A Xeno-Free Culture System for Human Microvascular Endothelial Cells from Various Sources -Toward Clinical Applications

Sharon Daniliuc, Mira Genser-Nir, Marina Tevrovsky, Roni Hazan Brill, Yuliya-Yael Miropolski, David Fiorentini

Biological Industries, Beit Haemek, Israel

BI Biological Industries *Culture of Excellence*

Scan Me

Abstract

Endothelial cells (EC) form the inner lining of a blood vessel, termed endothelium. They provide a selective permeability barrier and an anticoagulant barrier between the vessel wall and blood. Their unique location in the interface between the blood and surrounding tissue allows EC to detect local changes and blood-borne signals, and react by producing a wide range of vasoactive substances that regulate vascular tone, cellular adhesion, thromboresistance, smooth muscle cell proliferation, and vessel wall inflammation. Additionally, EC are pivotal in cancer research, angiogenesis, and vasculogenesis. Consequently, EC have become a key element in tissue engineering and cell therapy for improving graft implantation. Human microvascular EC (hMVEC) are the majority of the body's endothelium, and differ in morphology and properties according to the tissue containing the capillaries. Currently, there is no efficient xeno-free (XF) medium for the growth and expansion of hMVEC. Moreover, common culture media, as well as auxiliary solutions (for attachment, dissociation, and cryopreservation), are typically supplemented with serum or other xenogenic compounds, e.g., Bovian Brain Extract (BBE). A defined XF culture system optimized for hMVEC expansion would greatly facilitate development of robust, clinically accepted culture process for quality-assured cells. The present study evaluated a novel XF culture system comprising microvascular EC XF medium (EndoGo XF™) and auxiliary solutions for attachment, dissociation, and cryopreservation of hMVEC. The system was evaluated for long-term culturing of EC from various sources under XF culture conditions. Results show that the XF culture system for hMVEC efficiently supports optimal expansion of EC from various sources, while maintaining EC features: typical cobblestone-like cell morphology, phenotypic surface marker profile, and angiogenic differentiation capacity.

Materials and Methods

Cells
HMEC from a variety of sources: dermal (ATCC, Promocell), dermal lymph, dermal blood (Lonza), pulmonary and bladder (Lonza) were used in this study. Adipose-derived cells were freshly isolated.

Culture system
HMEC were cultured in a XF expansion medium (EndoGo XF™, BI) supplemented with 2% off the clot (OTC) human AB serum with or without human fibronectin pre-coating (hFN, BI) or commercial FBS-containing media. For expansion, cells were seeded at a concentration of 5000-6000 viable cells/cm^2 and harvested using recombinant Trypsin-EDTA solution (BI) and neutralized with Soybean Trypsin Inhibitor (SBTI, BI).

HAMEC Isolation:
For initial isolation (P0-P1), adipose tissue-derived cells (post-collagenase digestion) were seeded in EndoGo XF™ supplemented with OTC human AB serum. For enrichment and expansion (P2-P3), HAMEC were cultured as described in the culture system.

Real-time PCR:
HMEC were expanded for several passages in EndoGo XF™, followed by total RNA extraction (ReliaPrep™ RNA Cell Miniprep System, Promega), and total RNA was reverse transcribed (EZ-First Strand cDNA, BI). Quantitative real-time PCR was performed using TaqMan® Universal PCR Master Mix (Applied Biosystems), gene-specific TaqMan PCR probes and FAM primers. Each sample was tested in triplicates.

Immunofluorescence staining:
HMEC were expanded for several passages in EndoGo XF™, fixed and stained for the classical endothelial cells markers: CD31 (PECAM) (R&D Systems) and Von-Wilibrandt factor (vWF) (Santa Cruz) and counterstained with DAPI (MP Bioscience).

Immunophenotyping:
HMEC were expanded for several passages in EndoGo XF™, harvested and labeled with antibodies against CD31, CD144, and CD90, and fixed and analyzed by FACS .

Tube formation assay:
HMEC were expanded for several passages in EndoGo XF™, seeded (50k/well of 48 well plate) on BD Matrigel™ in EndoGo XF™ supplemented with 5% OTC human AB serum. Quality of tubes was assessed after 18-24h.

Abbreviations

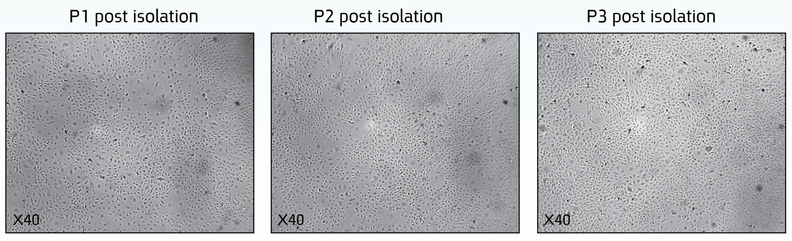
EC= Endothelial Cells
FBS= Fetal Bovine Serum
hFN= Human Fibronectin
hMEC= Human Microvascular Endothelial Cells
HAMEC= Human Adipose Microvascular Endothelial Cells
HBdMEC= Human Bladder Microvascular Endothelial Cells
HDBEC= Human Dermal Blood Microvascular Endothelial Cells
HDLEC= Human Dermal Lymph Microvascular **Endothelial Cells**
HDMEC= Human Dermal Microvascular Endothelial Cells
HPMEC= Human Pulmonary Microvascular Endothelial Cells
OTC= Off the Clot
TFA= Tube Formation Assay
XF= Xeno Free

Results

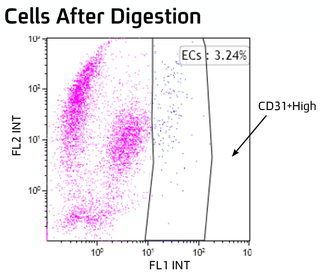
Isolation and Evaluation of HAMEC Using EndoGo XF™

Figure 1: Isolation of Microvascular EC from Adipose Tissue

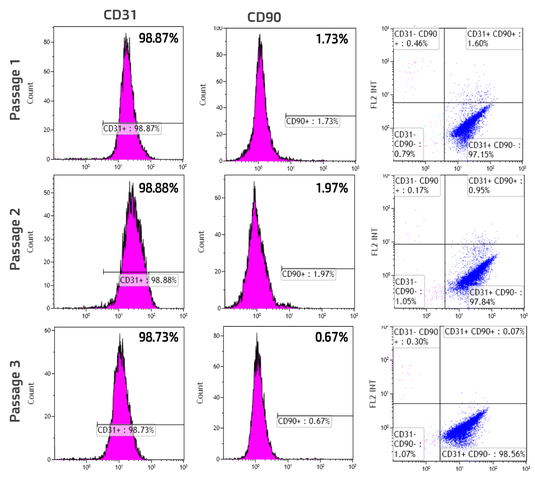
A. Morphology



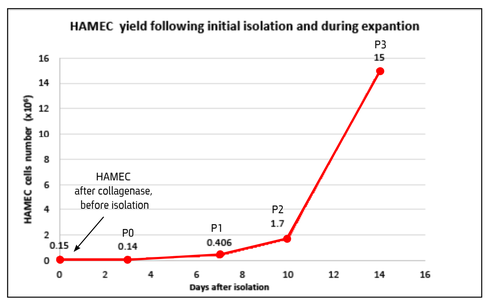
B. FACS Analysis of Total Cells After Digestion



C. FACS Analysis of HAMEC Post Purification



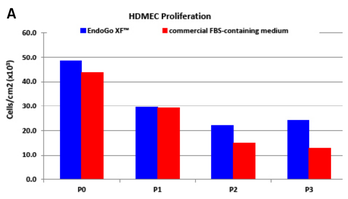
D. Expansion



Adipose tissue-derived cells were seeded in EndoGo XF™ for initial isolation (P0-P1), enrichment, and expansion (P2-P3). **(A)** Representative images of culture confluence and morphology at P1-P3 post-isolation. **(B)** Immunophenotyping results of total adipose-derived cells, and the HAMEC sub-population. **(C)** Immunophenotyping results of HAMEC during purification and expansion. **(D)** Cell counts during isolation (P0-P1), enrichment, and expansion (P2-3).
EndoGo XF™ supports successful isolation of pure microvascular EC from adipose tissue while maintaining high proliferation potential, typical morphology, and EC marker expression.

Expansion of hMEC Using EndoGo XF™

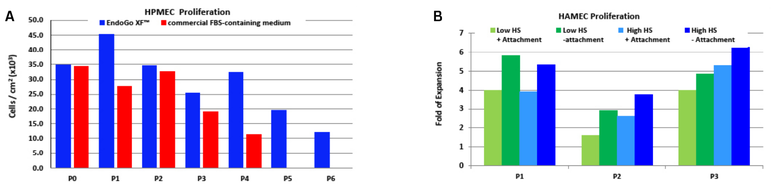
Figure 2: Proliferation and PDL of HDMEC



B	P0	P1	P2	P3	PDL
EndoGo XF™	2.07	2.56	2.15	2.26	9.04
Commercial FBS-containing medium	1.42	2.07	1.59	1.35	6.43

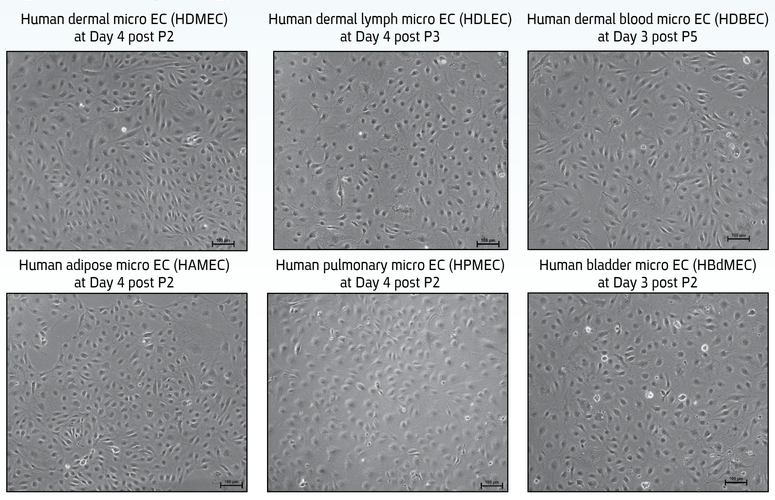
Cell counts and population doubling level of HDMEC expanded for several passages in EndoGo XF™ in comparison to commercial FBS-containing medium. Viable cells were counted using ChemoMetec Viability and Cell Count Assay.
(A) HDMEC cell counts (B) HDMEC Population Doubling Level (PDL).
Superior cell number and PDL of hDMEC in EndoGo XF™.

Figure 3: Proliferation of HPMEC and HAMEC



Cell counts of **(A)** HPMEC expanded for long-term culture using EndoGo XF™ in comparison to commercial FBS-containing medium, **(B)** HAMEC culture in EndoGo XF™ supplemented with different OTC human AB serum with and w/o pre-coating. Viable cells were counted using ChemoMetec Viability and Cell Count Assay.
EndoGo XF™ promotes higher cell number and longer cultivation. Elevation of the human serum concentration increases total cell yield. *HPMEC did not survive P5 in the FBS-containing medium.

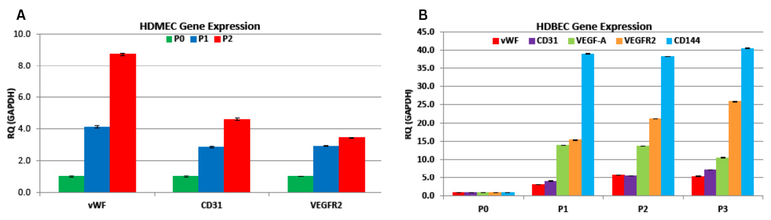
Figure 4: Morphology



HMVEC derived from a variety of sources were seeded (5-6k/cm²) on hFN- coated dishes and expanded in EndoGo XF™ for several sequential passages with equal seeding density at each passage. Representative images were taken at the indicated time points (x100).
EndoGo XF™ promotes proliferation of HMVEC from a variety of sources while maintaining classical EC morphology.

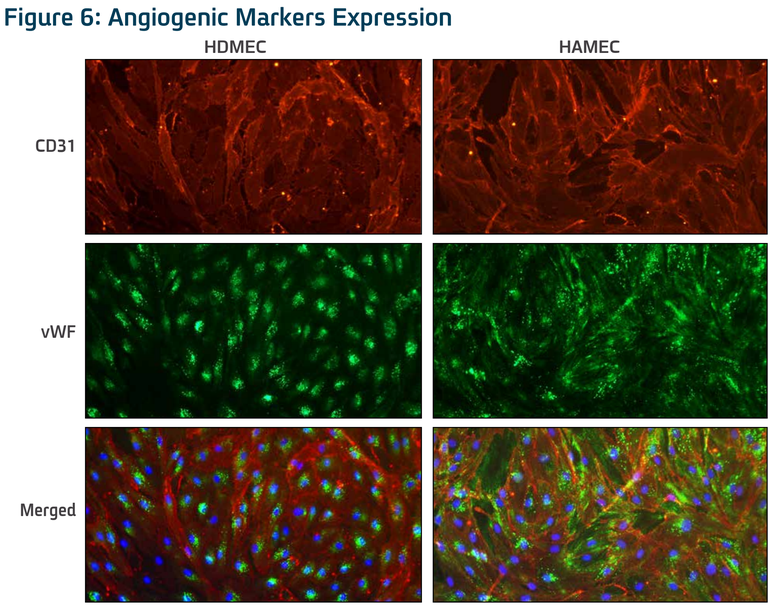
Characterization of hMEC Using EndoGo XF™

Figure 5: Gene Expression



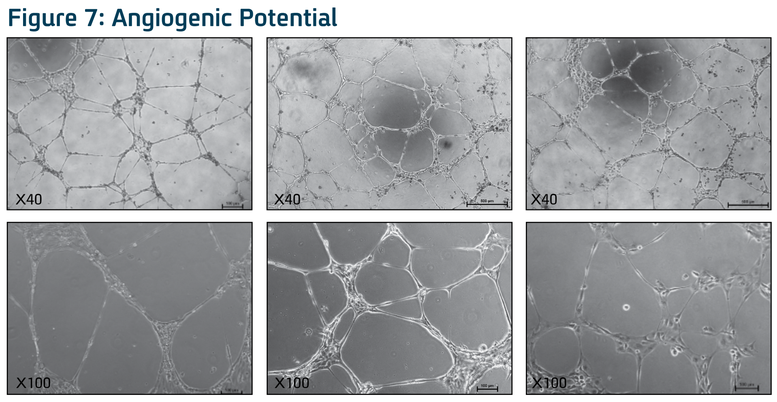
(A) Real-time results of HDMEC post P1 and P2 in EndoGo XF™ analyzed in comparison to P0 (original cell pellet w/o proliferation). **(B)** Real-time results of HDBEC post P1, P2, and P3 in EndoGo XF™ analyzed in comparison to P0 (original cell pellet w/o proliferation).
Microvascular EC cultured in EndoGo XF™ maintains a similar gene expression profile pattern of EC during passages.

Figure 6: Angiogenic Markers Expression



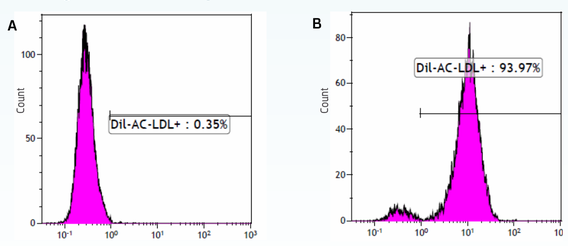
Immunofluorescence staining of hMVEC derived from dermal or adipose tissue after expansion in EndoGo XF™. Cells from P3 were fixed and stained for the classical endothelial cell markers: CD31 (PECAM) (red) and Von-Wilibrant factor (vWF) (green), and counterstained with DAPI (blue).
Microvascular endothelial cells expanded in EndoGo XF™ medium express high angiogenic markers expression

Figure 7: Angiogenic Potential



Tube Formation Assay (TFA) of cells after cultivation in EndoGo XF™. Cells were seeded on Matrigel™ in EndoGo XF™ (50k/well of 48wp). Representative image of: **(A)** HDMEC 18h, **(B)** HDBEC from P2 after 18h, **(C)** HAMEC from P2 after 18h.
Endothelial cells expanded in EndoGo XF™ preserved angiogenic potential to form capillary-like tubes.

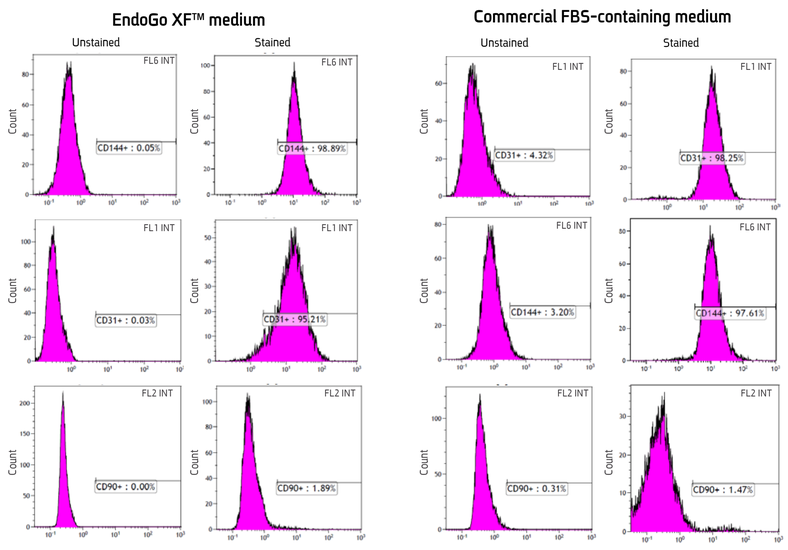
Figure 8: Acetylated LDL Uptake



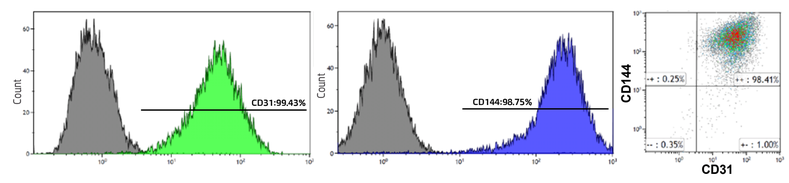
HAMEC expanded for 3 passages in EndoGo XF™ were exposed to Dil-AC-LDL for 2h (10ug/ml). Uptake results quantified by FACS (FL2).
Microvascular endothelial cells expanded in EndoGo XF™ preserved the ability to uptake acetylated LDL.

Figure 9: Immunophenotyping

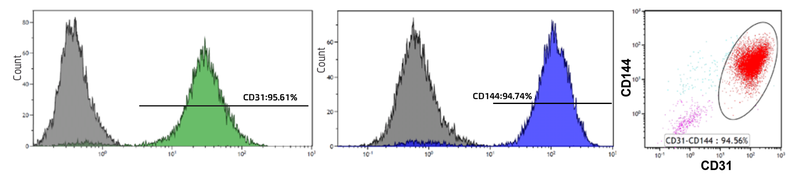
A. HDMEC



B. HDLEC



C. HAMEC



		CD144 (%)	CD31 (%)	CD90 (%)
HDMEC	EndoGo XF™	98.89	95.21	1.89
	Commercial 5% FBS-containing medium	97.61	98.25	1.47
HAMEC	EndoGo XF™	94.66	95.53	5.44
HDLEC	EndoGo XF™	98.75	99.43	--

Microvascular EC were expanded in EndoGo XF™ or 5% FBS-containing medium for several sequential passages. Cells were harvested and labeled with antibodies against CD31, CD144 and CD90, and analyzed by FACS. **(A)** HDMEC post 2 passages **(B)** HDLEC post 6 passages , **(C)** HAMEC post 3 passages.
Microvascular EC from a various tissue sources maintain a classical profile of EC markers (>95%) after expansion in EndoGo XF™.

Summary

- Microvascular EC from adipose tissue can be efficiently isolated using EndoGo XF™. Isolated HAMEC maintain high proliferation rate, typical morphology, and EC markers utilizing EndoGo XF™.
- Various sources of microvascular EC may be efficiently expanded using EndoGo XF™ supplemented with human AB serum.
- The highest proliferation rate of HMEC from a variety of sources was achieved using EndoGo XF™ in comparison to commercially available FBS-containing medium.
- HMEC expanded in EndoGo XF™ retain EC features: morphology, angiogenic gene expression, immunophenotyping, angiogenic potencial, and LDL uptake.