Cell Therapy Compliant Xeno-Free Culture System for Human Endothelial Cells

Sharon Daniliuc[1], Mira Genser-Nir[1], Yuliya-Yael Miropolski[1], Maria Sharovetsky[1], Marina Tevrovsky[1], Roni Hazan Brill[1], David Fiorentini[1], Ayokunle Ogunye[2], Ian McNiece[2], Joshua Kellner[2]

1 Biological Industries (BI), Beit Haemek, Israel
2 Department of Stem Cell Transplantation and Cellular Therapy, The University of Texas, MD Anderson Cancer Center, Houston, TX.

Abstract

Human Endothelial cells (hEC) form a single cell layer which lines the interior surface of blood and lymphatic vessels, termed endothelium. Their use in cellular therapy is promising as they can potentially be used to re-establish functional vasculature, support proper blood perfusion and tissue repair necessary in different conditions, including ischemia, heart disease, stroke and diabetes.

Currently, there is no efficient xeno-free (XF) medium for the growth and expansion of hEC. A defined XF culture system optimized for hEC 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 EndoGo™ XF medium and auxiliary solutions for attachment, dissociation, and cryopreservation of hEC.

Results show that the hEC XF culture system efficiently supports unique expansion of umbilical cord blood Endothelial Progenitor Cells (EPC) as well as hEC from large vessels (arteries and veins, e.g. HUVEC) and small vessels (lymph and blood). Expanded cells maintain endothelial cells features: typical cobblestone-like cell morphology, phenotypic surface marker profile, gene expression profile and angiogenic differentiation capacity.

EndoGo™ XF medium and auxiliary solutions provide a novel hEC XF culture system enabling the expansion of hEC suitable for cell therapy applications.

Materials and Methods

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

Culture system - Culture medium (EndoGo™ XF, BI) supplemented with 2-5% off the clot (OTC) human AB serum for XF culture system, 2-5% human platelet lysate (hPL) for serum-free (SF), XF culture system or commercial FBS medium. For expansion, cells were seeded with human fibronectin coating (hFN, BI) at a concentration of 5000-6000 viable cells/cm^2, harvested using recombinant Trypsin-EDTA solution (BI) and Soybean Trypsin Inhibitor (SBTI, BI). hEC were cryopreserved in ACF freezing medium (Serum-Free Cell Freezing Medium, BI).

Real-time PCR - hEC 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 triplicate.

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

Immunophenotyping - hEC were expanded for several passages in EndoGo™ XF, harvested, labeled with antibodies against CD31, CD144, and CD90. The cells were fixed, examined by flow cytometer (Stratedigm Inc.) and analyzed by FCS Express software.

Tube formation assay - hEC were expanded for several passages in EndoGo™ XF then harvested and 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.

CB EC isolation -
Cord Blood: Human umbilical cord blood (CB) was obtained with informed consent under The University of Texas M.D. Anderson Cancer Center Institutional Review Board (IRB) approved protocol. CB Mononuclear cells (MNCs) were obtained by layering CB over Histopaque and collecting the buffy coat. **EC isolation:** ECs were obtained by isolating the CD45- fraction of CB MNCs using CD45+ microbeads (Miltenyi) followed by CD34+ selection, of the CD45- fraction, using CD34+ microbeads (Miltenyi). Cells were placed into culture with endothelial culture medium (ECM) containing alpha MEM media (Gibco) supplemented with 20%FBS and 10 ng/ml EGF, 10 ng/ml VEGF and 20 ng/ml bFGF. Cell lines were established in three to four weeks. **EPC:** EPCs were obtained by sorting for CD45-CD34+CD31+CD144+CD42a- from CB MNCs. EPCs were placed into ECM for 7 days to expand initial colonies.

CB EC and EPC Immunophenotyping -
EC: ECs were stained using CD45, CD31 and CD34 antibodies (BD Biosciences). Samples were acquired either on FACSCalibur or LSR Fortessa (both from BD Biosciences).
EPC: EPCs were sorted using CD45, CD31, CD34, CD144 and CD42a (either eBioscience or BD Biosciences). Samples were sorted using Astrios (Beckman Coulter).

Abbreviations

CB	Cord Blood
EC	Endothelial Cells
ECM	Endothelial Culture Medium
EPC	Endothelial Progenitor Cells
FBS	Fetal Bovine Serum
hFN	Human Fibronectin
HS	Human Serum
hPL	Human Platelet Lysate
hMEC	Human Microvascular Endothelial Cells
HAMEC	Human Adipose 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

Evaluation of hEC expansion Using EndoGo™ XF

Figure 1: Morphology of hEC Using EndoGo™ XF

HUVEC and HDMEC were seeded (5-7k/cm^2) on hFN-coated dishes and expanded in EndoGo™ XF (with 5% hPL or 2% OTC HS) or FBS based medium for several sequential passages with equal seeding density at each passage. Representative images (x100).
EndoGo™ XF (with hPL or HS) promotes proliferation of both micro and macrovascular hEC while maintaining classical EC morphology

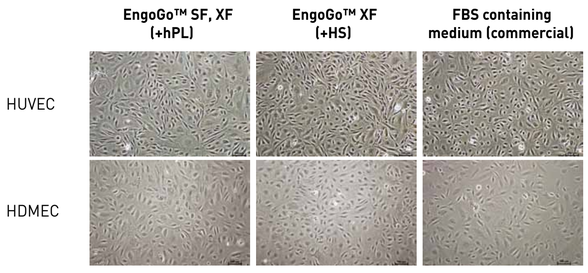
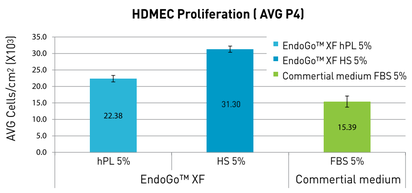


Figure 2: Proliferation and PDL of Microvascular hEC

Cell counts and PDL of microvascular hEC expanded for several sequential passages using EndoGo™ XF (with 5% hPL or 2% OTC HS) in comparison to commercial FBS-containing medium on hFN pre-coated plates with equal seeding density at each passage. Viable cells were counted using Chemometec Viability and Cell Count Assay. **(A)** HDMEC cell counts during 4 passages **(B)** HDMEC PDL **(C)** HPMEC cell counts during long term culture **(D)** HPMEC PDL. *HPMEC did not survive P5 in the FBS-containing medium.
EndoGo™ XF (with hPL or HS) promotes higher cell number and longer cultivation of microvascular hEC

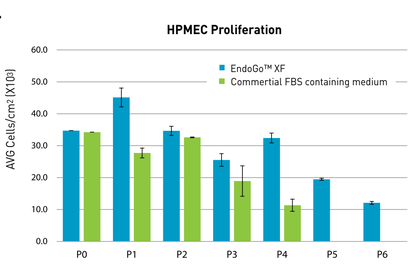
A.



B.

		Total HDMEC PDL
EndoGo™ XF	hPL 5%	6.7
	HS 5%	10.2
Commercial medium	FBS 5%	6.3

C.



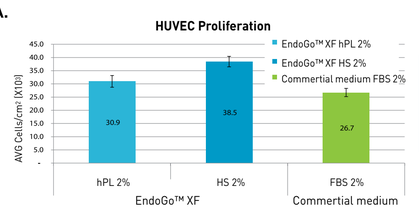
D.

		Total HPMEC PDL
EndoGo™ XF	HS 5%	15.3
Commercial medium	FBS 5%	8.8

Figure 3: Proliferation and PDL of Macrovascular hEC

Cell counts and population doubling level of HUVEC expanded for 4 passages using EndoGo™ XF (with 5% hPL or 2% OTC HS) in comparison to commercial FBS-containing medium on hFN pre-coated plates with equal seeding density at each passage. Viable cells were counted using Chemometec Viability and Cell Count Assay **(A)** HUVEC counts **(B)** HUVEC Population Doubling Level (PDL).
Excellent cell expansion and PDL of human macrovascular EC in EndoGo™ XF with HS or hPL

A.



B.

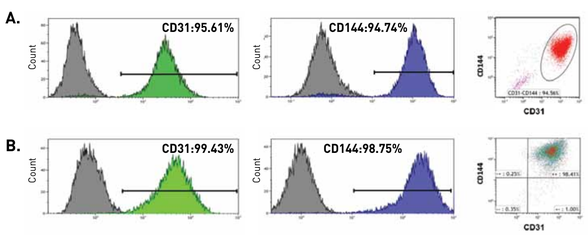
		Total HUVEC PDL
EndoGo™ XF	hPL 5%	10.5
	HS 5%	11.6
Commercial medium	FBS 5%	9.2

Characterization of hEC Using EndoGo™ XF

Figure 4: Immunophenotyping- Microvascular hEC

Microvascular hEC from various tissue sources were expanded in EndoGo™ XF with 5% OTC HS for several sequential passages. Cells were harvested and labeled with antibodies against CD31, CD144 and analyzed by FACS.
(A) HAMEC post 3 passages, **(B)** HDLEC post 6 passages **(C)** Summary of expression.
Microvascular hEC from various tissue sources maintain a classical profile of EC markers (>94%) after expansion in EndoGo™ XF

A.



B.

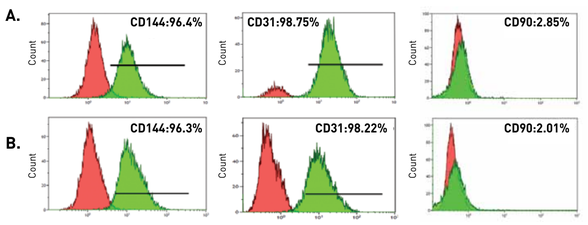
C.

	HAMEC	HDLEC
CD31%	95.61	99.43
CD144%	94.74	98.75

Figure 5: Immunophenotyping- Macrovascular hEC

HUVEC expanded for 4 passages in EndoGo™ XF with 2% OTC HS in comparison to commercial FBS-containing medium on hFN pre-coated plates with equal seeding density at each passage. Cells were harvested and labeled with antibodies against CD31, CD144 and CD90 and analyzed by FACS. **(A)** EndoGo™ XF with 2% OTC HS **(B)** Commercial FBS 2% **(C)** Summary of % expression.
HUVEC expanded in EndoGo™ XF with 2% human serum maintain a classical profile of EC markers expression (>96%)

A.



B.

C.

	EndoGo™ XF	Commercial medium FBS
CD31%	98.7	98.2
CD144%	96.4	96.3
CD90%	2.8	2.01

Figure 6: Gene Expression- macrovascular hEC

Real-time PCR results of HUVEC after expansion in EndoGo™ XF (with HS for XF system or hPL for SF, XF system) and FBS-containing medium analyzed in comparison to P0 (original cells w/o proliferation).

HUVEC expanded in EndoGo™ XF:
- **Maintain similar gene expression profile pattern during passages**
- **Higher angiogenic gene expression vs. FBS- containing medium**

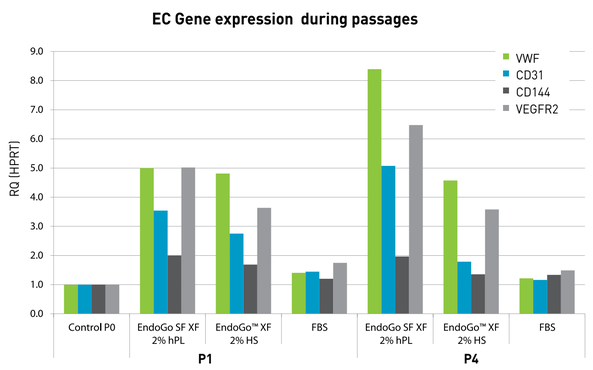


Figure 7: Angiogenic Markers Expression

Representative images of hEC derived from various tissue sources after expansion in EndoGo™ XF. Cells were fixed and stained for the classical endothelial cell markers: CD31 (PECAM) or CD144 (VE-CAD) (red), Von-Willebrand factor (vWF) (green), and counterstained with DAPI (blue).
Micro and macrovascular endothelial cells expanded in EndoGo™ XF medium express high angiogenic markers

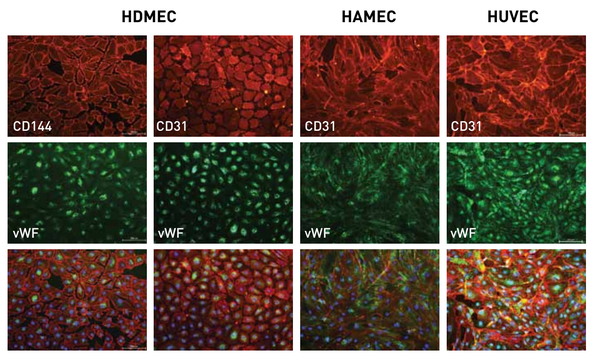


Figure 8: Angiogenic Potential

Tube Formation Assay (TFA) of cells from various tissue sources after cultivation in EndoGo™ XF medium with 2-5% OTC HS. Cells were harvested and seeded on MatrigelTM in EndoGo™ XF (50k/well, 48wp). Representative image of: **(A)** HDMEC P3 after 18h, **(B)** HDBEC from P2 after 18h, **(C)** HAMEC from P2 after 18h **(D)** HUVEC from P3 after 20h.
hEC expanded in EndoGo™ XF preserved angiogenic potential to form capillary-like tube structures

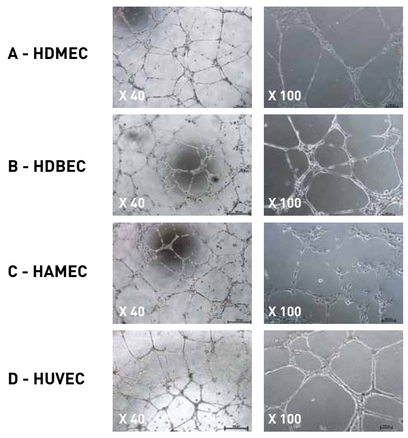
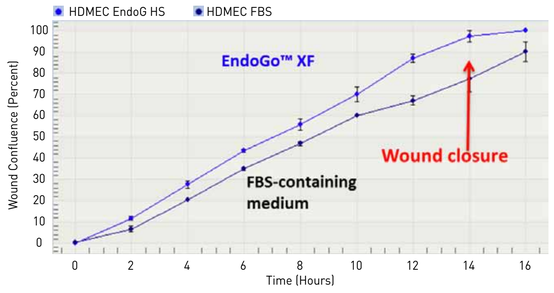


Figure 9: Wound Healing Assay

Confluence culture of HDMEC after expansion for 3 passages in EndoGo™ XF with OTC HS or FBS containing medium was scratched and analyzed for the rate of wound closure (IncuCyte®).
HDMEC expanded in EndoGo™ XF migrate faster than cells cultured in FBS-containing medium

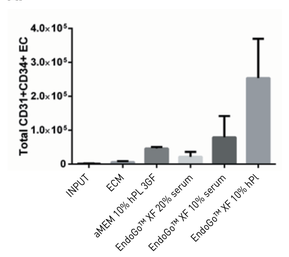


Evaluation of CB hEC and EPC Expansion Using EndoGo™ XF

Figure10: Isolation of hEC from Cord Blood

ECs expanded for 7 days in various culture medium. 1) ECM, 2) α-MEM +10% hPL + 20ng/ml bFGF + 10ng/ml VEGF + 10ng/ml EGF, 3) EndoGo™ XF with 20% FBS, 4) EndoGo™ XF with 10% FBS and 5) EndoGo™ XF with 10% hPL. **(A)** Quantitative analysis of day 7 CB EC CD31+CD34 + population. **(B)** Quantitative analysis of day 7 CB EC CD31+CD34- population. **(C)** Representative Flow Cytometric plots of ECs in ECM and EndoGo™ XF.
EndoGo™ XF with hPL significantly expands CB ECs

A.



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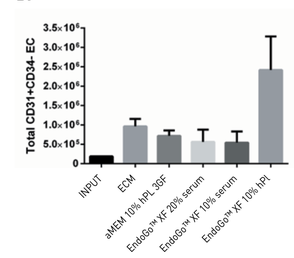


C.

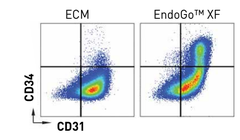
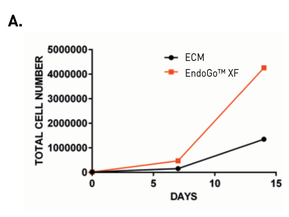


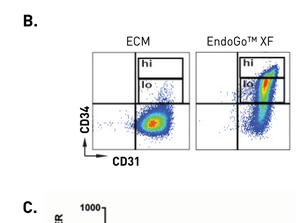
Figure 11: Expansion of CB EPC

Isolation and expansion of CB EPC with EndoGo™ XF. **(A)** Total cell expansion of early EPC outgrowth from CB using ECM and EndoGo™ XF with hPL. **(B)** Flow representation of ECM or EndoGo™ XF expansion of EPCs after 14 days. EPCs are contained primarily in the CD31hi CD34hi fraction. **(C)** Established primary CB EPC lines expanded in either EndoGo™ XF with hPL or ECM. Graph depicts total cell expansion of the EPC. (n=5).
EndoGo™ XF significantly expand day 14 EPC population (CD34hi CD31hi)

A.



B.



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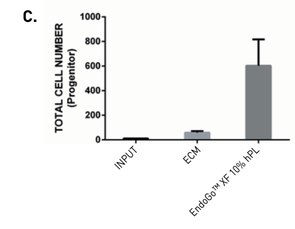

Summary

- Micro and macrovascular human EC from various sources were efficiently expanded using EndoGo™ XF supplemented with human AB serum in a xeno-free culture system or human PL for serum-free and xeno-free culture system.
- The highest proliferation rate of human EC from a variety of sources was achieved using EndoGo™ XF in comparison to commercially available FBS-containing medium.
- Human EC expanded in EndoGo™ XF with the addition of HS or hPL retain EC features: morphology, angiogenic gene expression, immunophenotyping, angiogenic potential (TFA), and migration (wound healing).
- Higher angiogenic gene expression and migration found in human ECs expanded in serum-free and xeno-free culture system.
- hEC from cord blood can be efficiently isolated and expanded using EndoGo™ XF + hPL. Superior expansion of total CB ECs as two distinct subpopulations: CD34+CD31+ and CD34-CD31+.
- CB EPC (CD34hi CD31hi population), with more progenitor-like tube initiating population