Technical Advance

Generation and Characterization of Telomerase-Transfected Human Lymphatic Endothelial Cells with an Extended Life Span

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The study of lymphatic endothelial cells and lymphangiogenesis has, in the past, been hampered by the lack of lymphatic endothelial-specific markers. The recent discovery of several such markers has permitted the isolation of lymphatic endothelial cells (LECs) from human skin. However, cell numbers are limited and purity is variable with the different isolation procedures. To overcome these problems, we have transfected human dermal microvascular endothelial cells (HDMVECs) with a retrovirus containing the coding region of human telomerase reverse transcriptase (hTERT), and have produced a cell line, hTERT-HDLEC, with an extended lifespan. hTERT-HDLEC exhibit a typical cobblestone morphology when grown in culture, are contact-inhibited, and express endothelial cell-specific markers. hTERT-HDLEC also express the recognized lymphatic markers, Prox-1, LYVE-1 and podoplanin, as well as integrin α9, but do not express CD34. They also form tube-like structures in three-dimensional collagen gels when stimulated with vascular endothelial growth factors -A and -C. Based on these currently recognized criteria, these cells are LEC. Surprisingly, we also found that the widely studied HMEC-1 cell line expresses recognized lymphatic markers; however, these cells are also CD34-positive. In summary, the ectopic expression of hTERT increases the life span of LECs and does not affect their capacity to form tube-like structures in a collagen matrix. The production and characterization of hTERT-HDLEC will facilitate the study of the properties of lymphatic endothelium *in vitro*. (Am J Pathol 2004, 165:11–24)

The blood and lymphatic vascular systems are anatomically and histologically closely related but distinct, and do not anastomose except at the level of the main lymphatic ducts. The blood vascular system supplies nutrients and oxygen to the body while the lymphatic vasculature is crucial for immune cell traffic and the uptake of extracellular fluid. The formation of new blood vessels from pre-existing blood vessels (angiogenesis) occurs in physiological (eg, postnatal growth, inflammation, and tissue repair) and pathological (eg, tumor recruitment of blood vessels) settings. Lymphangiogenesis is the formation of new lymphatic vessels from pre-existing lymphatic capillaries, and occurs in similar settings. Blood endothelial cells (BECs) and lymphatic endothelial cells (LECs) are quiescent cells that line the lumina of blood and lymphatic vessels.

The lymphatic system has traditionally been overshadowed by the greater emphasis placed on the blood vascular system. This has been due in part to the absence of suitable markers that distinguish lymphatic from blood vascular endothelium. In the past few years, this limitation has been overcome. Lymphatic markers include LYVE-1, a lymphatic endothelial receptor for the extracellular matrix/lymphatic fluid glycosaminoglycan hyaluronan;[1] Prox-1, a homeobox gene product involved in regulating early lymphatic development;[2] podoplanin, a glomerular podocyte membrane mucoprotein;[3] and vascular endothelial growth factor receptor-3 (VEGFR-3), a transmembrane tyrosine kinase receptor for vascular endothelial growth factors-C (VEGF-C) and VEGF-D.[4,5] Recent reports indicate that the expression of the integrin α9 subunit is restricted to lymphatic endothelium.[6]

Use of these markers has permitted the isolation of relatively pure populations of BECs and LECs using fluorescence-activated cell sorting (FACS)[7] or immunomag-

netic beads.[8,9] Initial characterization of these populations highlighted major differences between the two cell types. However, differences have also been observed in molecular expression and cellular function within the same cell type, depending on the isolation procedure used. An additional limitation remains the limited number of cells that can be obtained from a single isolation, and primary cultures of BECs and LECs enter senescence after 8 to 10 passages. Cell purity can also be a problem as contaminating cell types rapidly overgrow endothelial cell (ECs). Primary isolates, while most accurately representing the "physiological state", have the inherent problems of tissue availability and batch-to-batch variation, which can affect reproducibility of results.

Various approaches have been used to overcome EC senescence. These include ectopic expression of viral oncogenes[10–13] and spontaneous transformation.[10–13] However, the immortalized cell lines thus generated lose important EC functions and ultimately classical EC markers. An alternative way to overcome or delay senescence is by ectopic expression of human telomerase reverse transcriptase (hTERT). This approach has been successful for immortalizing fibroblasts and retinal pigment epithelial cells[14–16] and also for human dermal microvascular endothelial cells (HDMVEC),[17,18] and can be achieved without converting the cells to a transformed phenotype.

In the work presented herein, we have transfected commercially available primary HDMVEC with hTERT and compared these cells to a well-characterized immortalized cell line, HMEC-1.[10,19] HMEC-1 was generated by stably transfecting dermal microvascular endothelial cells (also isolated from human skin) with the coding region of the simian virus SV40 large antigen. We have found that our hTERT-HDLEC retain typical EC morphology and marker expression beyond 40 passages, express currently recognized LEC markers but not CD34, and retain the capacity to form capillary-like tubes in three-dimensional collagen gels. Interestingly, we also demonstrate that HMEC-1, which is used as a blood vascular-derived endothelial cell line, expresses many lymphatic markers and also CD34. Overall, a comparison of the panel of markers expressed by and functional characteristics of hTERT-HDLEC with the current LEC and BEC profiles, suggests that these cells are LEC.

Materials and Methods

Reagents

Recombinant human VEGF-A (165aa isoform) was purchased from PeproTech Inc. (Rocky Hill, NJ). Recombinant human FGF-2 was provided by Dr. P. Sarmientos (Farmitalia Carlo Erba, Milan, Italy), VEGF-C$_{\Delta N \Delta C}$ (designated VEGF-C from hereon) was provided by Dr. M. Skobe (Cancer Center, Mount Sinai Medical Center, New York), and mutant VEGF-C156[20] was provided by Dr. K. Alitalo (Biomedicum, Helsinki, Finland). Type I collagen was extracted from rat tail tendons as described previously.[21]

Cell Lines and Cell Culture

Primary HDMVEC were purchased from Clonetics (neonatal pooled, Cambrex Bio Science Inc, Walkersville, MD, USA) or PromoCell (PromoCell, Heidelberg, Germany) and cultured in EGM-2MV and ECGM-MV2 media, respectively. hTERT-transfected HDMVEC were cultured in EGM-2MV medium. HMEC-1 were kindly provided by Drs. T.J. Lawley and E.W. Ades (Centers for Disease Control, Atlanta, GA) and were grown in EBM131 supplemented with 1 μg/ml hydrocortisone, 10 ng/ml hEGF, and 10% donor calf serum (DCS). SkHep-1 were grown in DMEM (glucose 450 × g/L, Life Technologies Inc., Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (FCS).

Antibodies

Mouse mAbs anti-VE-cadherin,[22] -CD34 (QBEND-10), -VEGFR-2 (p1C11),[23] and -VEGFR-3 (p3C5)[24] were gifts from Dr. J. Gamble (Hanson Centre for Cancer Research, Adelaide, Australia), Dr. M. Greaves (Leukemia Research Fund Center, Chester Beatty Laboratories, London, UK), and Drs. D. Hicklin and B. Pytowski (ImClone Systems Inc., New York), respectively. Rat anti-CD44[25] and isotype control MEL14 were gifts from Dr. M. Aurrand-Lions (Department of Pathology, University Medical Center, Geneva, Switzerland). Biotinylated mAb anti-CD31 (Ancell, Bayport, MA, USA) was revealed by PE-conjugated streptavidin (Becton Dickinson, NJ, USA).

Polyclonal rabbit anti-podoplanin and -LYVE-1 were provided by Dr. D. Kerjaschki (Institute of Pathology, University of Vienna, Austria) and Dr. D.G. Jackson (MRC Human Immunology Unit, Institute of Molecular Medicine, Oxford, UK), respectively. Polyclonal rabbit anti-Prox-1 was provided by Dr. J. Wilting (Children's Hospital, University of Goettingen, Germany) and Reliatech (Braunschweig, Germany). Irrelevant mouse IgG1$_k$ (anti-Keyhole Limpet Hemocyanin (KLH) antibody) and normal rabbit immunoglobulins were purchased from Pharmingen and Santa Cruz, respectively. Rabbit polyclonal anti-human podoplanin antibody (no. 201853) was generated by Covalab (Lyon, France) by immunizing with peptides CDVVTPGTSEDRYKSG (RN16CG), CESTVHAQEQSPSATA (RN16CA), and CEGASTGQPEDDTETT (RN16CT), and was partially purified against immobilized peptides.

Cloning of Human Telomerase and Transfection of HDMVEC with hTERT

hTert was recloned from a human immortalized lymphocyte cell line by RT-PCR (details of the cloning can be obtained on demand). The full-length cDNA was verified by sequencing. It was then cloned into pBABEhygro vectors.[26] A stock of high-titer retrovirus was prepared by transfecting the Phoenix-Ampho packaging cell line as described.[27] Neonatal pooled HDMVEC (Clonetics) at passage 5 were plated the day before infection at 10^4 cells/cm^2 in 6-well plates. Virus stock at 10^6 CFU/ml was diluted threefold in ECGM-MV2 medium and was added

to the cells together with polybrene that was added to a final concentration of 8 μg/ml. The plate was centrifuged at 32°C for 45 minutes at 10,000 $\times$ g to increase infection efficiency and thereafter was incubated overnight in a CO_2 incubator at 37°C. Two days after infection, selection was initiated with 100 μg/ml hygromycin.

RT-PCR

Total RNA was extracted from human cell lines and tissues using Trizol (Life Technologies, Gaithersburg, MD, USA). Total RNA (2 μg) was reverse-transcribed (RT) using random hexanucleotides (Boehringer Mannheim, Mannheim, Germany) and Superscript II reverse transcriptase (Life Technologies). One twentieth of the RT products were amplified using Expand High Fidelity PCR System (Roche Molecular Biochemicals, Mannheim Germany) or with *Taq*DNA Polymerase (Invitrogen, Carlsbad, CA, USA). Amplification of the acidic ribosomal phosphoprotein P0 was used to control for RNA integrity and reverse-transcription efficiency.[28] Where indicated, RT was omitted. Equal volumes of PCR products were analyzed on 2% agarose gels. Bands representing the partial cDNAs of podoplanin and LYVE-1 were excised from the gel, subcloned into pGEMT-Easy vector (Promega, Madison, WI), and sequenced on both strands.

Immunocytochemistry

ECs were cultured on gelatin-coated dishes. Confluent cells were fixed with 4% paraformaldehyde and postfixed with cold methanol. ECs were incubated for 30 minutes in blocking buffer (1% bovine serum albumin (BSA) in phosphate- buffered saline (PBS)) before exposure to 5 μg/ml rabbit anti-Prox-1 or normal rabbit IgGs overnight at 4°C. Staining was revealed with Alexa 488-labeled secondary antibody (Molecular Probes, Leiden, The Netherlands). Nuclei were counterstained with 0.5 μg/ml 4′-6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO, USA).

FACS Analysis

Confluent monolayers were dissociated by gently pipetting in the presence of Cell Dissociation Solution (Sigma). Cell pellets were resuspended in PBS containing 0.2% BSA to a final cell number of 1 to 2 $\times$ 10^5 cells/sample, and incubated for 45 minutes on ice with 2 μg/ml anti-PECAM-1, 2 μg/ml CD34, 5 μg/ml CD44, or hybridoma supernatant (diluted 1:2) containing anti-VE-cadherin. Rabbit antisera were diluted as follows: 1:500 for anti-podoplanin (D.K.), 1:100 for anti-podoplanin 201853, and 1:100 for anti-LYVE-1. Primary antibody binding was revealed with an FITC-labeled goat anti-rabbit antibody (Biosys, Compiegne, France) or with streptavidin-phycoerythrin (PE) conjugate (Pharmingen, San Diego, CA, USA). Negative controls included omission of the first antibody for anti-PECAM-1 and anti-VE-cadherin, preimmune sera for rabbit antisera, and substitution of primary antibodies by appropriate irrelevant antibodies.

Cells were analyzed by flow cytometry using a FACScan instrument and CellQuest software, and overlays were executed with either CellQuest or Winmdi software.

RNase Protection Assay

RNase protection assays were performed as previously described.[29] Total cellular RNA was extracted using Trizol reagent (Invitrogen). [^{32}P]-dUTP cRNA probes were generated using cDNAs for human VEGFR-1, VEGFR-2, and VEGFR-3[30] and human acidic ribosomal phosphoprotein P0, the latter serving as an internal control.[31] Autoradiograms were scanned with a Laser Scan-Jet IIex Instrument (Hewlett Packard, Palo Alto, CA) and bands were quantitated using ImageQuant 3.3 software (Molecular Dynamics, Sunnyvale, CA).

Proliferation Assays

hTERT-HDLEC were seeded into 12-well plates at 10^4 cells per well and grown in VEGF-A- and FGF-2-depleted EGM-2MV medium (incomplete medium) for 24 hours; this was done to avoid interference by cytokines contained in the medium. The cells were left untreated or were treated with FGF-2 (10 ng/ml), or with VEGF-A (100 ng/ml), VEGF-C (100 ng/ml), or VEGF-C156 (100 and 500 ng/ml) alone or in combination with FGF-2 (10 ng/ml). Medium and cytokines were renewed every 2 days. After 6 days, the cells were harvested using trypsin and suspended in 1 ml, and were then counted for 1 minute using a FACScan instrument and CellQuest software. Results represent the mean of four independent experiments (passages 17–24) $\pm$ SEM per condition. Mean values were compared using Student's unpaired *t*-test, and a significant value was taken as $P < 0.05$.

In Vitro *Angiogenesis Assays*

The ability of HMEC-1 and hTERT-HDLEC to form capillary-like structures *in vitro* was assessed in three-dimensional collagen gel assays.[9,21,32] Cells were either allowed to form monolayers on top of collagen gels to assess their invasive capacity, or seeded as single cells in suspension within collagen gels, or "sandwiched" between two layers of collagen. Cells were seeded onto collagen gels in 16-mm wells at 1 $\times$ 10^5 cells/well for the invasion assay, at 0.5 $\times$ 10^6 cells/ml in the suspension assay and at a concentration of 3.4 $\times$ 10^4 cells/cm^2 in the sandwich assay. HMEC-1 and hTERT-HDLEC were cultured in EBM 131 or EGM-2MV medium (complete or incomplete), respectively. For cell suspension or sandwich assays, cells were treated after collagen polymerization, while for cells seeded onto collagen gels, treatment was begun only after the cells had reached confluence (approximately 1 week). Cells were treated with 10 ng/ml FGF-2, and 100 ng/ml VEGF-A, 100 ng/ml VEGF-C, 100 or 500 ng/ml VEGF-C156 alone or in combination with 10 ng/ml FGF-2. Media and cytokines were renewed every 2 to 3 days. After 7 days, cells were photographed under phase contrast microscopy using a

Nikon Diaphot TMD inverted photomicroscope (Nikon, Tokyo, Japan). Tube formation in the invasion assay was quantitated as described,[33] and results are expressed as mean additive sprout length $\pm$ SEM (in μm) from three fields per experiment for at least three experiments per condition. Mean values were compared using Student's unpaired *t*-test, and a significant value was taken as $P <$ 0.05.

Semi-Thin and Thin Sections

Collagen gel cultures were fixed *in situ* overnight with 2.5% glutaraldehyde in 100 mmol/L sodium cacodylate buffer (pH 7.4). After rinsing in the same buffer, the gels were cut into 2×2-mm fragments and post-fixed in 1% osmium tetroxide in Veronal acetate buffer for 60 minutes, stained *en bloc* with 2.5% uranyl acetate in 50% ethanol, dehydrated in graded ethanols, and embedded in Epon 812 in flat molds. Semi-thin (2 μm) and thin (40 nm) sections were cut with an LKB ultramicrotome (LKB Instruments, Gaithersburg, MD) and were stained with 1% methylene blue and photographed using a transmission light microscope (Carl Zeiss, Orberkochen, Germany).[32] Thin sections were stained with uranyl acetate and lead citrate and examined in a Philips CM10 electron microscope (Philips, Eindhoven, The Netherlands).

Zymography and Reverse Zymography

Matrix metalloproteinase (MMP) activity was analyzed using gelatin zymography.[34] Confluent monolayers of hTERT-HDLEC or HMEC-1 were washed with PBS and the cells incubated in their corresponding serum- and cytokine-free media. After a 15 hour incubation at 37°C, conditioned media were collected, supplemented with 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF) and 15 mmol/L N-(2-hydroxyethyl)piperazine-N'-(2 ethanesulfonic acid) (HEPES), centrifuged at $340 \times g$ for 5 minutes, and the resulting supernatants were stored at -80°C until use. Supernatants (30 μl) were electrophoresed in 10% SDS-PAGE gels co-polymerized with 1 mg/ml gelatin. After soaking in 2.5% Triton X-100 for 30 minutes, the gels were incubated in reaction buffer (50 mmol/L Tris-HCl, pH 8) containing 150 mmol/L NaCl, 10 mmol/L CaCl$_2$, and 0.02% NaN$_3$) at 37°C for 16 hours and stained with ethanol:acetic acid:water (30:10:60) containing 0.25% Coomassie Blue R250 for 4 hours. Gelatinolytic activity was detected as clear bands against a background of uniform staining. Conditioned media from MCF-7 and U937 cell lines, which secrete MMP-2 and MMP-9, respectively, were used as positive controls. For casein zymography and reverse zymography, the cells were treated as mentioned above, and cell extracts or supernatants were prepared and analyzed as previously described.[29,35,36]

Array Analysis

Targeted cDNA arrays designed to analyze adhesion molecule and protease expression (GEArray HGEA9913090 and HGEA 9914030) were purchased from SuperArray Bioscience Corporation (Frederick, MD). Probe synthesis and hybridization from total cellular RNA from HMEC-1 (passage 16) or hTERT-HDLEC (passage 21) were performed as a service by Artus-Biotech (Hamburg, Germany) according to the array manufacturer's instructions. Hybridization was quantitated using a Typhoon 9210 phosphoimager (Molecular Dynamics) and data were analyzed with ImageQuant 3.3 (Molecular Dynamics) software. Briefly, the lowest hybridization signal (considered as background) was subtracted from the average hybridization for each gene (duplicate spots for each gene), and values were normalized with respect to GAPDH. Any gene with a hybridization signal greater than two times that of pUC18 was deemed to be expressed. Results are summarized as supplementary data at http://ajp.amjpathol.org.

Results

hTERT-HDLEC Have a Markedly Extended Lifespan

HDMVEC were transduced with a plasmid containing hTERT cDNA. The cells were obtained from Clonetics (reference number CC-2516), and were pooled by the suppliers from at least three neonatal foreskins. Cells from each donor were isolated and grown separately before being pooled. Foreskins were enzymatically processed and endothelial cells were isolated using mesh strainers to catch clusters of endothelial cells together with differential split techniques (information obtained from the suppliers).

Similar to previous reports, we have found that human microvascular endothelial cells transduced with telomerase exhibit a longer life-span. The cells retained the characteristic endothelial cell cobblestone morphology and displayed contact-inhibited growth (Figure 1A). hTERT-transduced cells were analyzed for expression of the transduced hTERT gene by RT-PCR. Infected cells were found to express the hTERT cDNA (Figure 1B) and, as shown by TRAP assay (Figure 1C), also displayed detectable hTERT enzymatic activity on an artificial template. Cell growth was robust until about 40 passages, following which a subpopulation exhibited a flattened morphology typical of senescent cells (data not shown). Under the same culture conditions, the primary parental endothelial cells entered senescence after 8 to 10 passages. Thus, ectopic expression of hTERT in endothelial cells extended their replicative life span at least four times, technically defining these cells as immortalized.[37] There was no evidence for a transformed phenotype *in vitro*, since the cells retained their typical EC contact-inhibited phenotype and did not form colonies in soft agar (data not shown). All attempts to obtain independent populations by clonal expansion failed, as indicated by the fact that from the 20 colonies initially cloned using cloning rings, only three grew up to a maximum of five passages before exhibiting cell senescence.

Expression of EC markers was assessed by FACS. CD31 (PECAM-1), and VE-cadherin were expressed up
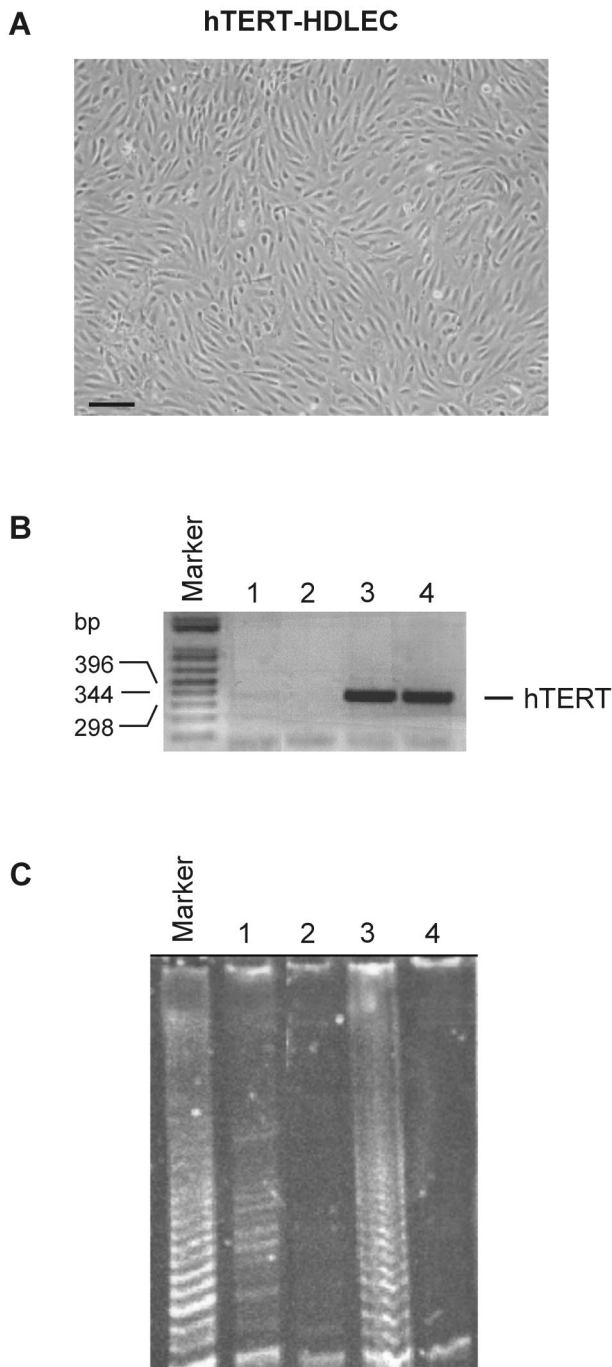
A

hTERT-HDLEC

B

C

Figure 1. Endothelial life span is increased by hTERT. **A:** Passage 27 hTERT-HDLEC were photographed under phase contrast microscopy. The cells retain their typical cobblestone morphology and are contact inhibited. **Bar**, 100 μm. **B:** RT-PCR assay. Samples were run in a 1.5% agarose gel. The expected 350-bp band is indicated. Marker (1-kb ladder, Invitrogen), primary HDMVEC (negative control, **lane 1**), H$_2$O (**lane 2**), RPE-hTERT (positive control, **lane 3**), hTERT-HDLEC, passage 9 (**lane 4**). **C:** Cell extracts were prepared and the TRAP assay was performed using the Intergen TRAPeze kit. Samples were run in an 8% acrylamide gel. Marker kit control, Mdx12 hTERT fibroblasts (positive control, **lane 1**), primary HDMVEC, passage 6 (**lane 2**), hTERT-HDLEC passage 9 (**lane 3**), H$_2$O (**lane 4**).

to the passages analyzed (passage 37). FACS profiles with both markers revealed the presence of a single population, and the relative expression of the two markers was similar at the different passages analyzed (Figure

2A). HMEC-1 cells were used as a positive control. Interestingly, CD34 was detected in both cell lines by RT-PCR while only HMEC-1 expressed detectable levels of the protein by FACS (data not shown). Similarly, von Willebrand factor was virtually undetectable in hTERT-HDLEC when compared to primary HDMVEC and human umbilical vein endothelial cells. We also found that both hTERT-HDLEC and HMEC-1 express CD44 as assessed by RT-PCR and FACS (data not shown).

hTERT-HDLEC Characterization

Neonatal HDMVEC purchased from Clonetics were pooled, by the supplier, from at least three donors; cells from each donor were isolated and grown separately before being pooled. It was thus likely that they contained both BEC and LEC populations. Transfection of neonatal pooled HDMVEC with hTERT could have immortalized either or both populations. Our first objective was to determine whether these cells express LEC markers. To this end, we generated a polyclonal antibody against peptides contained in the extracellular domain of human podoplanin (antibody characterization is shown as supplementary data at http://ajp.amjpathol.org). Both hTERT-HDLEC and HMEC-1 displayed similar FACS profiles, in that both cell lines contained only one population, which stained positively with three different antibodies (anti-podoplanin 201853, anti-LYVE-1, and anti-podoplanin (from D.K.)) to two reliable LEC markers (Figure 2B). Despite variations, hTERT-HDLEC expressed podoplanin up to at least passage 37 while LYVE-1 expression was lost between passages 35 and 37 where the mean fluorescence intensity reached a value of 1 (the value which was assigned to the mean fluorescence intensity of pre-immune serum) (Figure 2B).

We next assessed the expression of the transcription factor Prox-1, one of the most reliable lymphatic markers described to date.[38] RT-PCR using the human Prox-1-specific primers indicated in Table 1 amplified a band of the predicted size (333 bp) in both HMEC-1 and hTERT-HDLEC (Figure 3A). Immunocytochemistry was used to confirm Prox-1 expression at the protein level, and was repeated at least three times with HMEC-1 (passages 17–21) or with hTERT-HDMVEC (passages 21–37). Over 95% of nuclei stained positively for Prox-1 when compared to DAPI counterstaining (Figure 3B). (In fact, virtually 100% of the cells were positively stained; nuclei that gave a low signal were considered as negative.)

Both EC lines express reliable lymphatic markers, and thus could be defined as LECs when compared to current LEC and BEC molecular profiles. We wished to know however whether HDMVEC purchased from Clonetics acquire the capacity to express these markers in culture or are derived from a mixed population of BEC and LEC with BEC being lost during cell culture. To answer this question, we cultured primary endothelial cells purchased from Clonetics or PromoCell in their respective media and performed FACS analysis to determine the pattern of podoplanin expression at different passages. With regard to cells from PromoCell, 60 to 70% were
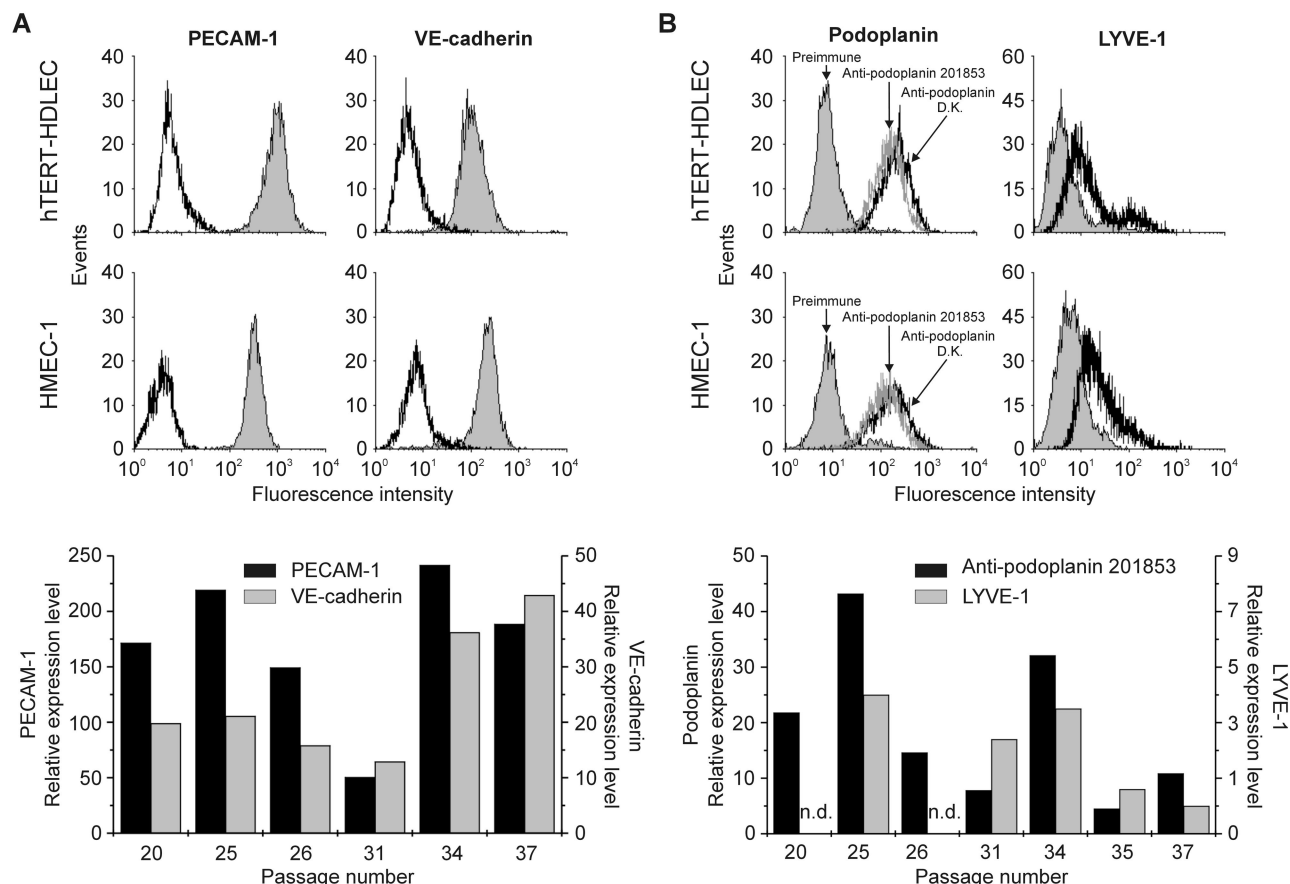
Figure 2. Expression of endothelial and lymphatic markers. **A:** Typical profiles obtained with the endothelial markers PECAM-1 and VE-cadherin on either hTERT-HDLEC (passage 26) or HMEC-1 (passage 18) (**top**). hTERT-HDLEC at different passages were stained with PECAM-1 (**black bars**, **left axis**) and VE-cadherin (**gray bars**, **right axis**) (**bottom**). The mean fluorescence intensities were normalized to the fluorescence with the secondary antibody alone (to which a value of 1 was assigned). **B:** Typical profiles obtained with the lymphatic markers podoplanin and LYVE-1 on either hTERT-HDLEC or HMEC-1. hTERT-HDLEC at different passages were stained with podoplanin (**black bars**, **left axis**) and LYVE-1 (**gray bars**, **right axis**), and the mean fluorescence intensities were normalized to fluorescence with pre-immune sera (to which a value of 1 was assigned). n.d., not determined.

podoplanin-positive, suggesting that these cells contain a mixed population of BECs and LECs, which is in accordance with previous reports.[38–40]

This ratio did not change with subsequent passaging suggesting that, at least in this passage range, the BEC did not acquire the capacity to express podoplanin. In addition, since the ratio of BEC:LEC did not change, we can conclude that BECs and LECs retain their molecular identities/characteristics in culture, and that neither population is lost or is overgrown by the other. Since the

Table 1. Sequences of PCR Primers, Length of PCR Product, Optimal Annealing Temperature and Number of Amplification Cycles

| Molecule	Sense (5′-3′)	Antisense (5′-3′)	Template (bases)	Annealing temperature (°C)	Number of cycles
Integrin α1	GCTACTGCTTCTTCTGGAGATGTG	TGAATTGTGCTGCCGAGATGAAC	345	55	30
Integrin α2	AGTGTGCTGTGTTCAGTTGATGTG	TGTGCGGATAGTGCCCTGATG	318	60	35
Integrin α5	ACCTGCTACCTCTCCACAGATAAC	TCACCAACAGCCACAGAGTATCC	327	55	30
Integrin α6	AGTCTCGTTCCTGTTCCTGCTAAC	CATCTGCCTTGCTGGTTCATGTAG	341	55	30
Integrin α9	ACTATGAAGCCGACCACATCCTAC	ATAAACCTTGCCGATGCCTTTGTC	386	60	40
Integrin β1	CAATGAAGGGCGTGTTGGTAGAC	TACAGACACCACACTCGCAGATG	383	55	30
hVEGF-C	TCCGGACTCGACCTCTCGGAC	CCCCACATCTATACACACCTCC	324	60	40
hLYVE-1	GGCAAGGACCAAGTTGACACAG	TGGAGCAGGAGGAGTAGTAGTAGG	376	55	35
hPodoplanin	AGCCAGAAGATGACACTGAGACTAC	ACCACAACGATGATTCCACCAATG	357	55	35
hProx-1	CCACCTGAGCCACCACCCTTG	GCTTGACGTGCGTACTTCTCCATC	333	60	35
hCD34	AAGCCTAGCCTGTCACCTGGAAATG	GGCAAGGAGCAGGGAGCATACC	300	60	35
hCD44	TGGGTTCATAGAAGGGCATGTGGTG	ATTCTGTCTGTGCTGTCGGTGATCC	420	60	35
hMMP-2	GCGACAAGAAGTATGGCTTC	TGCCAAGGTCAATGTCAGGA	390	62	40
hMMP-9	CGCAGACATCGTCATCCAGT	GATGCCATTCACGTCGTCCTTATG	829	60	40
hMMP-14	CAACACTGCCTACGAGAGGA	GTTCTACCTTCAGCTTCTGG	380	60	40
hTERT	CTCTCCCCCTTGAACCTCCTCTTTC	AGGACACCTGGCGGAAGGAG	350	55	35
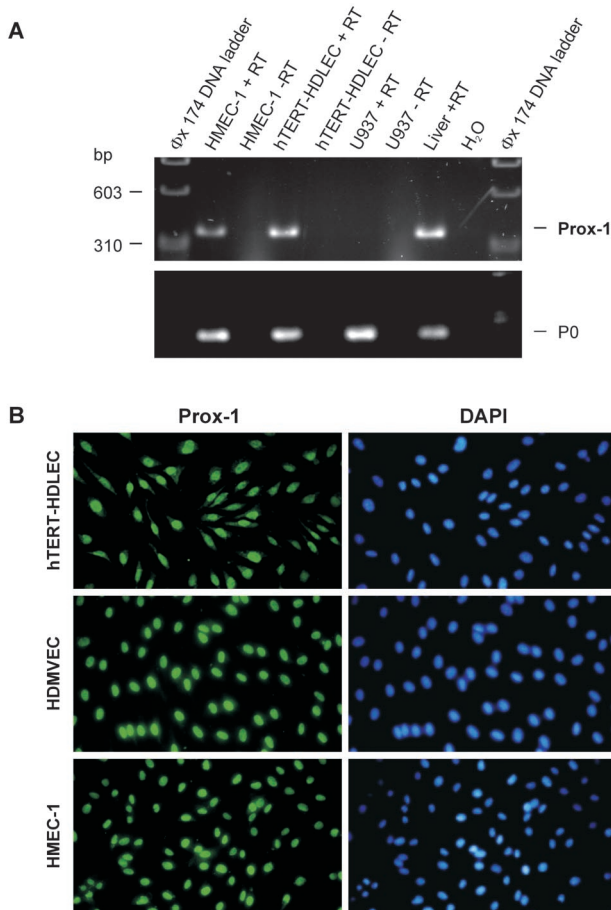
Figure 3. Expression of Prox-1. **A:** The pair of primers indicated in Table 1 was used to amplify partial cDNAs encoding for Prox-1, and equal volumes of the PCR products were analyzed on 2% agarose gels. cDNA fragments of ± 330 bp were amplified from hTERT-HDLEC, HMEC-1 and human liver (used as a positive control) but not from U937 cells. This fragment corresponds in size to the predicted Prox-1 amplification product. Amplification of the acidic ribosomal phosphoprotein P0 was used as a control for RNA integrity and reverse-transcriptase efficiency. Where indicated, RT was omitted to exclude genomic contamination of the signal. **B:** Nuclear localization of Prox-1 (**left**) was detected by immunocytochemistry in hTERT-HDLEC (**top**), neonatal pooled HDMVEC (Clonetics, **middle**) and HMEC-1 (**bottom**). Nuclei were counterstained with DAPI (**right**). One representative staining out of at least three independent experiments is shown.

Figure 4. PromoCell *versus* Clonetics: podoplanin expression. Human dermal microvascular endothelial cells purchased from PromoCell or Clonetics were cultured in their respective media. Podoplanin expression was determined by FACS analysis. The cells were incubated with control IgGs or with affinity-purified anti-podoplanin 201853, and labeling was revealed with a FITC-conjugated secondary antibody. **A:** Cells from PromoCell. **B:** Cells from Clonetics. **C:** The experiment was performed at different passages and the percentage of podoplanin-positive cells for each passage is indicated. n.d., not determined.

HDMVEC purchased from PromoCell are isolated from a single donor, we performed the experiment on three different batches. Similar results were obtained with each batch, and one representative experiment is shown in Figure 4. Experiments performed on neonatal-pooled ECs from Clonetics showed that over 98% of the cells expressed podoplanin from passage 6 (Figure 4). Since the cells are grown in different media, it is possible that one medium might favor LEC growth and therefore increase the LEC to BEC ratio. To address this question, we performed the same experiments but switched the media: PromoCell cells were cultured in Clonetics medium and vice versa. This gave similar results as described above when cells were cultured in their own media (data not shown). This suggests that once the cells are established in culture (ie, when they are received from the supplier), the medium does not have a significant effect on the growth of one population *versus* the other. Further-
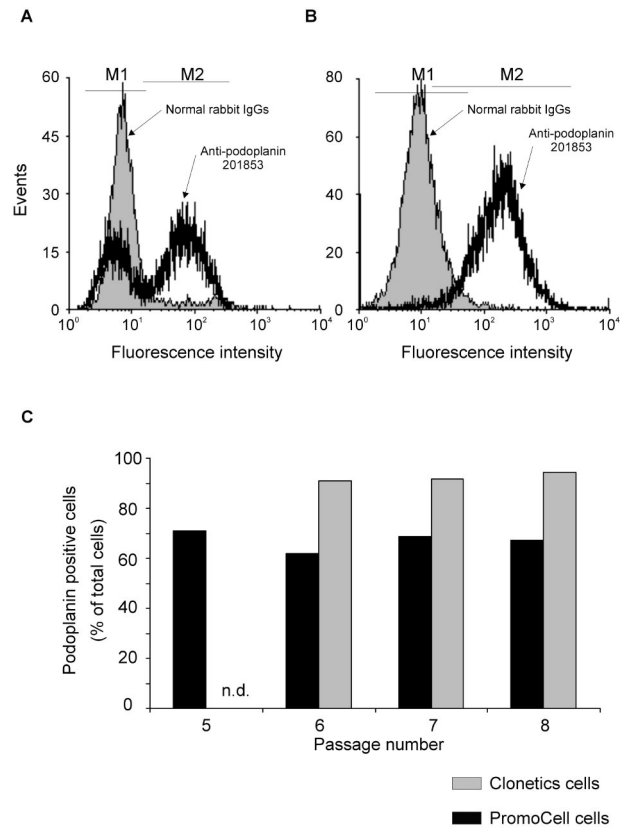
more, experiments performed in our lab in which a heterogeneous population of cells derived from neonatal foreskins were grown for two weeks, gave rise to a 99% or 60% pure CD31+/podoplanin+ population when grown in EGM-2MV (Clonetics) or ECGM-MV2 (PromoCell) media, respectively (data not shown). Furthermore, and of major significance for our study, Clonetics cells are initially LEC-enriched. This is likely to explain why virtually all of the cells in our hTERT-HDLEC line express characteristic LEC markers.

Cytokine-Induced hTERT-HDLEC Proliferation

A characteristic of endothelial cell lines is their ability to respond mitogenically to angiogenic or lymphangiogenic cytokines. Thus, we treated hTERT-HDLEC with different cytokines in FGF-2- and VEGF-A-depleted EGM-2MV medium. FGF-2, VEGF-A, and VEGF-C significantly increased cell proliferation. VEGF-A and VEGF-C alone induced a similar response and did not synergize with FGF-2 (Figure 5A). Interestingly, VEGF-C156 induced a mild, but significant, effect at 500 ng/ml[39] and at 100 ng/ml VEGF-C156 only potentiated FGF-2 response (Figure 5B). These results suggested hTERT-HDLEC express
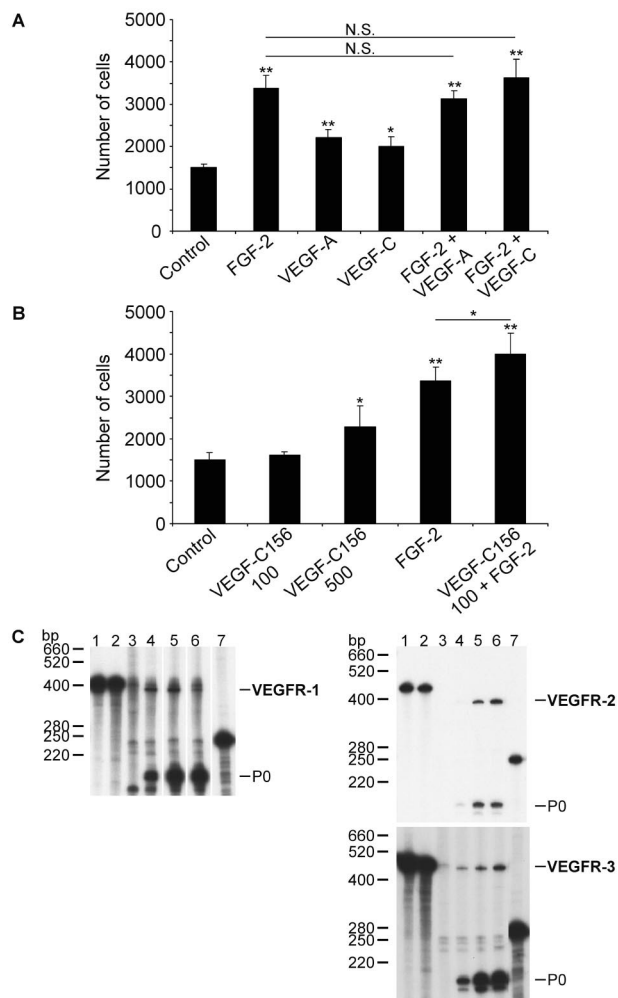
Figure 5. Cytokine-induced hTERT-HDLEC proliferation. hTERT-HDLEC were seeded into 12-well plates at 10^4 cells per well and starved for 24 hours in EGM-2MV medium, from which VEGF-A and FGF-2 had been omitted. **A:** The cells were left untreated or were treated with FGF-2 (10 ng/ml), VEGF-A (100 ng/ml), or VEGF-C (100 ng/ml) alone or in combination with FGF-2 (10 ng/ml). **B:** The cells were treated with FGF-2, VEGF-C156 (100 ng/ml or 500 ng/ml), or VEGF-C156 (100 ng/ml) in combination with FGF-2 (10 ng/ml). Medium and growth factors were renewed every 2 days. After 6 days, the cells were harvested and counted. Values are the mean from four independent experiments ± SEM. Mean values were compared using Student's unpaired *t*-test (*, $P < 0.05$; **, $P < 0.005$). **C:** RNase protection analysis for VEGFR-1, -2, and -3 mRNA in HMEC-1 and hTERT-HDLEC. Purified ^{32}P-labeled cRNA probes (**lane 1**) were hybridized to hybridization mix (**lane 2**), yeast tRNA (**lane 3**), 10 μg of total RNA from human liver (**lane 4**), HMEC-1 (**lane 5**) or hTERT-HDLEC (**lane 6**). As an internal control, an equal amount of the P0 cRNA probe was included in all samples or loaded onto the gel (**lane 7**). One of two experiments with similar results is shown.

VEGF receptors and were confirmed by the detection of VEGFR-1 (Flt-1), VEGFR-2 (Flk-1), and VEGFR-3 (Flt-4) transcripts by RNase protection assay (Figure 5C).

In Vitro *Tubulogenesis Assays*

In agreement with previous reports,[17,18] cytokine treatment induced HMEC-1 and hTERT-HDLEC lines to form tube-like structures. With regard to hTERT-HDLEC, the same results were obtained when the cells were treated in complete or FGF-2- and VEGF-A-depleted EGM-2MV. Thus, the concentration of FGF-2 and VEGF-A contained
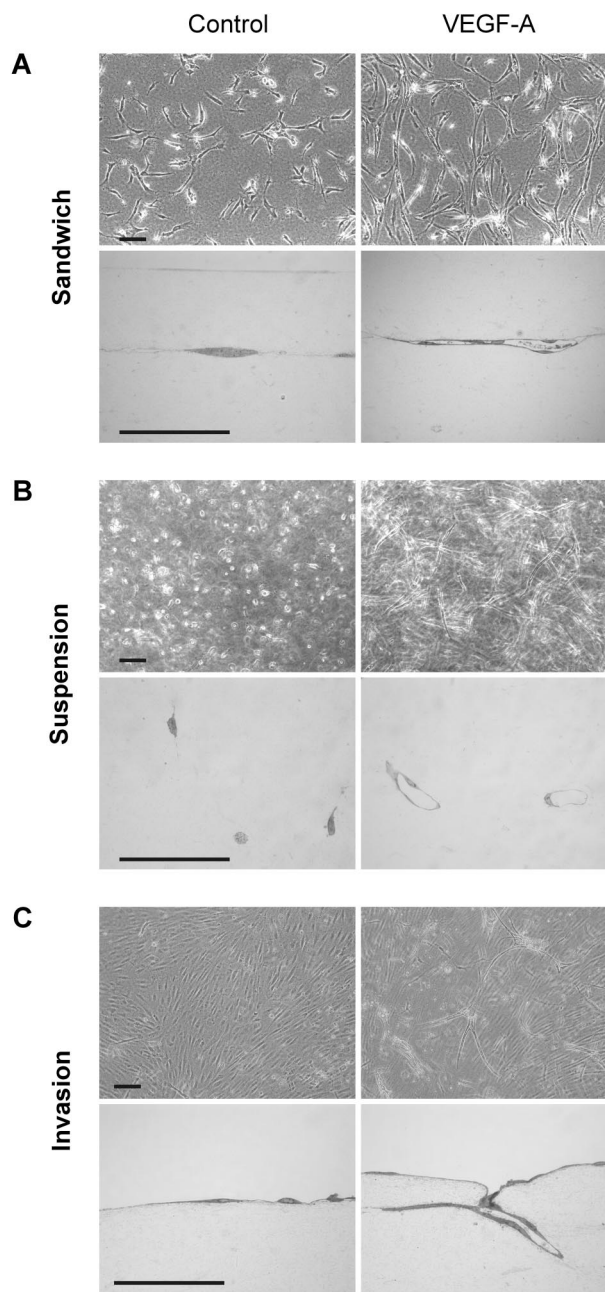

Figure 6. Tube formation by hTERT-HDLEC and HMEC-1 in collagen gels. Cells were either sandwiched between two collagen gels (**A**), seeded in suspension as isolated single cells in a collagen gel (**B**), or seeded on the surface a collagen gel (**C**). Cells were left untreated (**left**) or treated with the indicated cytokines (**right**). After 5 to 7 days of culture, cells were photographed, and semi-thin sections (**bottom**) were analyzed to assess whether tube formation had occurred. **Bar**, 100 μm. Endothelial cell responses to cytokines are summarized in Table 2.

in commercial medium appears to be insufficient to induce major morphological modifications. Cytokine stimulation of both cell lines when "sandwiched" between two three-dimensional collagen layers (Figure 6A) or in suspension as single isolated cells in a collagen gel (Figure 6B), promoted cell survival and increased the formation of tube-like structures. Tube formation was confirmed by semi-thin and thin section. Electron microscopic analysis revealed that hTERT-HDLECs delimited a patent lumen
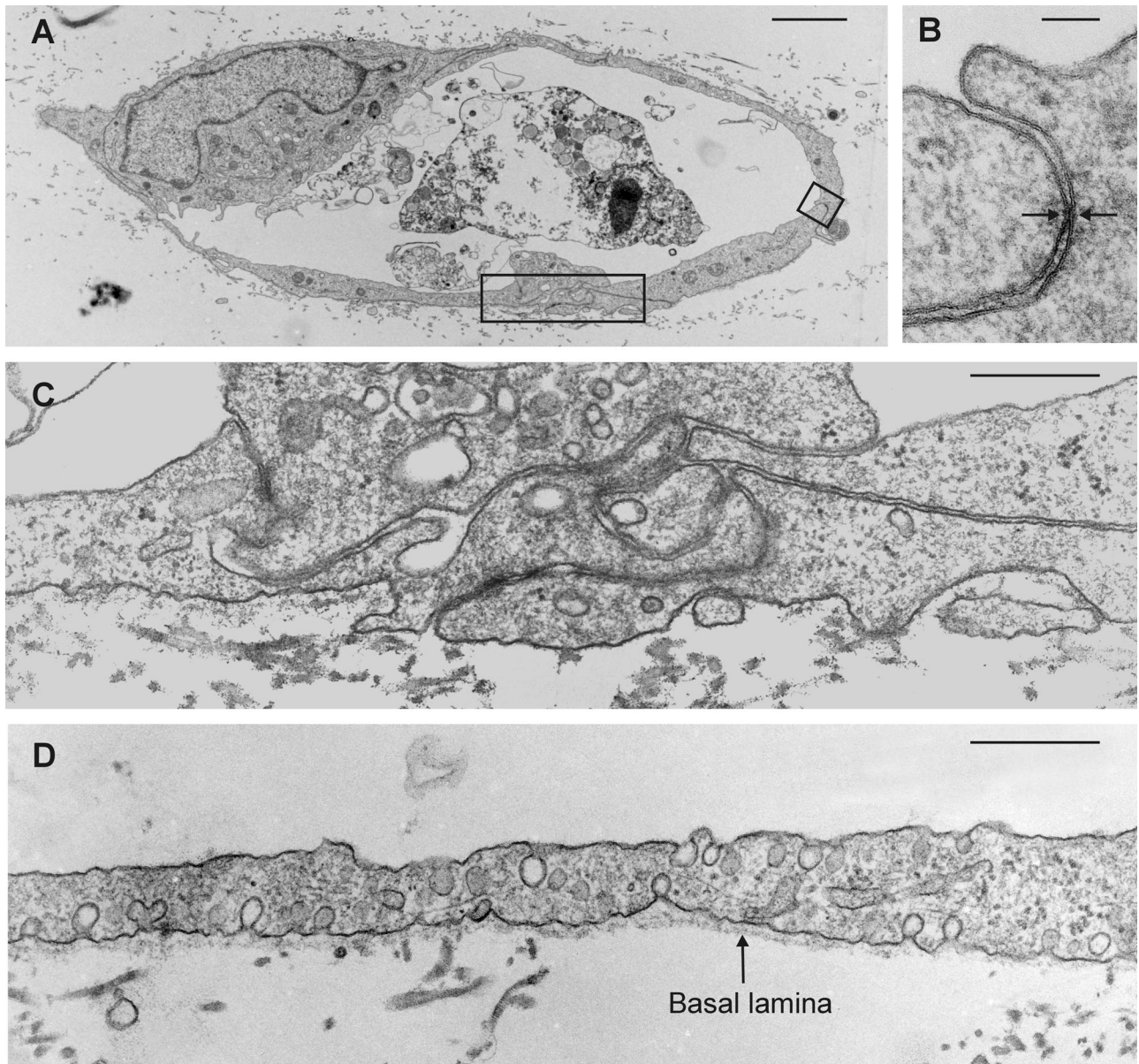
Figure 7. Ultrastructural analysis of endothelial tubes. **A–C:** hTERT-HDLECs were sandwiched between two collagen layers and grown for 7 days in the presence of 100 ng/ml VEGF-A. **A:** Cells delimit a patent lumen containing cell debris. **Bar**, 2 μm. **B:** Higher magnification of the area outlined in **A**. A tight junction (**arrows**) is recognizable as a focal pentalaminar fusion of the plasma membranes of two adjacent endothelial cells. Bar, 0.1 μm. **C:** Higher magnification of the area outlined in **A**. A long overlapping interdigitated junction, typically found in lymphatics, is clearly visible. **Bar**, 0.5 μm. **D:** hTERT-HDLECs were suspended in a three-dimensional collagen gel and grown for 7 days in the presence of 100 ng/ml VEGF-A. The abluminal plasma membrane of an endothelial cell delimiting a tube-like structure is underlined by a basal lamina showing focal discontinuities. **Bar**, 0.5 μm.

containing cell debris (Figure 7A) and formed tight junctions at sites of intercellular contact (Figure 7B). Furthermore, long overlapping interdigitating junctions were observed (Figure 7C) as well as focal discontinuities in the basal lamina underlying the abluminal plasma membrane (Figure 7D). These are two characteristic morphological features of lymphatics.[41] Results obtained with the different cytokines are summarized in Table 2. When grown on the surface of three-dimensional collagen gels, limited superficial spontaneous invasion by isolated HMEC-1 was observed, which was slightly increased by cytokine treatment. Similarly, minimal superficial spontaneous invasion of isolated hTERT-HDLEC was observed. In con-

trast, cytokine-stimulated hTERT-HDLEC reproducibly and extensively invaded the underlying collagen gels, within which they formed clearly distinguishable tube-like structures (Figure 6C, Figure 7A, and Table 2). VEGF-A induced a maximal response after 3 days and tube regression began after 7 days. FGF-2 and VEGF-C increased invasion and also induced tube formation, albeit to a lesser degree than VEGF-A. VEGF-A and -C both synergized with FGF-2 (Figure 8A). In contrast, VEGF-C156 never induced invasion or tube formation in any of the three collagen models, even after 2 weeks of culture at 100 ng/ml and 1 week at 500 ng/ml. However, as seen with proliferation assays, VEGF-C156 at a concentration

Table 2. Effect of Angiogenic and/or Lymphangiogenic Cytokines on HMEC-1 and hTERT-HDLEC in Three-Dimensional Collagen Gel Tubulogenesis Assays

hTERT-HDLEC (treatment ng/ml)	Sandwich	Suspension	Invasion
Control	+	+	−
FGF-2 (10)	++	++	+
VEGF-A (100)	+++	+++	+++
VEGF-C (100)	++	++	+
VEGF-C156 (100 or 500)	+	+	−
FGF + VEGF-A (10 + 100)	n.d.	++++	++++
FGF + VEGF-C (10 + 100)	n.d.	n.d.	+++
FGF + VEGF-C156 (10 + 100)	n.d.	n.d.	+

HMEC-1	Sandwich	Suspension	Invasion
Control	−	−	−
FGF-2 (10)	n.d.	n.d.	−
VEGF-A (100)	+	+	−
VEGF-C (100)	+	+	−
VEGF-C156 (100 or 500)	n.d.	n.d.	n.d.
FGF + VEGF-A (10 + 100)	n.d.	n.d.	n.d.
FGF + VEGF-C (10 + 100)	n.d.	n.d.	n.d.
FGF + VEGF-C156 (10 + 100)	n.d.	n.d.	n.d.
PMA (10)	n.d.	n.d.	−

+, tube formation; −, absence of tube formation; n.d., not determined.

of 100 ng/ml slightly but significantly potentiated the effect of FGF-2 (Figure 8C).

Since VEGF-C activates both VEGFR-2 and -3, and VEGF-C156 has no effect by itself, VEGF-C-driven invasion is likely to be due to activation of VEGFR-2. To investigate this hypothesis, we stimulated hTERT-HDLEC with VEGF-C alone or in combination with neutralizing antibodies to VEGFR-2 (p1C11) or VEGFR-3 (p3C5); an isotype-matched IgG was used as a control (Figure 8C). VEGFR-2 blockade reduced invasion to untreated levels, whereas blockade of VEGFR-3 alone had no effect. These results demonstrate that VEGF-C drives hTERT-HDLEC invasion via the activation of VEGFR-2. Interestingly, similar invasion profiles were observed with cells at passages 36–38 (data not shown), albeit to a lesser degree than in Figure 8A and Figure 8B, demonstrating that the cells still respond to angiogenic or lymphangiogenic cytokines even at high passage numbers.

Molecular Characterization of hTERT-HDLEC

During invasion and tube formation, endothelial cells degrade their basement membrane, and proliferate and migrate into the surrounding collagen-rich matrix. This complex process requires the coordinated activities of many different molecules, including VEGFRs, integrins, and proteinases. RNase protection assays confirmed expression of all three VEGFRs in both HMEC-1 and hTERT-HDLEC (Figure 5C). VEGFR-2 expression on the surface of the two lines was confirmed by FACS analysis (data not shown). Metalloproteinase and integrin expression were analyzed using SuperArray filters (see supplementary data at http://ajp.amjpathol.org) and several molecules implicated in the invasive process were subsequently validated by RT-PCR. hTERT-HDLEC express mRNA for integrin subunits responsible for interactions with type-I collagen, namely $\alpha1\beta1$ and $\alpha2\beta1$, the fibronectin receptor $\alpha5\beta1$ and the laminin receptor $\alpha6\beta1$ (Figure 9A). Interestingly, we detected expression of the transcript coding for the $\alpha9$ integrin subunit which has previously been reported to be restricted to lymphatic endothelium.[6] Furthermore, we could not detect expression of VEGF-C, which previous reports have indicated is restricted to BECs. hTERT-HDLEC also expressed $\alpha v\beta3$ and $\alpha v\beta5$ integrins, as assessed by FACS analysis using LM609 and P1F6 antibodies (data not shown). These integrins have previously been reported to mediate angiogenesis.[42,43]

We next assessed whether hTERT-HDLEC express proteolytic enzymes implicated in angiogenesis/lymphangiogenesis.[44,45] With regard to MMP expression, we detected mRNA for MMP-2, MMP-9, and MMP-14 by RT-PCR (Figure 9,A). While MMP-2 activity was also detected in conditioned media by gelatin zymography, MMP-9 was not detected and may be expressed at levels below the detection limit (Figure 9B). Zymography and reverse zymography demonstrated hTERT-HDLEC express urokinase and tissue-type plasminogen-activators (uPA and tPA) as well as PA inhibitor-1 (PAI-1) (Figure 9C). The identity of uPA was confirmed by its inhibition by amiloride (data not shown). As previously described,[45] VEGF-A, and to a lesser extent VEGF-C, up-regulate both uPA and tPA activities with no effect on PAI-1. In contrast, only VEGF-A induced a small increase in tPA activity in HMEC-1 with no measurable effect on uPA (one of three experiments with similar results is shown).

Discussion

The major objectives of this study were to generate endothelial cell lines with an extended lifespan by ectopic expression of hTERT in HDMVEC and to study their molecular profile and their ability to respond to angiogenic and/or lymphangiogenic cytokines. We report the gener-
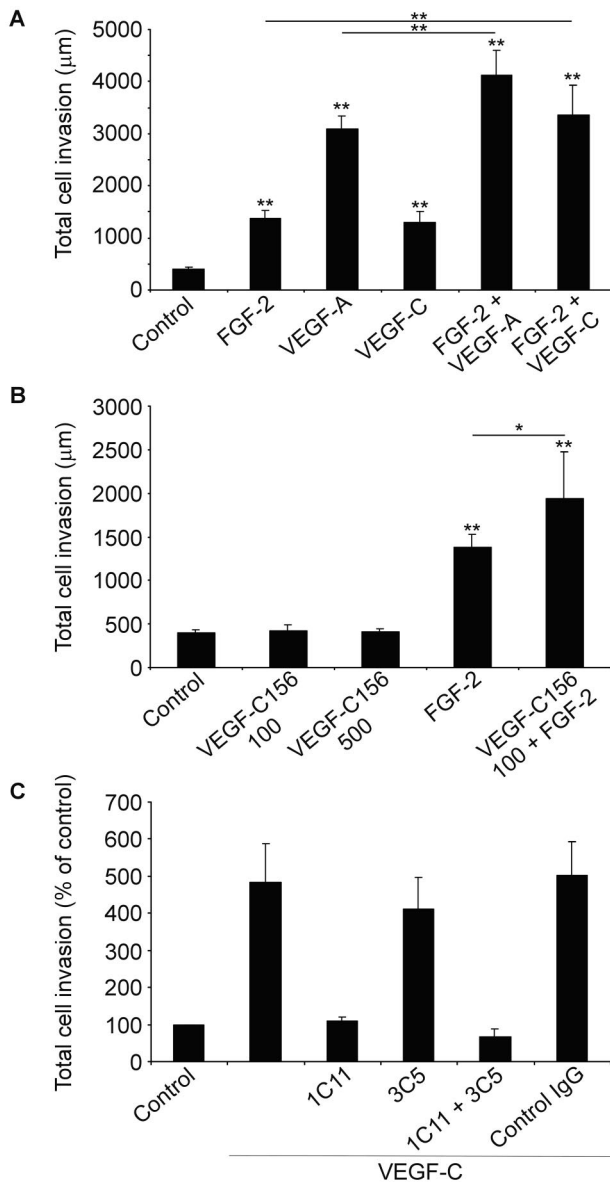
A

B

C

VEGF-C

Figure 8. Quantitation of cytokine-induced hTERT-HDLEC invasion of collagen gels. Confluent monolayers of hTERT-HDLEC (between passage 18 and 25) on three-dimensional collagen gels were left untreated or were treated (**A**) with FGF-2 (10 ng/ml), VEGF-A (100 ng/ml), VEGF-C (100 ng/ml) alone or in combination with FGF-2 (10 ng/ml) or (**B**) with FGF-2, VEGF-C156 (100 ng/ml or 500 ng/ml), or VEGF-C156 (100 ng/ml) in combination with FGF-2 (10 ng/ml). Cells were cultured for 7 days; media and growth factors were renewed every 2 days. Data are shown as mean values, at least three experiments per condition (at least three fields per condition per experiment) ± SEM. Mean values were compared using Student's unpaired *t*-test (*, $P < 0.5$; **, $P < 0.005$). **C:** hTERT-HDLEC were left untreated or were treated with VEGF-C (100 ng/ml) alone or together with 20 μg/ml p1C11 (anti-VEGFR-2), 20 μg/ml p3C5 (anti-VEGFR-3) or both antibodies in combination; anti-KLH 20 μg/ml was used as an isotype control. Data are shown as mean percentage of total cell invasion with respect to untreated cells (100%), ± SEM.

ation of hTERT-immortalized human dermal lymphatic endothelial cells (hTERT-HDLEC). The cells have been grown to 44 passages (at least a fourfold increase over parental cells) and maintain a phenotype that is similar to the primary cells from which they were derived. Senescence was observed at higher passage numbers, which may explain our inability to clone these cells.

Detailed phenotypic and molecular analysis of hTERT-HDLEC revealed several interesting features. First, they retain typical EC cobblestone morphology, and in agreement with other reports,[18] hTERT expression did not induce EC instability or a transformed phenotype. Also, hTERT-HDLEC exhibit contact inhibition, ie, cell density-induced proliferation arrest, and do not form colonies when cultured in soft agar. Second, the cells stably express typical EC markers including PECAM-1 and VE-cadherin. However, they do not express CD34 protein, which is in accord with the currently accepted notion that CD34 is strongly expressed by BEC but not by LEC.

In fact, several reports have demonstrated that CD34 is specifically expressed by blood vascular but not by lymphatic endothelium in neonatal foreskins[8,9] and rodent skin.[2] However, a recent report[7] showed co-expression of CD34 and podoplanin in a subpopulation of endothelial cells in adult human skin. These discrepancies might be related to the age of the donor and/or the donor site (neonatal foreskins *versus* breast reduction or abdominoplasty). Third, hTERT-HDLEC form tube-like structures in collagen gels when stimulated with the angiogenic and/or lymphangiogenic cytokines FGF-2, VEGF-A, and VEGF-C. Fourth, hTERT-HDLEC express many molecules that have been shown to mediate angiogenesis and/or lymphangiogenesis[42,43] such as VEGFRs, integrins, MMPs, and members of the PA family of proteases. Finally, both hTERT-HDLEC and the well-characterized HMEC-1 line express all of currently recognized lymphatic markers analyzed (ie, Prox-1, podoplanin, LYVE-1, VEGFR-3, and integrin α9). The lack of CD34 expression in hTERT-HDLEC supports our conclusion that they are a pure population of lymphatic endothelial cells. In contrast, the fact that HMEC-1 express lymphatic markers as well as CD34 and PAL-E[10] suggests that these cells may be hybrid BEC-LEC. Whether this difference between hTERT-HDLEC and HMEC-1 is due to the different immortalization techniques (hTERT *versus* SV40) is not known.

Expression of CD44 by hTERT-HDLEC may appear to contradict their definition as lymphatic, since it appears that *in vivo*, expression is restricted to blood vessels. However, recent microarray reports have indicated that cultured LECs express CD44[8,9] (confirmed by real-time PCR in the latter report). Griffioen et al[46] and have suggested that since CD44 is not expressed by HUVECs *in vivo*, expression *in vitro* is artifactual. In keeping with this idea is the observation that CD44 expression is increased with passage number and by FGF-2 or VEGF-A in HUVECs and microvascular endothelial cells isolated from the dermis. These observations might be relevant to hTERT-HDLEC.

hTERT was chosen for immortalization since spontaneous transformation or ectopic viral oncogene expression[10–13] had previously been shown to generate cells which lose many EC markers and functional characteristics. Furthermore, recent reports have demonstrated successful immortalization of human microvascular EC using hTERT. These cells formed tube-like structures either in three-dimensional collagen or fibrin gels or on matrigel,[18] and behaved as early passage primary EC *in vitro*. Inter-
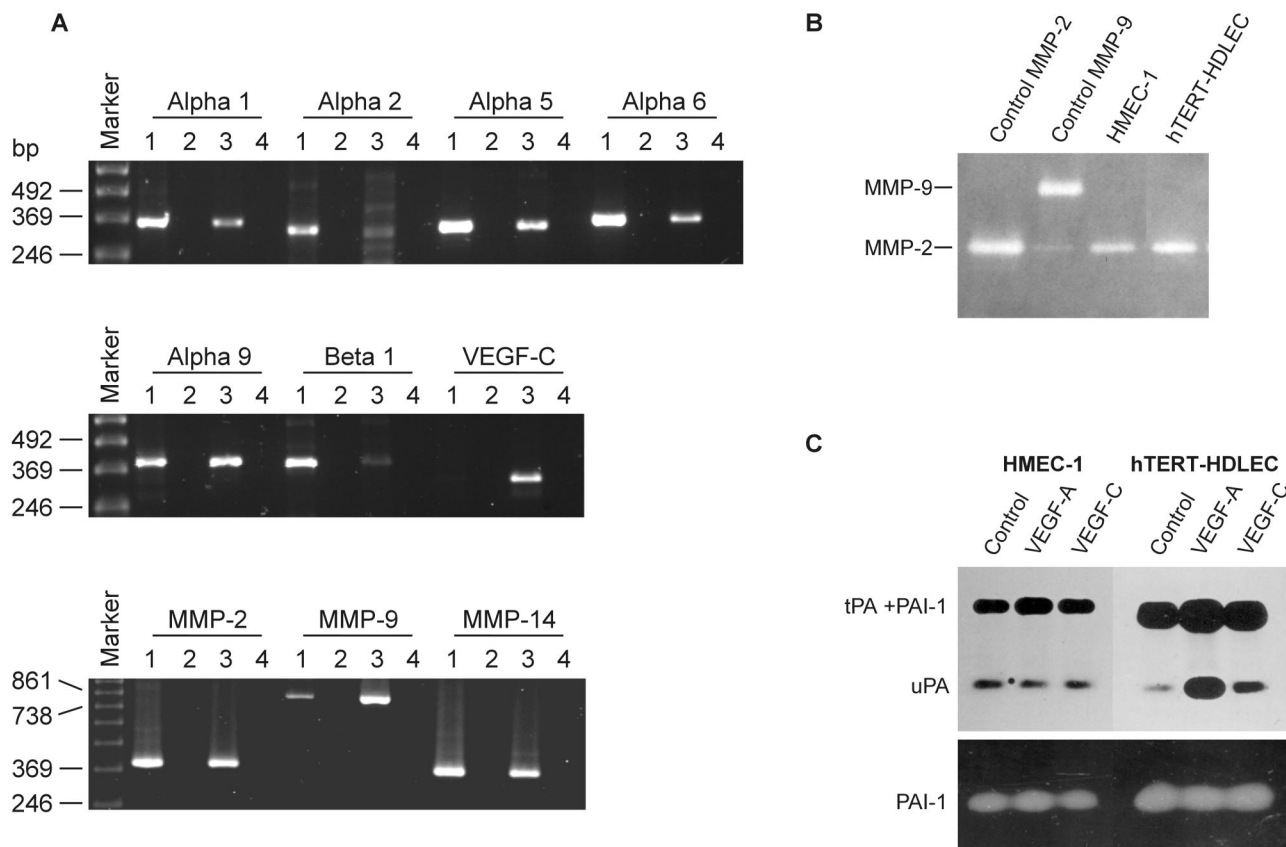
Figure 9. Molecular characterization of h-TERT-HDMVEC. **A:** hTERT-HDLEC integrin and MMP expression were assessed by RT-PCR. The total cellular RNA, isolated from confluent hTERT-HDLEC monolayers (at passage 26) cultured in EGM-2MV Bullet kit medium, was reverse-transcribed and amplified by PCR. The specific conditions and primers pairs are indicated in Table 1. Equal amounts of PCR products were analyzed on a 2% agarose gel. Marker (123-bp DNA ladder), hTERT-HDLEC + RT (**lane 1**), hTERT-HDLEC − RT (**lane 2**), human liver + RT (**lane 3**), H$_2$O (**lane 4**). The expression of $\alpha v \beta 3$ and $\alpha v \beta 5$ integrin complexes was confirmed by FACS analysis (data not shown). **B** and **C:** Zymographic analysis of HMEC-1 and hTERT-HDLEC. Cells were cultured in serum-free media lacking FGF-2 or VEGF-A. Confluent monolayers in 35-mm tissue culture dishes were left untreated (control) or exposed to 100 ng/ml VEGF-A or 100 ng/ml VEGF-C for 15 hours. Supernatants were analyzed by gelatin (**B**) or casein (**C**) zymography. uPA activity was confirmed by its inhibition with amiloride (data not shown).

estingly, hTERT-immortalized human microvascular endothelial cells have been reported to form functional capillaries *in vivo*.[47] However, blood vascular *versus* lymphatic phenotypic characterization was not assessed in these cells.

Comparative experiments were carried out on endothelial cells obtained from PromoCell and Clonetics in their respective media, and also by switching media. Once the cells are established in culture (ie, on receipt from the manufacturer), we have observed that BEC do not acquire the capacity to express lymphatic markers with increasing passage. The media do not drastically alter the growth rate of one population with respect to the other, as the proportion of both populations remains constant with increasing passage number (PromoCell cells) independent of the culture medium. Our hTERT-HDLEC are a pure population of LEC because they were derived from EC that were already a relatively pure LEC population. This fact, taken together with our own (unpublished) observations on primary EC isolation, suggests that some of the currently used isolation procedures give rise to a greater proportion of LEC than BEC (also reported by Kriehuber et al[7]), and that the choice of medium may be a critical determinant in early passages (below passage 5).

Both HMEC-1 and hTERT-HDLEC exhibited interesting functional properties. Both responded to angiogenic cytokines, such as FGF-2 and VEGF-A, when sandwiched between two collagen gels or grown in suspension in a three-dimensional collagen matrix. However, we were unable to induce HMEC-1 invasion or tube formation when these cells were seeded on the surface of a collagen gel, irrespective of the angiogenic stimulus (which also included phorbol myristate acetate (PMA)). In contrast, hTERT-HDLEC reproducibly formed measurable tube-like structures when seeded on the top of collagen gels. The robust induction of hTERT-HDLEC invasion by VEGF-A and the synergistic effect of FGF-2 with VEGF-A and VEGF-C is in line with other reports performed on bovine primary EC.[33,48] hTERT-HDLEC formed tube-like structures in the sandwich assay or when grown in suspension in collagen gels when stimulated with the lymphangiogenic cytokine VEGF-C. Our data with VEGFR-2-neutralizing antibodies and with VEGF-C156 suggest that the effect of VEGF-C on invasion was mediated by VEGFR-2. VEGFR-3 activation induced proliferation but not tube formation in the three different models. However, specific stimulation of VEGFR-3 potentiated FGF-2-induced proliferation and invasion. Taken together, these

findings suggest that VEGFR-3 activation is necessary for proliferation, and also modulates signal transduction pathways induced by other cytokines. More specifically, VEGFR-3 signaling may be linked to the FGF-2 pathway (also reported by Kubo et al[49]).

Using recently identified markers of lymphatic endothelium, we have characterized two immortalized human microvascular endothelial cell lines namely hTERT-HDLEC (described herein) and HMEC-1.[10] From their molecular expression profiles we conclude that hTERT-HDLEC are derived from lymphatic endothelium and represent an immortalized LEC line. HMEC-1 may represent a hybrid BEC-LEC line. hTERT-HDLEC will be useful for the further investigation of lymphatic endothelial physiology and pathology, including lymphangiogenesis.

Acknowledgments

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