatic marker that can cleave certain oligopeptides, including chemokines, and is also expressed by LECs [44]. CD26 was also analyzed for expression by 293

and 293T cells to compare the expression levels of LEC-associated markers between 293, 293T, and HDLECs. Two additional LEC-related markers, CCL20 and CD209 were also included to compare expression by 293, 293T, and HDLECs. CCL20 is a chemokine similar to CCL21, which is expressed in mucosal surfaces and aids in the trafficking of CCR6 positive cells, often DCs, T cells, and B cells, to the lymphatic endothelium [18]. On the other hand, CD209, also known as DC-SIGN, is a marker typically expressed by antigen presenting cells (APCs), including DCs and macrophages, that aids in cell adhesion and trafficking [45]. Figures 9 and 10 show mRNA expression levels for LEC markers, podocyte markers, CD26, CCL20, CD31, and CD209 by 293 and 293T cells respectively. Using this approach, I found that 293 and 293T cells express four of the five LEC markers, three of which were expressed to levels nearly equivalent to GUSB. Similarly, both 293 and 293T cells expressed WT1, Synpo, CD31 and CD26. As a control population of known LECs, HDLECs were also analyzed for mRNA expression of the described genes and results are represented in Figure 12. Comparing Figures 9, 10, and 11 reveals that 293 and 293T cells express Prox-1 and VEGFR-3 to similar levels as HDLEC and that all three cell populations express podocyte markers WT1 and Synpo.

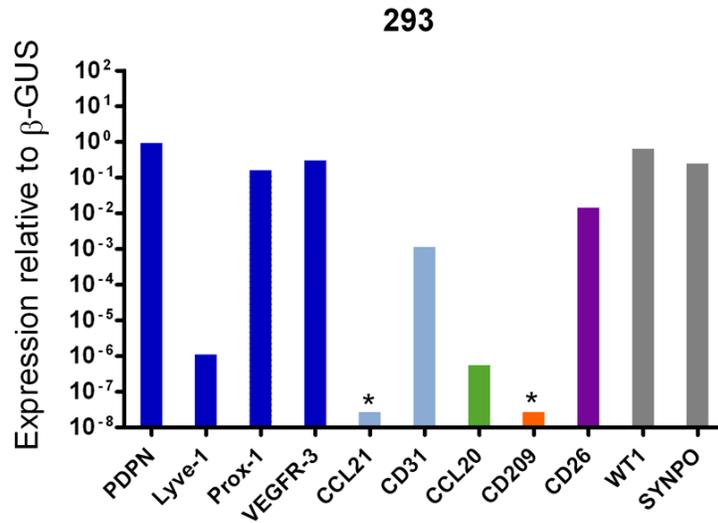


Figure 9. Real-time RT-PCR results for 293 cells.

Results reveal 293 cells express LEC markers: PDPN, Lyve-1, Prox-1, CCL21, and VEGFR-3 as well as CD26, CD31, CCL20, CD209, and podocyte markers, WT1 and Synaptopodin. Asterisks designate that CT values were not obtained within 50 cycles. Real-time RT-PCR was run using purchased Taqman primer/probes or SYBR green assay detection for WT1 and Synpo. Bars represent $2^{-\Delta Ct}$ values calculated using mean Ct values from duplicate wells.

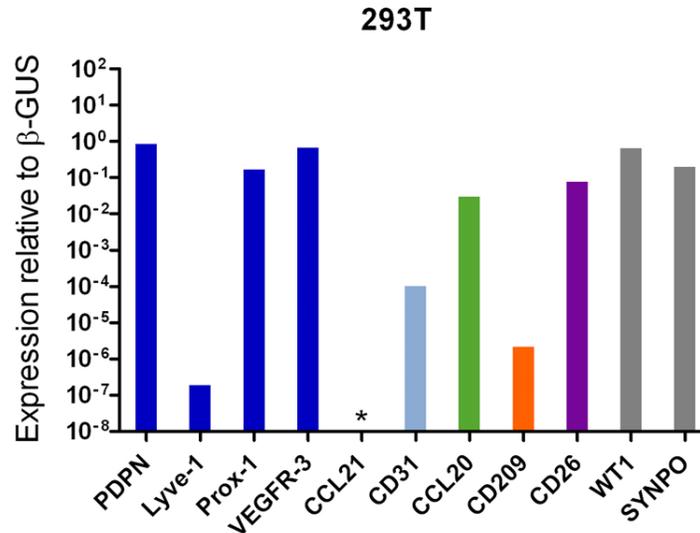


Figure 10. Real-time RT-PCR results for 293T cells.

Results reveal 293T cells express LEC markers: PDPN, Lyve-1, Prox-1, CCL21, and VEGFR-3 as well as CD26, CD31, CCL20, CD209, and podocyte markers, WT1 and Synaptopodin. Asterisks designate that CT values were not obtained within 50 cycles. Real-time RT-PCR was run using purchased Taqman primer/probes or SYBR green assay detection for WT1 and Synpo. Bars represent $2^{-\Delta Ct}$ values calculated using mean Ct values from duplicate wells.

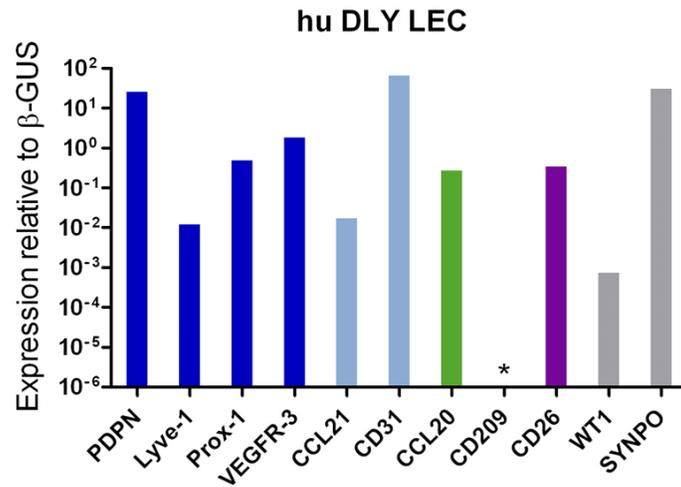


Figure 11. Real-time RT-PCR results for HDLECs.

Results reveal HDLECs express LEC markers: PDPN, Lyve-1, Prox-1, CCL21, and VEGFR-3 as well as CD26, CD31, CCL20, CD209, and podocyte markers, WT1 and Synaptopodin. Asterisks designate that CT values were not obtained within 50 cycles. Real-time RT-PCR was run using purchased Taqman primer/probes or SYBR green assay detection for WT1 and Synpo. Bars represent $2^{-\Delta Ct}$ values calculated using mean Ct values from duplicate wells.

Table 8. Tissue, Cell Type, and Disease State of Incorporated Cell Lines.

Cell Line	Culture Medium	Received From
Human dermal LECs	EGM-2MV	In house stock
HEK293	DMEM (5%FBS, 1X L-glut, 1X Pen/Strep)	In house stock
HEK293T	DMEM (5%FBS, 1X L-glut, 1X Pen/Strep)	In house stock
Caco-2	DMEM (10%FBS, 1X L-glut, 1X Pen/Strep)	Dr. Tanya Wang
A549	RPMI 1640 (10% FBS, 1X L-glut, 1X Pen/Strep)	Dr. Tianyi Wang
786-O	RPMI 1640 (10% FBS, 1X L-glut, 1X Pen/Strep)	Dr. Tianyi Wang
Tzm-bl	DMEM (5%FBS, 1X L-glut, 1X Pen/Strep)	In house stock
Thp-1	RPMI 1640 (10% FBS, 1X L-glut, 1X Pen/Strep)	In house stock
CEM	RPMI 1640 (10% FBS, 1X L-glut, 1X Pen/Strep)	In house stock
CEMx174	RPMI 1640 (10% FBS, 1X L-glut, 1X Pen/Strep)	In house stock
Jurkat	RPMI 1640 (10% FBS, 1X L-glut, 1X Pen/Strep)	In house stock
U937	DMEM (5%FBS, 1X L-glut, 1X Pen/Strep)	In house stock

Table 9. Cell Markers, Typical Expression, and Function.
[2, 10, 13, 18, 23, 24, 26, 44-48]

Gene of Interest	Typical Cellular Expression	Function
PDPN	LECs, podocytes, thymic epithelial cells, fibroblast reticular cells	Binds to CLEC-2 and is involved in platelet aggregation of lymphatic vessels
Prox-1	Liver, pancreas, lymphatic systems	Acts as a transcriptional regulator for LEC markers
VEGFR-3 (FLt-4)	LECs,	Receptor for VEGF-C/VEGF-D and might be essential for LEC sprouting to occur
Lyve-1	LECs, Liver sinusoidal cells,	Hyaluronan receptor
CCL21	LECs,	Aids in trafficking of DCs to the lymph via expression of CCR7 receptor
CCL20	LECs,	Aids in trafficking cells expressing the receptor CCR6
CD31 (PECAM-1)	ECs, lymphocyte, DCs	Platelet function
CD206	Macrophages, LECs	Leukocyte trafficking and may contribute to metastatic development of cancer within lymphatics
CD209	APCs	Adhesion molecule
CD40	ECs,	Produces adhesion molecules, cytokines, and chemokines
CD26	Th 17 cells	Cleaves oligopeptides, including chemokines
IL-7	ECs	Recruitment of macrophages and monocytes
IL-7R α	Monocytes and macrophages	Necessary for some T cell development
SPIN1-2	ECs,	Transport of sphingosine-1 phosphate
IL-3	LECs,	Shown to induce expression of Prox-1 and PDPN in LECs and BECs
IL-3R α	ECs	Receptor for IL-3, might respond to IL-3 through autocrine signaling
NEFL-L	Neuronal cells, 293 cells	Neuron specific intermediate filament
Rpl13a	Common among all cell types	Endogenous control expression
GUSB	Common among all cell types	Endogenous control expression

To investigate more fully the mRNA expression profiles of 293 and 293T cells, a total of 12 cell populations were included in a larger analysis. Table 8 lists the tissue, disease state, and cell type for each of the cell lines included in the mRNA analysis and Table 9 lists the genes investigated and example functions. Real-time RT-PCR was performed and analyzed relative to the previously validated endogenous control, GUSB. Figure 12 is a block diagram representing the mRNA expression level for each gene of interest, relative to GUSB expression, for each cell population. Results were concordant with standard RT-PCR results that revealed 293 and 293T cells are not the only cell lines to express multiple LEC markers. However, 293 and 293T cells relative level of expression of LEC markers is more similar to HDLEC relative expression levels of LEC markers than any other cell type.

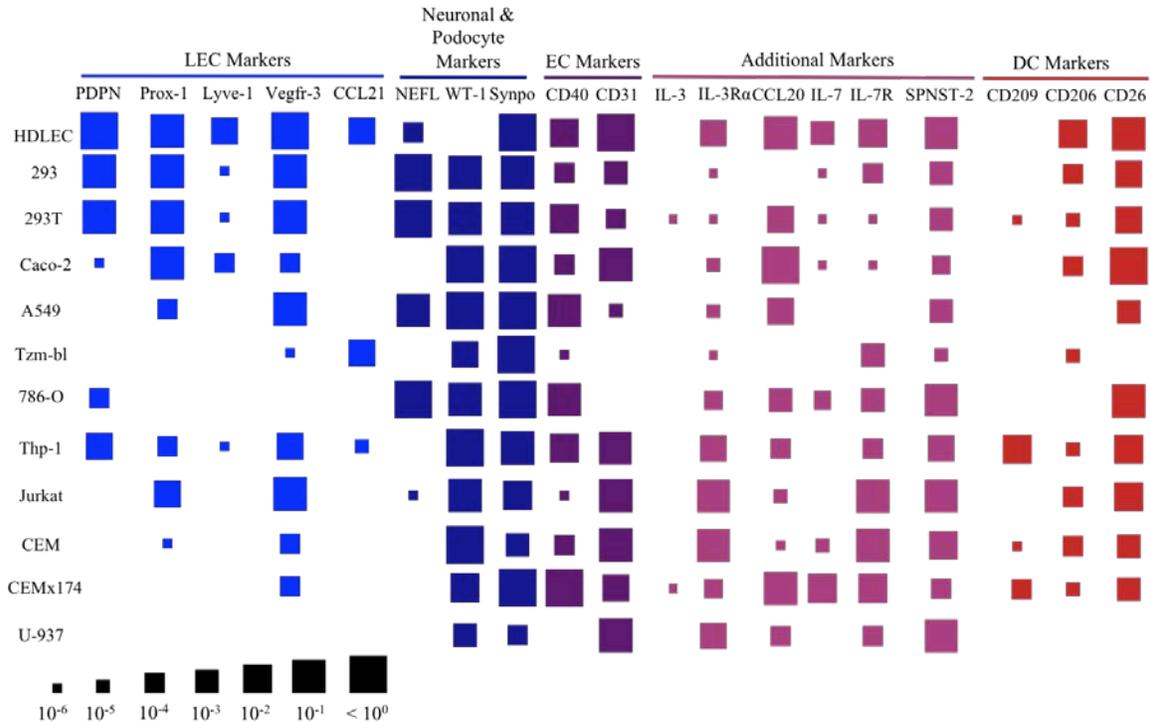


Figure 12. Block Diagram Representing mRNA Expression for a Panel of Genes.

Cell lines were analyzed using Taqman primer/probes or SYBR Green Assay (WT1 and Synpo). HDLEC act as positive control LEC population. Table 8 lists the tissue, disease state, and cell type of each cell line included.

Reactions were prepared in 96-well plates including duplicate RT wells and single NRT wells. Each block represents the relative expression of each gene to endogenous control, GUSB. Absence of a block designates that CT values were not obtained within 50 cycles.

The genes included in Figure 12 can be grouped into several categories including: LEC markers (PDPN, Prox-1, VEGFR-3, Lyve-1, and CCL21), additional markers (CCL20, IL-7, IL-7R, IL-3, IL-3Ra, SPNS-2), endothelial cell markers (CD40, CD26, and CD31) DC-associated markers (CD209 and CD206), and podocyte and neuronal markers (NEFL, WT1, and Synpo). Results indicate that all but one cell line expressed at least one LEC marker, indicating that expression of one LEC marker is not uncommon. Similarly, all of the included cell lines expressed either WT1 or Synpo, revealing that expression of these markers does not strictly indicate a cell line is podocytic and that 293 and 293T cells are not likely to be podocytes despite expression of these markers. Furthermore, 293 and 293T cells express endothelial markers

CD40 and CD31, although slightly lower than HDLEC, which again suggests their possible LEC-like lineage. When comparing 293 and 293T cells to one another, real-time analysis reveals a slight difference in mRNA expression between the two cell lines including expression for CCL20, CD40, and CD209. A reasonable suggestion for this difference in expression might be due to the presence of SV40 T antigen within 293T cells and not 293 cells. Additionally, the lack of expression of CCL21, a known chemokine expressed by LECs, might propose a lack of LEC function by 293 and 293T cells, despite the presence of many LEC markers. However, results also suggest that 293 and 293T cells express markers that might have functional properties including CD26, an enzymatic marker that can cleave chemokines and spinster 2 (SPNST-2), a transporter of lipid mediator, sphingosine-1 phosphate, which acts to transport lymphocytes during inflammation [44, 49, 50]. Further functional analysis of 293 and 293T cells for example, involving chemotaxis assays, would further reveal their functional potential.

Taken together, these results show the unique levels of mRNA expression by 293 and 293T cells and their resemblance to HDLEC when comparing LEC markers VEGFR-3, PDPN, and Prox-1, suggesting that phenotypically 293 and 293T cells strongly resemble LECs. This argument is strengthened by the addition of nine other cell lines of differing origin, which do not show unique levels of mRNA expression comparable to HDLECs. Secondly, real-time RT-PCR results reveal that almost all cell types investigated expressed at least one LEC marker, leading to the conclusion that expression of one or two markers, even to higher levels, is not a strong indicator of LEC origin. Even more interesting was the common expression of WT1 and Synpo among all cell types, suggesting that they are typical markers expressed by cells in culture.

4.3 SPEARMAN CORRELATION ANALYSIS REVEALS STRONG RELATIONSHIPS BETWEEN LEC MARKER EXPRESSION LEVELS

The correlation between the expression levels of two markers can help shed light upon the relation between those markers and the importance of correlated marker expression. A non-parametric Spearman analysis was performed using the $2^{-\Delta Ct}$ values to investigate the relationships among the expression levels of multiple markers. These analyses revealed that among the markers analyzed the strongest correlations were among LEC markers, VEGFR-3 and Prox-1, and PDPN and Lyve-1, with R-values of 0.776 and 0.797, respectively (Table 10, Figures 13 and 14). As R-values approach one, they have a more linear relationship, indicating a strong positive correlation. The opposite is also true as, R-values approach negative one it indicates there is a strong negative correlation between expression levels of the two markers analyzed. Furthermore, p-values were analyzed and the relationship between VEGFR-3 and Prox-1 revealed a significant p-value of 0.004 and the relationship between PDPN and Lyve-1 revealed a significant p-value of 0.003 (Table 10). Figures 13 and 14 represent the relationship between LEC markers and revealed that 293 and 293T cells are among the top three cell lines, along with the HDLECs expressing high levels of LEC markers. The significant positive relationship between PDPN and Lyve-1 also reveals that cell types can be placed into two different groups: a group of cells expressing PDPN and Lyve-1 to low levels and a group of cells expressing high levels of PDPN and Lyve-1. The HDLEC, are among the cells expressing high levels of both Lyve-1 and PDPN, suggesting that LEC phenotype is associated with higher levels of both PDPN and Lyve-1 expression.

Table 10. Spearman Correlation Analysis R-Values and Associated P-values.

	PDPN	Prox-1	Lyve-1	Vegfr3	CCL21	CD26	CD209	WT1	SYNPO	CD31	CCL20	IL-7	IL-7R	SPNST-2	NEFL	CD206	CD40	IL-3	IL-3Ra
PDPN	1.000	0.622 0.0346	0.797 0.0029	0.371 0.238	0.196 0.543	0.552 0.666	-0.182 0.573	-0.189 0.546	0.287 0.366	0.098 0.766	0.210 0.513	-0.105 0.749	-0.252 0.430	0.329 0.297	0.545 0.070	0.175 0.588	0.294 0.354	-0.014 0.973	-0.329 0.297
Prox-1		1.000	0.692 0.015	0.776 0.004	0.042 0.902	0.615 0.027	-0.168 0.603	0.035 0.916	-0.042 0.903	0.448 0.147	0.266 0.404	-0.210 0.513	-0.154 0.653	0.189 0.357	0.217 0.499	0.727 0.009	0.000 1.00	-0.371 0.256	-0.042 0.903
Lyve-1			1.000	0.301 0.342	0.448 0.147	0.392 0.209	-0.147 0.209	0.224 0.630	0.252 0.576	0.273 0.483	0.273 0.410	-0.147 0.391	-0.343 0.630	0.105 0.276	0.175 0.749	0.301 0.588	0.084 0.342	-0.189 0.800	-0.112 0.357
Vegfr3				1.000	-0.105 0.749	0.336 0.286	-0.119 0.715	-0.042 0.890	-0.070 0.834	0.469 0.127	0.091 0.782	-0.224 0.485	0.098 0.766	0.189 0.557	0.322 0.308	0.636 0.299	0.112 0.732	-0.224 0.485	0.056 0.869
CCL21					1.000	-0.035 0.920	0.056 0.869	-0.333 0.282	0.294 0.354	0.168 0.603	0.126 0.699	-0.098 0.766	0.119 0.715	0.133 0.683	-0.252 0.430	-0.154 0.635	0.042 0.903	-0.231 0.470	0.392 0.209
CD26						1.000	-0.224 0.483	0.214 0.502	0.392 0.209	0.196 0.543	0.538 0.074	0.119 0.715	-0.014 0.973	0.378 0.227	0.301 0.542	0.364 0.246	0.378 0.227	-0.322 0.508	-0.231 0.470
CD209							1.000	0.203 0.523	-0.231 0.470	0.238 0.457	0.392 0.683	0.133 0.619	-0.161 0.619	-0.161 0.184	-0.413 0.443	-0.245 0.404	0.266 0.588	0.175 0.588	0.371 0.236
WT1								1.00	-0.046 0.881	-0.147 0.638	-0.028 0.925	-0.224 0.473	-0.396 0.576	-0.175 0.466	-0.228 0.508	-0.207 0.906	0.004 0.966	-0.347 0.262	-0.130 0.678
SYNPO									1.000	-0.329 0.650	0.490 0.800	0.392 0.098	0.035 0.093	-0.084 0.200	0.287 0.079	-0.105 0.366	0.790 0.749	0.042 0.903	-0.308 0.331
CD31										1.000	0.147 0.850	0.084 0.098	0.503 0.093	0.510 0.200	-0.399 0.079	0.531 0.782	-0.091 0.255	-0.357 0.085	0.762 0.005
CCL20											1.000	0.538 0.074	-0.140 0.667	-0.021 0.935	0.091 0.782	0.161 0.619	0.706 0.012	0.189 0.557	-0.056 0.869
IL-7												1.000	0.343 0.276	0.294 0.354	0.056 0.869	0.049 0.886	0.538 0.074	0.531 0.079	0.189 0.557
IL-7R													1.000	0.462 0.134	-0.301 0.542	0.336 0.286	0.042 0.905	-0.224 0.485	0.657 0.022
SPNST-2														1.000	0.210 0.513	0.035 0.920	-0.014 0.973	0.070 0.834	0.378 0.227
NEFL															1.000	-0.091 0.366	0.287 0.079	0.531 0.011	-0.713 0.011
CD206																1.000	-0.070 0.834	-0.406 0.192	0.203 0.527
CD40																	1.000	0.252 0.430	-0.168 0.603
IL-3																		1.000	-0.287 0.366
IL-3Ra																			1.000

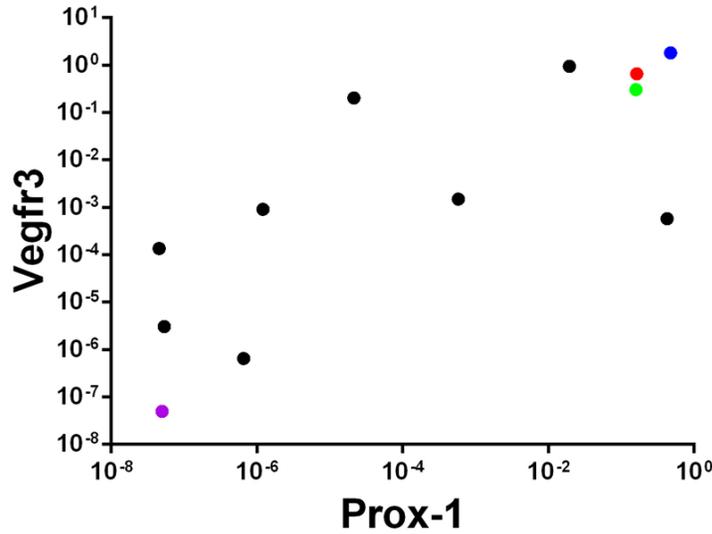


Figure 13. XY Data Plot of Correlation between VEGFR-3 and Prox-1 mRNA Expression.

Spearman Correlation analysis was performed on $2^{-\Delta Ct}$ values of 12 cell lines for VEGFR-3 and Prox-1. Analysis revealed a strong positive correlation between VEGFR-3 and Prox-1 with an R -value of 0.776 and a p-value of 0.004. Blue dot represents HDLEC, red dot represents 293T cells, green dot represents 293 cells, and purple dot represents 786-O cells, a cell line which has low mRNA expression of both Prox-1 and VEGFR-3.

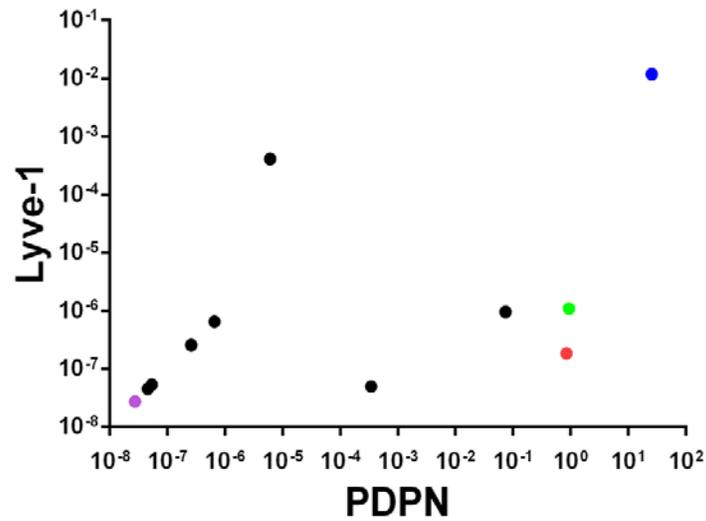


Figure 14. XY Data Plot of Correlation between PDPN and Lyve-1 mRNA Expression.

Spearman Correlation analysis was performed on $2^{-\Delta C_t}$ values of 12 cell lines for PDPN and Lyve-1. Analysis revealed a strong positive correlation between PDPN and Lyve-1 with an R-value of 0.797 and a p-value of 0.003. Blue dot represents HDLEC, red dot represents 293T cells, green dot represents 293 cells, and purple dot represents CEM cells which have low levels of PDPN and Lyve-1 mRNA expression.

Furthermore, these results indicate that there might be some mechanisms involved in regulation of LEC markers, dependent on other LEC marker expression. In particular, Prox-1 mRNA expression has a significant positive correlation with VEGFR-3, PDPN, and Lyve-1 mRNA expression. This supports the finding that Prox-1 regulates VEGFR-3 expression amongst LECs [51]. Additionally, among all of the genes analyzed, there are only 10 significant correlations, with five of them being between LEC markers and only one being a negative correlation between IL-3R α and NEFL.

4.4 FLOW CYTOMETRIC ANALYSIS OF LEC MARKERS ON 293T CELLS FURTHER CONFIRMS THEIR LEC-LIKENESS

Results from both standard RT-PCR and real-time RT-PCR, (Figures 1-11) revealed that 293 and 293T cells expressed multiple LEC markers at the mRNA level to a similar degree as HDLECs. To extend the mRNA analyses, flow cytometric staining was performed to measure the protein expression on 293T cells for LEC markers, Lyve-1 and PDPN. Previous experiments have been performed using HDLECs to demonstrate PDPN protein expression using flow analysis [26]. PDPN was chosen as an appropriate protein for staining due to its high level of mRNA expression by 293T cells (Figure 12), giving an anticipated result of high levels of protein staining. In contrast, Lyve-1 levels were measured by 293T cells leading to an anticipated low level of Lyve-1 protein staining. Figure 15 shows the gating strategy and results for both Lyve-1 and PDPN individual staining, as well as Lyve-1/PDPN dual staining. Given that 293T cells are a homotypic population, the gating strategy included gating on the population of interest through analyzing the forward and side scatter properties and excluding debris, dead cells, and doublets (Figure 15, top panel). A total of 30,000 cells were acquired for each sample and positively stained cells were gated based on staining of an aliquot of cells in parallel with isotype-matched controls.

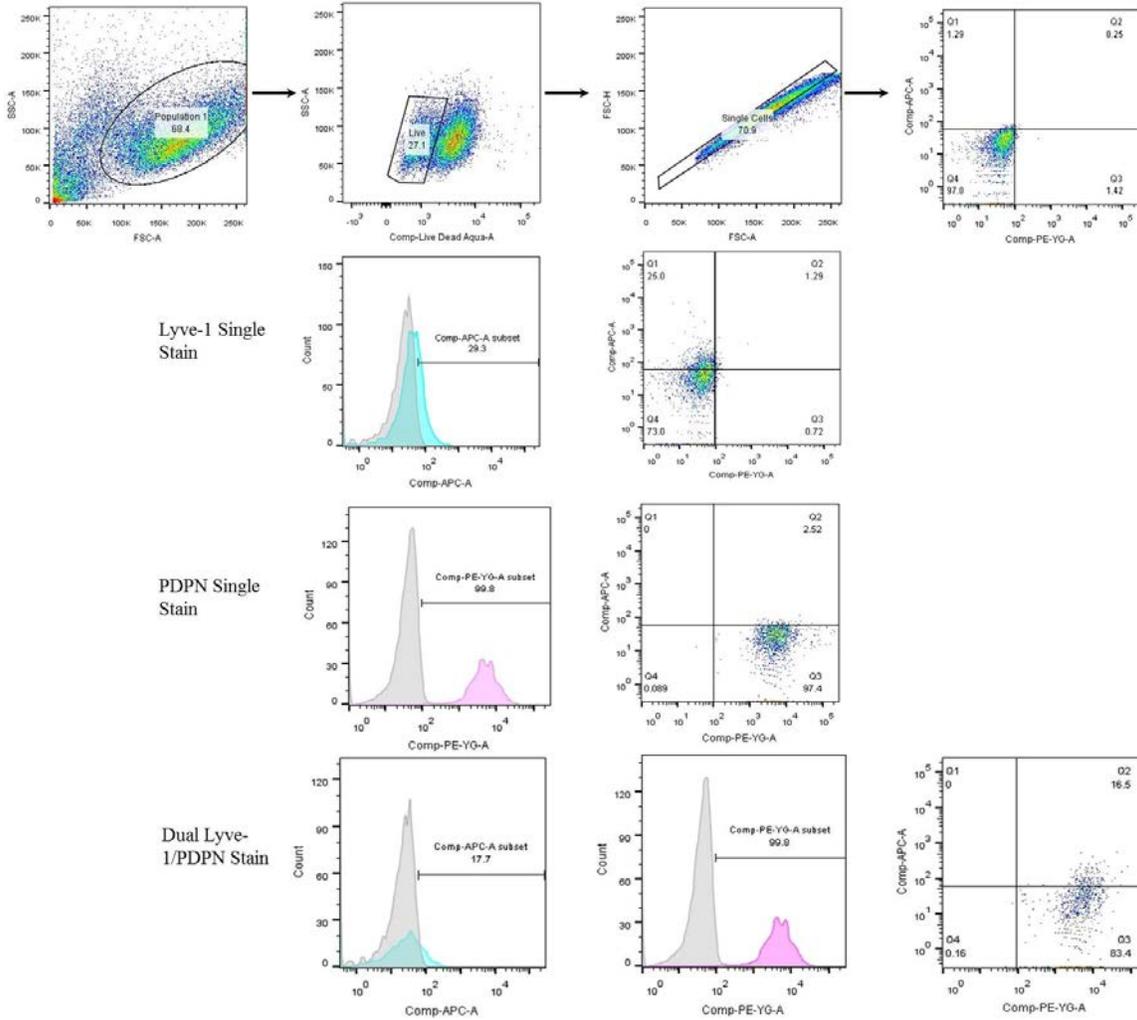


Figure 15. Flow Cytometry Double and Single Staining for PDPN and Lyve-1 Expression by 293T cells.

293T cells were stained were single stained or double stained with Lyve-1 or PDPN antibodies. Gating strategy (top panel) is shown using Isotype control, Rat IgG 2a-PE. 293T cells stained for Lyve-1 expression with anti-human lyve-1-APC (R&D, cat no. FAB20892A) showed 29.3% positive stain and cells stained for PDPN expression with anti-human podoplanin-PE (Angiobio, cat no. 11-009PE) showed 99.9% positive stain after gating on respective Isotype controls. Dual staining for Lyve-1 and PDPN revealed 99% positive PDPN staining and 19% positive Lyve-1 staining for 293T cells.

Overall 98% of 293T cells stained strongly for PDPN and approximately 20% stained for Lyve-1 when cells were stained with individual antibodies (Figure 15). Double stained cells showed that all Lyve-1 positive cells were also PDPN positive, but not all PDPN positive cells were Lyve-1 positive (Figure 15). This is concordant with mRNA expression levels for PDPN

and Lyve-1 (Figure 12). These results also reveal that high PDPN mRNA expression is not from a small subset of cells, but is consistently expressed throughout the 293T cell population.

This experiment further reveals the phenotypic resemblance between 293T cells and HDLEC through not only mRNA expression, but also through cell surface protein expression. Moreover, it confirms that 293T cells consistently express LEC markers Lyve-1 and PDPN at the mRNA and protein levels.

4.5 TREATMENT OF 293 AND 293T CELLS WITH VEGF-C FAILS TO SHOW UPREGULATION OF A VEGF-C INDUCED GENE

Isolated primary LECs are typically cultured in the presence of VEGF-C due to its necessity for attachment and survival in culture settings. As previously stated, VEGF-C is one of the two ligands for VEGFR-3, a marker expressed by LECs. The functional role of VEGF-C for LECs has been thoroughly investigated with results pointing to VEGFR-3's early role as an LEC marker and the necessity of VEGF-C for LEC growth, which can be produced in vivo by BECs, smooth muscle cells, and mesenchymal cells [52]. Previous studies have explored the impact of VEGF-C treatment on LECs through microarray analysis, finding an up-regulation in Apolipoprotein L2 (Apol L2), amongst other mRNAs, although Apol L2 was most strongly upregulated [37]. Given that 293 and 293T cells express VEGFR-3 and that VEGF-C is a necessary supplement for primary cultured LECs, 293 and 293T cells were cultured in the presence of VEGF-C to observe if they respond similarly to LECs in their upregulation of Apol L2 [37]. Secondly, since VEGFR-3 is an early marker expressed by LECs and LECs are grown in the presence of VEGF-C, signaling through VEGFR-3 might impact the expression levels of

other LEC markers. Lastly, since 293 and 293T cells are embryonic in origin, they might exhibit some plasticity allowing them to be pushed into a more LEC-like phenotype, as revealed by higher mRNA expression of LEC markers in the presence of VEGF-C. To investigate the impact VEGF-C treatment might have on Apol L2 and LEC marker mRNA expression levels, 293 and 293T cells were seeded at a density of 200,000/25cm². Cells were allowed to attach overnight and medium was supplemented with a final concentration of 0.2µg/mL recombinant human VEGF-C for 48 hours. Results shown in Figure 16 indicate that there was no significant change in mRNA expression levels of Apolipoprotein L2 in the presence of VEGF-C, unlike previous findings, which suggest a four-fold change in LEC upregulation of Apol L2 after six hours of treatment (Figure 16) [37]. Similarly, there was not a significant change in LEC marker mRNA expression after VEGF-C treatment in 293 and 293T cells. When comparing 293 and 293T cell up-regulation of LEC markers, 293 cells show a higher change in both VEGFR-3 and Prox-1 mRNA expression of approximately 1-fold, compared to relatively no fold change in 293T cell mRNA expression, suggesting that 293 cells might have a greater potential to change under adverse culture conditions (Figure 17).

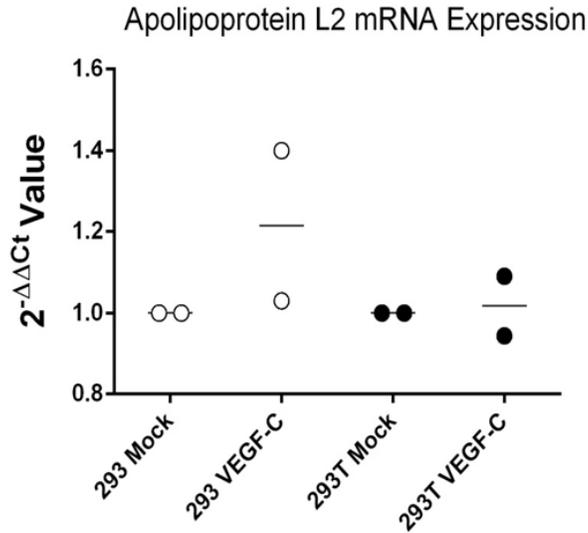


Figure 16. Change in Apolipoprotein L2 After Treatment with VEGF-C.

293 and 293T cells were seeded at a density of 200,000 cells/T25 flask. Cells were allowed to attach overnight, then treated with 0.2ug/mL rh-VEGF-C for 48 hours. Student's paired T-test reveals no significant change in Apolipoprotein L2 expression after treatment for 48 hours with VEGF-C by 293 or 293T cells. This figure is a representation of two independent experiments where bars represent mean values and points represent individual experimental values.

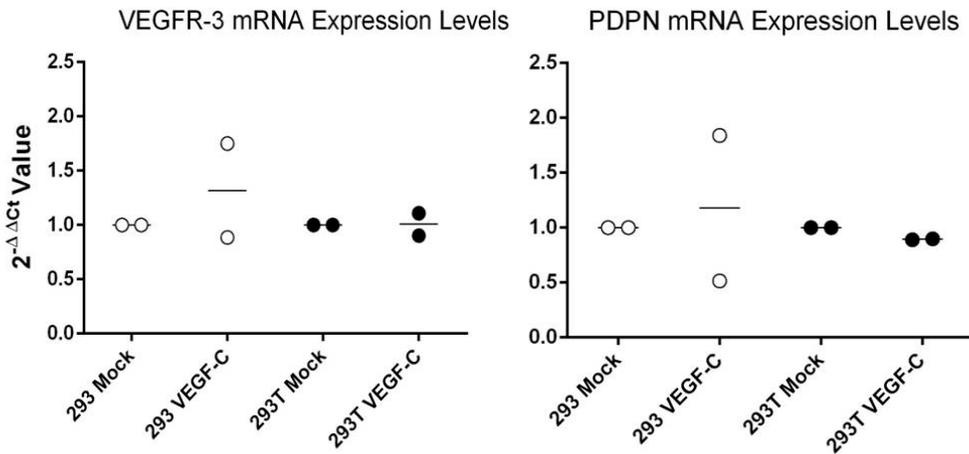


Figure 17. Change in LEC Marker Expression After Treatment with VEGF-C.

293 and 293T cells were seeded at a density of 200,000 cells/T25 flask. Cells were allowed to attach overnight, then treated with 0.2ug/mL rh-VEGF-C for 48 hours. Points represent 2^{-ΔΔCt} value calculated using the average Ct value from duplicate wells. This figure is a representation of two independent experiments where bars represent mean values and points represent individual experimental values. Student's paired t-test revealed no significant change in VEGFR-3 or PDPN mRNA expression after treatment for 48 hours with VEGF-C.

Despite a lack of change in Apol L2 and LEC marker mRNA expression upon treatment with VEGF-C, 293 and 293T cells still maintain their expression of LEC markers. A lack of up-regulation of Apol L2 in 293 and 293T cells could be explained by continuous culture in the absence of VEGF-C and therefore loss of dependence on it, leading to a more defined phenotype and signaling pathway than previously thought. Further studies will be needed to more fully determine the reason for a lack of response in 293 and 293T cells following VEGF-C treatment.

4.6 3D CULTURE SHOWS LITTLE CHANGE IN DE-DIFFERENTIATION MARKER EXPRESSION WITH SIMULTANEOUS MAINTENANCE OF LEC MARKER EXPRESSION BY 293 AND 293T CELLS

For many years, two-dimensional cell culture of adherent cells, which includes the interaction between cells and a plastic surface on which to attach and grow, was used for in vitro study. More recently, however, three-dimensional (3D) culture has been developed that involves interaction between neighboring cells and their environment, more accurately mimicking the in vivo environment in which most cells will exist. Three-dimensional culture of adherent cells in environments of low to no attachment causes the formation of spheroids or multi-cellular aggregates, which can change cell-cell interactions, cell morphology, and cell matrix interactions [40]. One example of how 3D culture can affect cellular marker expression was shown with embryonic stem cells and how they maintained their stem phenotype [40]. Other studies have shown the ability to differentiate stem cells into LECs through use of treatment with growth factors through three dimensional formation of embryoid bodies [44]. Likewise, 293 cells grown in three-dimensional culture have the ability to up-regulate stem markers and cell specific

differentiation markers [40]. For the purpose of this study, two different methods were attempted to form spheroids using 293 and 293T cells to analyze the effects of such culture conditions on mRNA expression of stem markers and LEC markers. De-differentiation of 293 or 293T cells would provide the opportunity to specifically re-differentiate them using LEC growth conditions, such as treatment with VEGF-A and VEGF-C, perhaps resulting in cells that are even more LEC-like [44].

4.6.1 Low Attachment Method

One method used to form spheroids requires the use of low attachment substrates so that cells are not able to adhere to the culture plate [40]. This method has been used to study the change in stem properties of non-stem cell lines after grown in low attachment conditions. They found that 293 and RT4 cells could form spheroids after 24 hours of incubation using low attachment dishes (Figure 18) [40]. Analysis of stem-like properties revealed approximately a 1,000-fold increase in Nanog expression after five or 10 day incubation of 293 cells. Similarly, they reported a 14-fold change in ectoderm marker, Nestin, by 293 cell aggregates, which indicates a de-differentiation of the cell line due to its origin from mesoderm [40]. To further investigate if 293 and 293T cells could undergo reprogramming to a de-differentiated state, cells were grown in low attachment dishes on agarose for seven or six days, respectively and then analyzed using real-time RT-PCR for Nanog and Nestin mRNA expression. Similarly, real-time RT-PCR was performed for LEC markers, Lyve-1 and Prox-1 to determine the impact of low attachment culture on LEC marker expression. Results show that spheroid formation using low

attachment dishes does not induce an up-regulation of de-differentiation marker expression or a change in expression of LEC marker, Prox-1, by 293 or 293T cells (Figures 19 and 20).

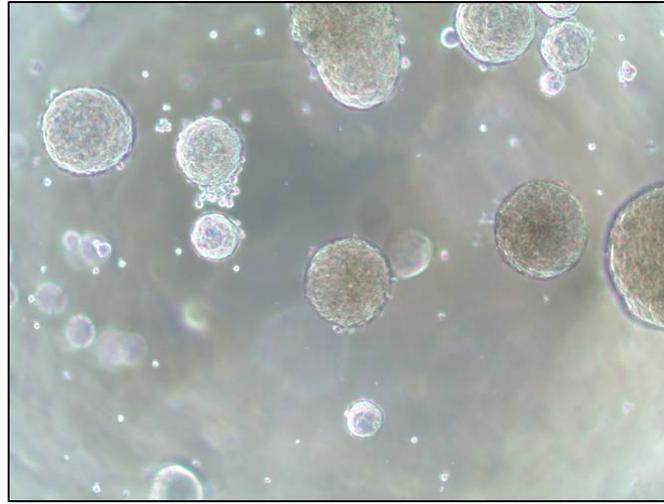


Figure 18. 293 Spheroids Formed using Low Attachment Method.

293 cells were seeded at a density of 3×10^6 on 66mm petri dishes on high glucose DMEM/agar for 6 days to form spheroids. Image was captured using IDEA Spot Camera at 100X.

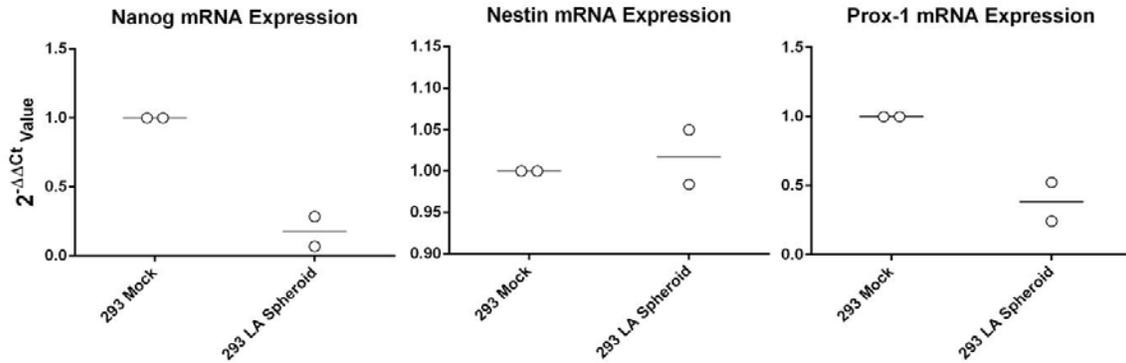


Figure 19. Change in Stem Markers and Prox-1 mRNA Expression in 293 Spheroids Formed in Low Attachment Dishes.

293 cells were seeded at a density of 3×10^6 on 66mm petri dishes on high glucose DMEM/agar for 7 days to form spheroids. Real-time RT-PCR was performed for stem marker, Nanog, de-differentiation marker, Nestin, and LEC markers Prox-1 and Lyve-1 for changes in mRNA expression. Points represent the $2^{-\Delta\Delta Ct}$ value calculated from the mean of duplicate wells. Mock Ct values were used for both low attachment analysis and hanging drop analysis. Student's paired T-test revealed no significant difference between mock and low attachment spheroid mRNA expression for Nanog, Nestin, or Prox-1. Lyve-1 Ct values were undetected (CT values above 50) and were not included in this analysis.

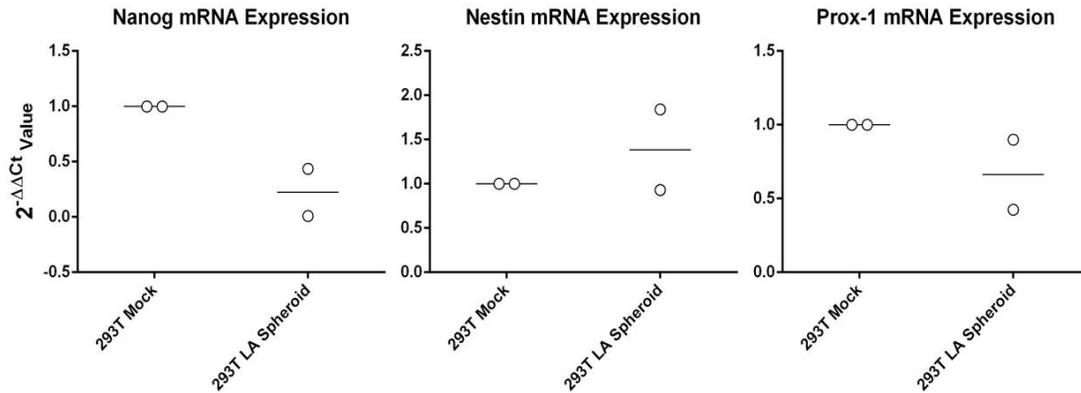


Figure 20. Change in Stem Markers and Prox-1 mRNA Expression in 293T Spheroids Formed in Low Attachment Dishes.

293T cells were seeded at a density of 3×10^6 on 66mm petri dishes on high glucose DMEM/agar for 6 days to form spheroids. Real-time RT-PCR was performed for stem marker, Nanog, de-differentiation marker, Nestin, and LEC markers Prox-1 and Lyve-1 for changes in mRNA expression. Points represent the $2^{-\Delta\Delta C_t}$ value calculated from the mean of duplicate wells. Mock Ct values were used for both low attachment analysis and hanging drop analysis. Student's paired T-test revealed no significant difference between mock and low attachment spheroid mRNA expression for Nanog, Nestin, or Prox-1. Lyve-1 Ct values were undetected (CT values above 50) and were not included in this analysis.

Although, 3D growth of 293 and 293T cells in low attachment conditions showed no significant change in stem marker expression, a second approach was pursued to attempt replicating previous results [40].

4.6.2 Culture of 293 and 293T Cells Using The Hanging Drop Method

One method for forming spheroids in vitro involves the use of methylcellulose and gravity to discourage attachment of cells to a culture dish surface. To form hanging drops spheroids, 293 and 293T cells were seeded at a density of 500 cells per 25 μ L of methylcellulose supplemented complete DMEM media for 24 hours on plastic square petri dishes. Both 293 and 293T cells formed spheroids after 24 hours, similar to previous studies using HUVECs [39]. To investigate spheroid morphology, 293T spheroids were grown for seven days, then fixed, processed, sectioned and stained with hematoxylin and eosin (Figure 21).

Recent studies have shown the up-regulation of stem marker and de-differentiation marker mRNA expression after culture of 293 cells as spheroids for seven days [40]. To replicate this study using the hanging drop method, 293 and 293T cells were seeded at a density of 500 cells per 25 μ L and incubated for a total of seven days. Real-time RT-PCR was performed on purified RNAs to investigate changes in mRNA expression levels of Nanog, a stem marker, and Nestin, a de-differentiation marker [40]. Similarly, LEC marker expression was investigated to determine if 3D culture had an ability to up-regulate expression. Results showed a slight increase in expression of Nanog, with an average of a 3-fold change by 293T spheroids, but relatively no change in 293 cells. A paired student's T-test revealed no significant change in Nanog, Nestin, or Prox-1 expression for 293 and 293T cells. However, 293 cells revealed approximately a 400-fold difference in expression of Nestin in one replicate. To determine if that experiment is an outlier, a third independent experiment would need to be completed and statistical tests performed for Nestin mRNA expression by 293 cells. Results reveal that 293T cells have a consistent expression of Nestin, and approximately 2-fold change in Prox-1 expression when cultured in hanging drops (Figures 23 and 24).

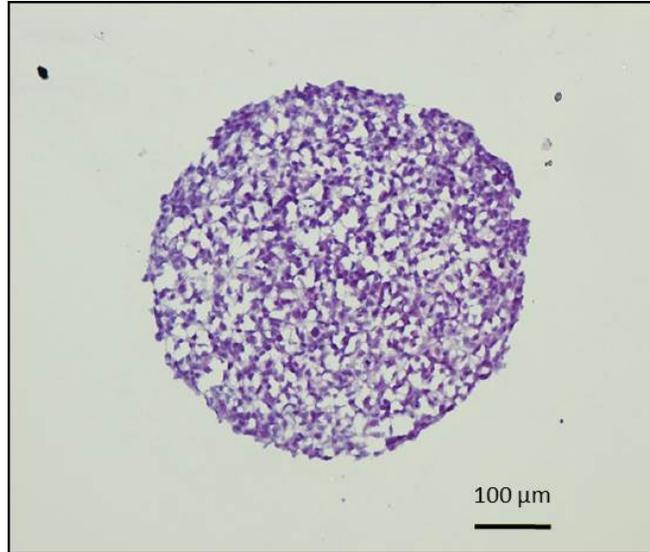


Figure 21. 293T Hanging Drop Spheroid.

293T cells were seeded at a density of 500 cells/25 μ L DMEM/methylcellulose media and incubated at 5% CO₂, 37°C, for seven days. Spheroids were cryosectioned and stained with Hematoxylin and Eosin.

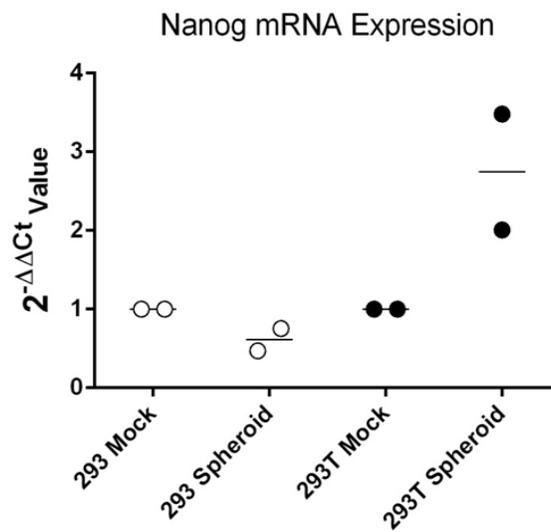


Figure 22. Change in Nanog mRNA Expression in Spheroids Formed in Hanging Drops.

293 and 293T cells were seeded at a density of 500 cells/25 μ L DMEM/methylcellulose media and incubated at 5% CO₂, 37°C, for seven days. Student's paired T-test revealed no significant change in mRNA expression levels for Nanog by 293 or 293T spheroids formed in hanging drop culture.

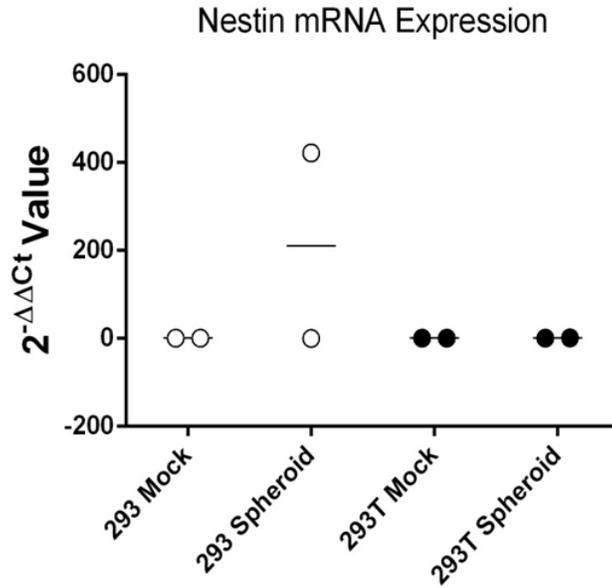


Figure 23. Change in Nestin mRNA Expression in Spheroids Formed in Hanging Drops. 293 and 293T cells were seeded at a density of 500 cells/25 μ L DMEM/methylcellulose media and incubated at 5% CO₂, 37°C, for seven days. Student's paired T-test revealed no significant change in mRNA expression levels for Nestin by 293 or 293T spheroids formed in hanging drop culture.

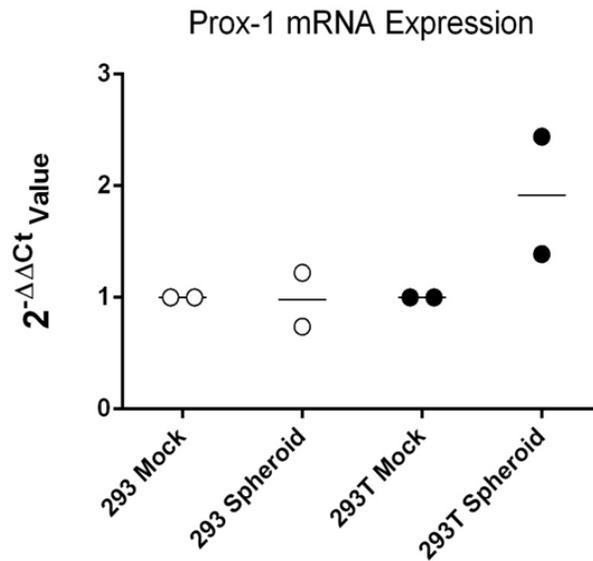


Figure 24. Change in Prox-1 mRNA Expression in Spheroids Formed in Hanging Drops. 293T cells were seeded at a density of 500 cells/25 μ L DMEM/methylcellulose media and incubated at 5% CO₂, 37°C, for seven days. Student's paired T-test revealed no significant change in mRNA expression levels for Prox-1 by 293 or 293T spheroids formed in hanging drop culture.

In an attempt to increase expression of de-differentiation markers and reveal a more stem-like phenotype, 293 and 293T cells were grown in two different environments that induce spheroid formation. Results from both the hanging drop method and the low attachment method reveal that 293 and 293T cells did not de-differentiate in our hands and that under these 3D culture conditions they nevertheless maintained their LEC-like phenotype (Figures 19-24).

4.7 TRANSFECTION OF A PLASMID CONTAINING PROX-1 REVEALS LITTLE CHANGE IN LEC MARKER EXPRESSION

The function of Prox-1 within the lymphatic endothelium has been investigated in several contexts revealing that it is the master regulator of lymphatic differentiation, has the ability to guide sprouting and polarity of growth from the cardinal vein, and can induce VEGFR-3 expression [23, 51, 53]. Given Prox-1's dynamic function and presence within LECs (Figure 12) transfection studies were performed on 293 and 293T cells with a plasmid containing a cDNA encoding the Prox-1 open reading frame (ORF) in an attempt to overexpress Prox-1 within 293 and 293T cells and to create a more LEC-like phenotype. Cells were seeded onto 12 well plates and treated with a Polyjet-plasmid DNA mixture according to the manufacturer's recommendations. Cells were checked for transfection efficiency using pEGFP-N1 transfected wells as a positive control and were analyzed by real-time RT-PCR. In response to transfection with a plasmid expressing Prox-1, 293 and 293T cells did not show a robust change in LEC markers VEGFR-3 or Lyve-1 (Figures 25 and 26). Changes in the levels of Prox-1 signals could be due to contaminating, undigested Prox-1 plasmid DNA, despite DNase treatment. Two forms of plasmids containing Prox-1 were used to transfect 293 and 293T cells, including versions

expressing the human and rhesus derived ORFs. Transfected cells were compared to mock transfected cells, which received treatment of Polyjet alone and empty vector control, pcDNA3.1.

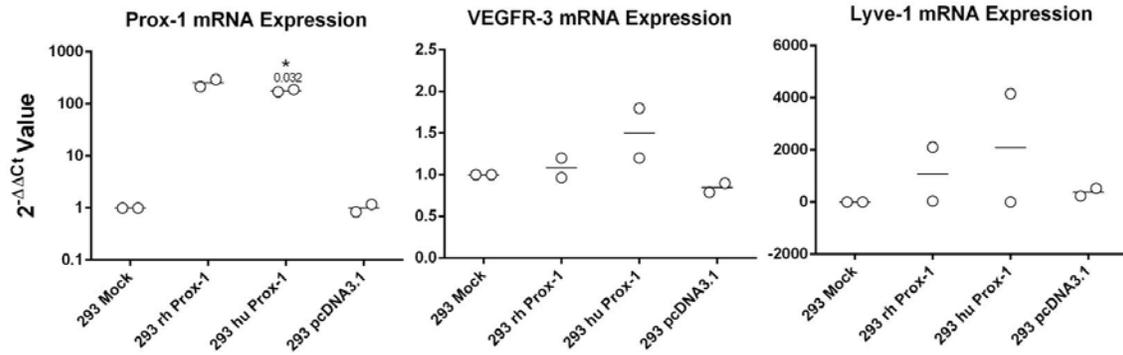


Figure 25. Change in LEC Marker Expression by 293 Cells Transfected with Prox-1.

293 cells were seeded into 12 well plates and transfected with plasmids containing rhesus or human Prox-1. Transfection of plasmid containing Prox-1 was calibrated to mock transfected wells, which contained Polyjet reagent, but no DNA. Empty vector, pcDNA3.1 also served as a negative control. Cells were harvested 48 hours post transfection and checked for transfection efficiency using peGFPN1 transfected wells (data not shown). Human Prox-1 transfected 293 cells showed a significant upregulation of Prox-1 mRNA expression with a p-value of 0.032. Points represent independent experiments and bars represent the mean of two independent experiments.

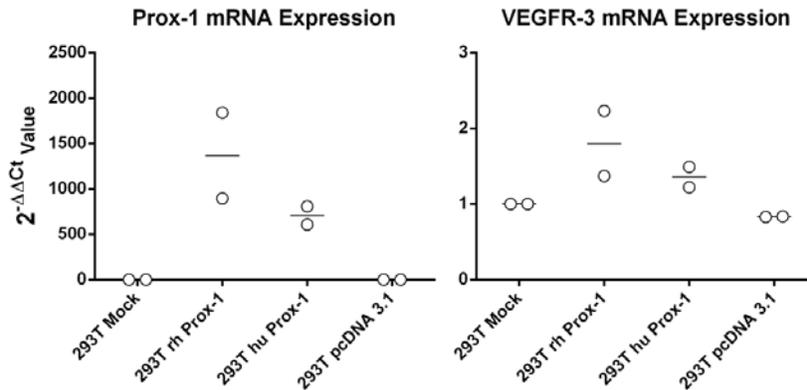


Figure 26. Change in LEC Marker Expression by 293T Cells Transfected with Prox-1.

293T cells were seeded into 12 well plates and transfected with plasmids containing rhesus or human Prox-1. Transfection of plasmid containing Prox-1 was calibrated to mock transfected wells, which contained Polyjet reagent, but no DNA. Empty vector, pcDNA3.1 also served as a negative control. Cells were harvested 48 hours post transfection and checked for transfection efficiency using peGFPN1 transfected wells (data not shown). 293T cells did not reveal expression of Lyve-1 in this study regardless of treatment (Ct values over 50). Points represent independent experiments and bars represent the mean of two independent experiments.

Transfected 293 cells revealed a significant change in mRNA expression of LEC marker, Prox-1, albeit NRT controls also amplified with NRT Ct values varying from between 4 and 12 cycles from cDNA reactions including RT (Figure 25 and data not shown). Furthermore, one experiment using 293 cells revealed a robust change in Lyve-1 expression suggesting that in some circumstances Prox-1 might have the ability to up-regulate mRNA expression levels of Lyve-1 (Figure 25). Real-time results also revealed that cells transfected with plasmid encoding Prox-1 consistently expressed LEC markers VEGFR-3 and Lyve-1 (Figures 25 and 26). This lack of LEC marker expression change in response to overexpression of Prox-1 might indicate that Prox-1 is at its maximal responsiveness in 293 and 293T cells.

4.8 RESULTS CONCLUSION

The above studies outline an attempt to phenotypically characterize 293 and 293T cells and attempt to further LEC-likeness through various culture conditions. Standard and real-time RT-PCR revealed that 293 and 293T cells express four of the five LEC markers and that their expression levels are similar to that of HDLECs (Figure 1-12), revealing that 293 and 293T cells have very similar phenotypes to LECs. Similarly, to investigate protein expression by 293T cells, flow cytometry staining was performed for LEC markers, Lyve-1 and PDPN. Results confirmed real-time RT-PCR mRNA expression showing that approximately 99% of 293T cells stained positively for PDPN expression and 28% of cells stained positively for Lyve-1 protein expression (Figure 15). An attempt at finding an appropriate culture condition in which 293 and 293T cells would most resemble LECs was completed using a variety of culture conditions. Results from two different 3D models suggest that 293 and 293T cells might not have an

influenced phenotype and that they exhibit a more stable LEC-like phenotype (Figures 19-24). Furthermore, these analyses reveal that 293 and 293T cells have the potential to serve as model LECs due to their phenotypic resemblance.

5.0 DISCUSSION

HEK293 and 293T cells are a typical cell line used widely in the field of research primarily for the production of recombinant proteins or investigation into cellular function. Though frequently used, the type of cell they are and their phenotypic characterization remains incompletely understood. Despite previous research that suggested 293 cells are neuronal in origin, more recent studies have observed expression of a LEC marker, suggesting a need for deeper analysis. This study focuses on the phenotypic characterization of 293 and 293T cells in the context of LEC marker expression. In addition to phenotypic analysis, 293 and 293T cells were treated through manipulation of culture conditions in an attempt to further differentiate them into LECs or to de-differentiate them for the purpose of controlled differentiation further into LECs. The results presented open a variety of avenues for interpretation and discussion in regard to what defines the LEC phenotype, whether 293 cells are LEC-like, troubleshooting areas of skepticism, and what the implications are for characterization of 293 and 293T cells as LECs.

5.1 DEFINING A LEC PHENOTYPE

LECs, as previously discussed, make up the lining of the lymphatic vasculature and have multiple functions within the lymphatic system. Until the discovery of LEC markers VEGFR-3,

Lyve-1, Prox-1, and PDPN, it was difficult to study the function of LECs. More recently however, it has been observed that from an immunologic perspective they aid in trafficking of DCs to the lymph node and express multiple TLRs [19]. Defining LECs however, has become a difficult process that relies heavily upon expression of multiple cellular markers, which in turn, contribute to function. Though there is no strict defining factor, a general understanding is that they are a subset of cells that are derived from the cardinal vein to make up the lymphatic system and express multiple sub-specific markers to high levels, the most influential being VEGFR-3, Lyve-1, Prox-1, PDPN, and CCL21. Several papers discuss the necessity of Prox-1 expression, particularly in early states of differentiation. In addition, it has been shown that Prox-1 expression is necessary for the maintenance of the LEC phenotype, as loss of Prox-1 causes LECs to phenotypically change into BECs [54]. Secondly, another reference for defining LECs comes from the isolation of primary LECs for study in culture. Typically, LECs are isolated using one or multiple cellular markers such as PDPN and Lyve-1. Similarly, the real-time RT-PCR data from the findings presented suggest that expression of one or even two LEC markers is not unique to many cell lines. Taken together, this suggests that currently, the best way to characterize LECs is by the measurement of the expression levels of multiple LEC markers, including VEGFR-3, Lyve-1, PDPN, and Prox-1.

5.2 CHARACTERIZING 293 AND 293T CELL PHENOTYPE

The need to explore the phenotype of 293 and 293T cells became apparent when we first found expression of the LEC marker, PDPN by 293 cells (Berendam, unpublished data) [36]. As already discussed, I found that expression of one LEC marker was not sufficient enough to

characterize cells as LEC-like. This study reveals the expression of multiple LEC markers by 293 and 293T cells, which strongly suggests we need to re-characterize 293 and 293T cells as LEC-like. Here, real-time RT-PCR analysis revealed that 293 and 293T cells express VEGFR-3, Prox-1, PDPN, and Lyve-1. This expression of multiple LEC markers, though it might not be unique given that two other cell lines express four LEC markers, is strongly indicative of LEC-likeness due to the high levels of expression of VEGFR-3, Prox-1, and PDPN, which nearly matches the expression levels of the HDLECs, which are bona fide LECs.

5.2.1 Caco-2 and Thp-1 Reveal LEC Marker Expression

Due to the expression of several LEC markers, by multiple cell lines, it becomes imperative to take into consideration the level of expression. Out of the 12 cell lines analyzed five expressed at least three LEC markers including HDLECs, 293, 293T, Caco-2, and Thp-1. LEC marker expression among 293 and 293T cells was challenged when several, otherwise defined, cell types also showed expression of LEC markers. However, like previously stated, the level of marker expression is substantially different when comparing Thp-1 and Caco-2 cells to HDLECs. Secondly, overall marker expression of these cells must be compared to HDLECs. For instance, Thp-1 cells express CD209, whereas HDLECs do not. This also is true for the expression of IL-7 by HDLECs, but not by Thp-1 cells and expression of CCL21 by HDLECs and not by Caco-2 cells. Similar to marker expression is the importance in the level of LEC marker expression in deciding that 293 and 293T cells can be re-defined as LEC-like and that Thp-1 and Caco-2 cells cannot be re-defined as LEC-like. Thirdly, the origin of the cells must be considered when re-evaluating LEC marker expression. Caco-2 cells are human colon adenocarcinoma cells that are used in research to study intestinal barrier function [55]. It has

been observed that Caco-2 cells during normal growth show the presence of sub-populations, each exhibiting a different morphology [55]. This suggests that expression of multiple LEC markers could be due to a small sub-population of Caco-2 cells and that all cells might not consistently express LEC markers. This is in contrast to PDPN protein expression by 293T cells, which is consistent throughout the population (Figure 15) [55]. The second cell type expressing multiple LEC markers are Thp-1 cells which are monocytic precursor cells used as model monocyte-derived macrophages after treatment with phorbol esters [56]. Post differentiation they resemble macrophages morphologically and exhibit similar functions to macrophages such as phagocytosis [56]. Likewise, LEC marker expression has been observed on normal macrophages, antigen stimulated macrophages, and tumor associated macrophages (TAM) [56, 57]. Furthermore, Lyve-1 staining has been observed on normal macrophages isolated from Prox-1 heterozygous animals, LPS stimulated macrophages have shown binding of CLEC-2 to PDPN, and VEGFR-3 protein expression has been observed on tumor-associated macrophages [53, 57]. Analogously, HDLECs have been shown to express macrophage markers such as macrophage mannose receptor, also known as CD206, revealing an overlap of markers between the two cell populations [47]. Therefore, LEC marker expression by Thp-1 cells is not indicative that this cell line should be redefined as LEC-like – it could rather reveal an overlap of both macrophage and LEC marker expression between both cell types.

5.2.2 Low Lyve-1 Expression is Not Unique Among LECs

Another caveat to redefining 293 and 293T cells as LEC-like is the comparatively low level of Lyve-1 mRNA and protein expression. Flow cytometry results suggest that only 20-30% of 293T cells are expressing Lyve-1 protein (Figure 15). Like 293 and 293T cells,

HDLECs also do not express Lyve-1 to a similar level as other LEC markers including VEGFR-3, Prox-1, and PDPN when compared to GUSB. However, low Lyve-1 mRNA and protein expression is not unique to LECs and has been shown among isolated cells from animals developing AIDS [58]. Other research has found that depending on the type of lymphatic vessel, deep, superficial, or subcutaneous, expression of Lyve-1 might vary [59]. For example, Lyve-1 is often found in dermal tissue of the human skin (deep and superficial layers) but can be completely absent in subcutaneous vessels [59].

Previous research reveals a difference in Lyve-1 expression dependent on the level of inflammation in the local environment and many studies do not specifically define expression level of Lyve-1, but merely the presence as dictating LEC phenotype [28, 58]. These findings together suggest that expression of Lyve-1 to any level, in conjunction with expression of other LEC markers, contributes to defining a LEC-like phenotype.

5.2.3 293 and 293T Cells Are Not Definitively Neuronal or Podocyte-like

Previous studies have identified expression of neuronal proteins by 293 and 293T cells in a study of adenovirus transformation. Their results concluded that neuronal cells were more easily transformed by adenovirus, suggesting that HEK293 cells were neuronal in origin [10]. To determine the impact of neurofilament expression by 293 and 293T cells, 10 other cell lines were analyzed for mRNA expression of the neurofilament light subunit (NEFL). Results revealed that 293 and 293T cells were not unique in their expression of NEFL mRNA (Figure 12). Three of the 10 cell lines expressed NEFL to a similar degree as 293 and 293T cells including, A549, 786-O, and HDLECs. The significance of NEFL expression by other cell lines suggests that NEFL expression is not restricted to neuronal cells. Furthermore, the expression of

NEFL by HDLECs, suggests that NEFL is widely expressed by cultured cells. Interestingly, studies have shown that Prox-1 can be found in the nuclei of neuroblastomas, suggesting there might be a link between LEC marker expression, particularly Prox-1, and neuronal marker expression [60].

To determine resemblance to podocytes 293 and 293T cells were also investigated for expression of podocyte markers WT1 and Synpo [43]. Podocytes are typical human kidney cells that also express the LEC marker PDPN [61]. Real-time RT-PCR analysis revealed that WT1 and Synpo expression by 293 and 293T cells was not unique and that many other cell lines expressed mRNAs for WT1 and Synpo (Figure 12). Similar to neurofilament expression, podocyte marker expression is not specific enough to define cultured cells, including 293 and 293T cells, as podocytes.

The expression of neuronal and podocyte marker mRNA does not define 293 and 293T cell phenotype due to similar expression by all of the other cell types. The expression of NEFL by HDLECs, along with previous findings of Prox-1 expression by neuroblastomas, suggests that there might be a connection between LEC marker expression and neuronal marker expression. Taken together, the expression of NEFL, WT1, and Synpo by 293 and 293T cells is not definitive of a neuronal or podocyte-like characterization, leaving the argument that these two cell lines are LEC-like intact.

5.3 FUTURE IMPLICATIONS FOR 293 CELLS AS LEC-LIKE

The re-characterization of HEK293 and 293T cells as LEC-like brings implications and potential to the cell line that was previously unrevealed. One implication is the need to re-

evaluate previous research using 293 and 293T cells. In particular, studies designed using 293 or 293T cells as a neuronal cell type should be re-evaluated due to the commonality in expression of neuronal mRNA by several cell lines. Similarly, studies using 293 and 293T cells as neuronal cells must be re-evaluated due to the ability to redefine 293 and 293T cells as LEC-like. Along with re-evaluation of previous research, this study has revealed the necessity to use one or even two markers to define cell type. This is especially the case for cells like LECs that do not have a robust functional method for characterization and must rely heavily upon the expression, in particular the level of expression, of multiple known cellular markers. Lastly, characterization of 293 and 293T cells as LEC-like provides the potential to use these cells in LEC studies. As more is revealed about the cellular markers expressed and the function of LECs, characterization of 293 and 293T cells will need to be further evaluated. Deeper analysis would also reveal differences between LECs and 293 cells as well as their potential use, such as investigation into the expression and functionality of TLRs by 293 and 293T cells. Results from experiments such as these will continue to determine the degree to which 293 cells can be used as model LECs and if they have the potential to become more LEC-like.

5.4 293 AND 293T CELLS AS MODEL LECs

One difference between 293 or 293T cells and LECs is their growth in cell culture. For example, 293 and 293T cells have a doubling time between 24 and 35 hours in comparison to LECs, which have a doubling time of approximately 2-5 days [62]. Due to this increased proliferation and uninhibited growth, 293 and 293T cells might be more suitable for 3D culture. One area of study for the lymphatics includes the attempt to form lymphatic vessels in vitro [63].

Since 293 and 293T cells phenotypically express many LEC markers and to equal levels, they can be re-defined as LEC-like. Taken together, this provides an opportunity for 293 and 293T cells to be used as model LECs in an attempt to form in vitro lymphatic vessels. Due to 293 and 293T cell success in 3D culture in this study, a proposed model for forming lymphatic vasculature would be with scaffolding modeling lymphatic vasculature formation. If 293 and 293T cells formed 3D vasculature it could stand as a model system representing lymphatic vasculature response in vivo. The protein and mRNA expression levels of 293 and 293T cells reflect the phenotype of LECs and thus, provide the opportunity to be utilized as model LECs in future lymphatic related research, in particular 3D culture to represent in vivo environments.

6.0 PUBLIC HEALTH SIGNIFICANCE

The public health significance of this research is applicable on multiple levels from making researchers aware of the need for characterization of previously undetermined cell types to describing a new potential for a popularly used cell line. As previously stated, it is of high importance that within the field of basic research, the most accurate system is used to mimic human cellular responses. One way in which scientists can ensure accurate results is through the use of a characterized cell type that bears significant resemblance to the *in vivo* cell population. This research provides an example of a previously un-determined cell type that is highly used and presents convincing data for a new characterization of 293 and 293T cells as LEC-like. Similarly, the lymphatics play a crucial role in maintaining fluid balance and can play a key role in metastatic spread of cancer through the lymphatic response to VEGF-C [59]. For example, lymphedema, a condition in which there is excess fluid in tissues due to malfunction of the lymphatic system, is common in women diagnosed and treated for breast cancer [64, 65]. Furthermore, understanding the role of the lymphatics in metastatic spread is essential in uncovering new forms of cancer therapy and treatment. One of the greatest public health advancements within the twentieth century has been the development and implementation of vaccines within the community. Recent studies have suggested that LECs play a critical role in vaccine response through the trafficking of DCs and T cells to the lymph node and through MHC class II antigen presentation [46]. Specific functions and the extent to which LECs play a role in

lymphatics are still being investigated. The identification of an LEC cell line, 293 and 293T cells, provides a new avenue through which to further study the role of LECs. The ease of which 293 and 293T cells are cultured, and the timeliness of division provides unlimited potential for initial studies into understanding the function of LEC markers, interaction with antigen, and chemotactic properties. Continued study into the functional similarity between 293, 293T cells, LECs will further reveal their potential as model LECs.

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