StemBioSys®

Technical Bulletin

CELLvo™ Human Cord Blood-Endothelial Progenitor Cells are Highly Proliferative and Angiogenic

Introduction

Endothelial cells from the umbilical vein (hUVECs), artery (hAECs), and microvasculature (hMVECs) have been important in elucidating the pathways involved in angiogenesis and may have important clinical applications in the treatment of ischemic tissues or in the vascularization of graft tissues. In 2004, Mervin Yoder first described a novel population of rare circulating putative endothelial progenitor cells (EPCs) found in human umbilical cord blood and, less abundantly, in human adult peripheral blood [1]. These cells were highly proliferative relative to more commonly utilized endothelial-type cells. Yoder dubbed these putative endothelial progenitor cells as endothelial colony forming cells for their astounding capacity to form large primary, secondary, and tertiary colonies, as compared to other endothelial cell types. In addition to having superior colony forming ability, these cells have been shown to possess greater angiogenic potential *in vitro* as well as *in vivo* and it has been suggested that they may actually be progenitors of other endothelial cell types [2]. These properties make these cells especially interesting for research on angiogenesis and for potential therapeutic applications.

A major obstacle to the widespread adoption of these cells for research use and therapeutic development has been their relative rarity, and difficulty isolating these cells from primary tissues. The use of hCB-EPCs in basic and translational research makes this both practical and affordable.

Here, we directly compare StemBioSys CELLvo™ hCB-EPCs to commercially available hUVECs. Our results support what has already been reported in literature by multiple groups. CELLvo™ hCB-EPCs have similar morphology and immunophenotype to other endothelial cells, but contrast to mature endothelial cells in other ways. They are more proliferative; CELLvo™ hCB-EPCs form secondary and tertiary colonies, whereas hUVECs only consistently form primary colonies. They are more angiogenic; in an in vitro vessel formation assay, CELLvo™ hCB-EPCs form more vessels relative to hUVECs. Lastly, they are more pro-angiogenic; a comparison of conditioned media from CELLvo™ hCB-EPCs and hUVECs reveals that CELLvo™ hCB-EPCs have a more pro-angiogenic paracrine profile.

Taken together, these data suggest the CELLvo™ hCB-EPCs have significant advantages relative to hUVECs in basic and translational research and may be a promising alternative to more mature endothelial cell types.

Materials and Methods

Study Design

CELLvo™ hCB-EPCs previously isolated from fresh human umbilical cord blood and expanded for three passages prior to cryogenic storage and hUVECs purchased from a large cell manufacturer and similarly stored were thawed and expanded on StemBioSys' CELLvo™ Matrix plates. Cells were compared based on proliferation, phenotype, clonal expansion, vessel formation, and angiogenic paracrine profile.

Substrate and Media Preparation

CELLvo™ Matrix was prepared as previously described [3]. Briefly, bone marrow mesenchymal stem cells (BM-MSCs) were seeded onto fibronectin-coated 6-well plates. After being induced to secrete abundant matrix protein on the surface of the dish, the plates were decellularized using low concentration detergent. The remaining decellularized matrix was washed and then dried for future use. CELLvo™ Matrix stored at 4°C, is a ready-for-use product and has a shelf-life of up to 12 months.

Cell Culture

Cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20% fetal bovine serum, 1% anti-biotic/anti-mycotic, 1% L-Glutamine, 10ng/mL basic fibroblast growth factor, and 20ng/mL epithelial growth factor. Cells were seeded at 6,000 cells/cm^2 and passaged at 90% confluence. For colony formation assays, one-half media was replaced with fresh media every 3 to 4 days.

Phenotyping

Immunophenotype was determined by flow cytometry. Immediately after counting cells, approximately 10^5 cells per marker to be measured were collected and washed twice using FACS buffer (Hank's buffered saline solution + 5% fetal bovine serum + .1% sodium azide). After washing, cells were resuspended in FACS buffer and aliquoted into individual tubes for each marker and appropriate controls (100uL/tube). Primary antibody was added to each marker and incubated at 4°C on a rocker plate for at least 1 hour before being washed and stained with secondary antibody at 4°C. After incubation, cells were either analyzed immediately or fixed using 2% paraformaldehyde and kept at 4°C protected from light until analyzed. For intracellular markers, cells were fixed

with 80% methanol and permeabilized using 1% tween in PBS, prior to addition of the primary antibody.

Colony Formation Assay

CELLvo™ hCB-EPCs or hUVECs were seeded onto CELLvo™ Matrix in 6-well plates at 20 cells/cm2. One half media was replaced every 3-4 days with fresh media. When dense distinct colonies were observable by bright field microscopy, full media was aspirated and previously marked colonies were detached and reseeded into a single well of a 6-well plate (one colony to one well). Whenever possible, single colonies were isolated. This was not always possible due to the proximity of multiple colonies.

Vessel Formation Assay

CELLvo™ hCB-EPCs or hUVECs were seeded onto hESC-qualified Matrigel® (Corning®) at 20,000 cells/cm^2 in 24-well plates. After 18 hours, cells were stained with calcein AM fluorescent dye and 5 images were taken in bright field and under fluorescence.

Angiogenic Cytokine Array

Cells were cultured to confluence and then switched to low serum media for 24 hours. After 24 hours, media was collected and stored at -20°C for future use. To measure the pro-angiogenic cytokine secretion of CELLvo™ hCB-EPCs and hUVECs we utilized the RayBiotech Human Angiogenesis Cytokine Array.

Results

CELLvo™ hCB-EPCs are phenotypically similar to hUVECs

CELLvo™ hCB-EPCs display a quintessential endothelial cell phenotype. Similar to hUVECs and other more familiar endothelial cell types, CELLvo™ hCB-EPCs are small in size where colonies exhibit the characteristic cobblestone morphology (Figure 1). Additionally, these cells express surface markers typical of endothelial cells including CD31, CD105, and von-Willebrand Factor (Table 1). Importantly, these cells are also positive for CD146, distinguishing them from myeloid angiogenic cells (MACs) which may also be isolated from cord blood and are also pro-angiogenic, but do not possess the intrinsic vessel formation capacity of EPCs.

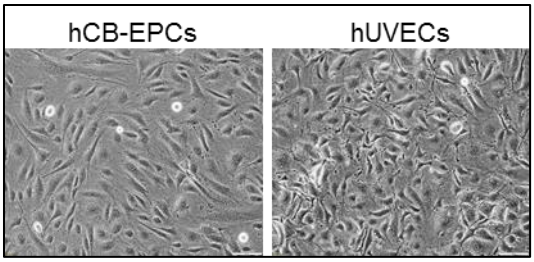

Figure 1. CELLvo™ hCB-EPCs and hUVECs cultured on CELLvo™ BM-Matrix are phenotypically indistinguishable. Both cell types are small and exhibit a cobblestone morphology that is typical of endothelial cells as shown by the bright field images.

Phenotypic Characterization	
CD31	Positive
CD34	Negative
CD90	Negative
CD105	Positive
CD146	Positive
acLDL uptake	Positive
vWF	Positive

Table 1. The immunophenotype of CELLvo™ hCB-EPCs is characteristic of endothelial cells and does not distinguish them from other, more mature, endothelial cell types.

CELLvo™ hCB-EPCs are uniquely capable of clonal expansion

Previously, it had been reported that a key distinguishing characteristic of EPCs relative to more mature endothelial cell types (i.e. hUVECs) is their high proliferative potential, especially as it relates to clonal expansion. To test this, passage-matched CELLvo™ hCB-EPCs and hUVECs were seeded onto CELLvo™ Matrix at clonal density (20 cells/cm^2). After colonies formed, cloning rings were used to detach individual colonies to be reseeded for secondary colony formation. This process was repeated for tertiary colony formation (Figure 2). Interestingly, all wells in both groups consistently yielded primary colonies. However, CELLvo™ hCB-EPC colonies tended to be larger and more numerous. When individual colonies were reseeded to form secondary colonies more differences emerged. While over 80% of CELLvo™ hCB-EPC primary colonies were capable of secondary colony formation, roughly 20% of hUVECs were capable of the same feat. Of those colonies, nearly all of the CELLvo™ hCB-EPC secondary colonies that were reseeded formed tertiary colonies (16/18), but only 11% of hUVEC secondary colonies were able to form tertiary colonies. In figure 2, the yields are reported as a percent of original colonies capable of forming tertiary colonies.

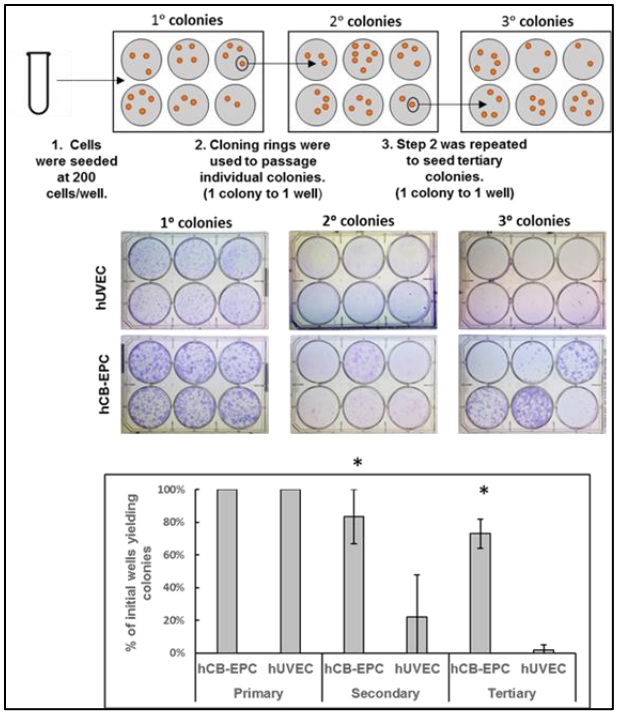

Figure 2. CELLvo™ hCB-EPCs form secondary and tertiary colonies. CELLvo™ hCB-EPCs and hUVECs were seeded at clonal density on CELLvo™ Matrix. Individual colonies were passaged using cloning rings to form secondary and tertiary colonies as shown in the diagram (top). CELLvo™ hCB-EPCs formed larger, denser, and more numerous primary colonies. Additionally, CELLvo™ hCB-EPCs consistently formed secondary and tertiary colonies, as shown by the representative images (middle) and the graph describing the percentage of originally seeded wells that yielded primary, secondary, and tertiary colonies (bottom). * p<.05 vs. hUVEC.

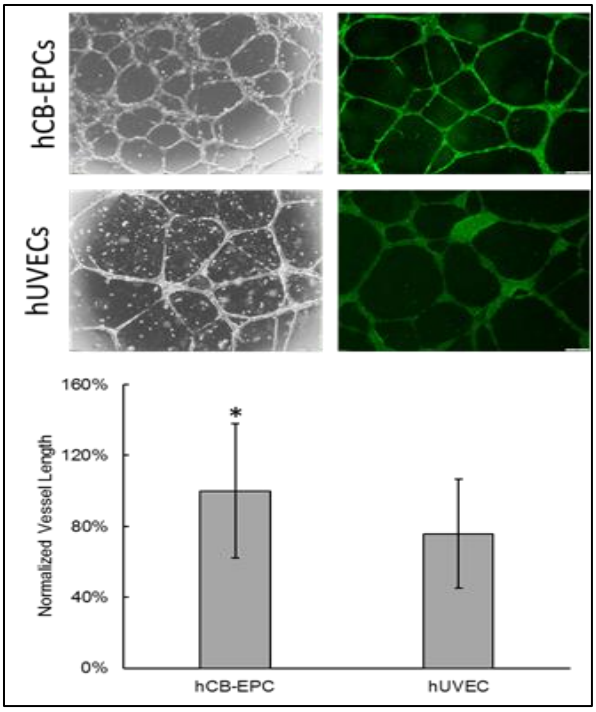

Figure 3. CELLvo™ hCB-EPCs have greater intrinsic vessel formation capacity relative to hUVECs. CELLvo™ hCB-EPCs and hUVECs were seeded at high density onto Matrigel® and incubated for 18 hours. Spontaneous vessel formation was observed by microscopy (top). Vessel formation was quantified (in terms of tube length) using the NIH angiogenesis analyzer ImageJ plug-in (bottom). * p<.05 vs. hUVEC.

CELLvo™ hCB-EPCs display increased angiogenesis in vessel formation assay

To measure intrinsic angiogenic properties of CELLvo™ hCB-EPCs, they were compared to hUVECs in a Matrigel® vessel formation assay. Cells were seeded onto Matrigel® at (20,000 cells/cm^2) and incubated for 18 hours. During that time, vessels spontaneously form in the Matrigel® (Figure 3). Images of the vessel formation were analyzed using the NIH angiogenesis analyzer plug-in for ImageJ for non-biased quantification. 22 total images from 3 different lots of hUVECs and CELLvo™ hCB-EPCs were analyzed. After elimination of outliers using Rosner's test, CELLvo™ hCB-EPCs were significantly more angiogenic than hUVECs (as measured by total tube length per image), forming approximately 25% more vessels (p=.025).

CELLvo™ hCB-EPCs exhibit a more pro-angiogenic paracrine profile

In addition to intrinsic vessel formation capacity, it may be therapeutically important for cells to secrete pro-angiogenic factors that may induce capillary formation via paracrine effects. To test this, we used the RayBiotech Human Angiogenic Cytokine Array to examine the secretion of 43 pro-angiogenic cytokines into conditioned media of CELLvo™ hCB-EPCs and hUVECs. Interestingly, of the 43 cytokines, 17 were expressed at higher levels by CELLvo™ hCB-EPCs relative to hUVECs. Only three were expressed at higher levels in conditioned media from hUVECs. There was no significant difference between the other 23 cytokines. Figure 4 shows the normalized expression of all 43 cytokines illustrated in a heat map. Darker colors illustrate greater concentrations of the cytokines in conditioned media. Large differences were observed between the CELLvo™ hCB-EPCs and hUVECs in expression of VEGF, VEGF-R2, VEGF-R3, b-FGF, IL-10, GM-CSF, and I-309, among others.

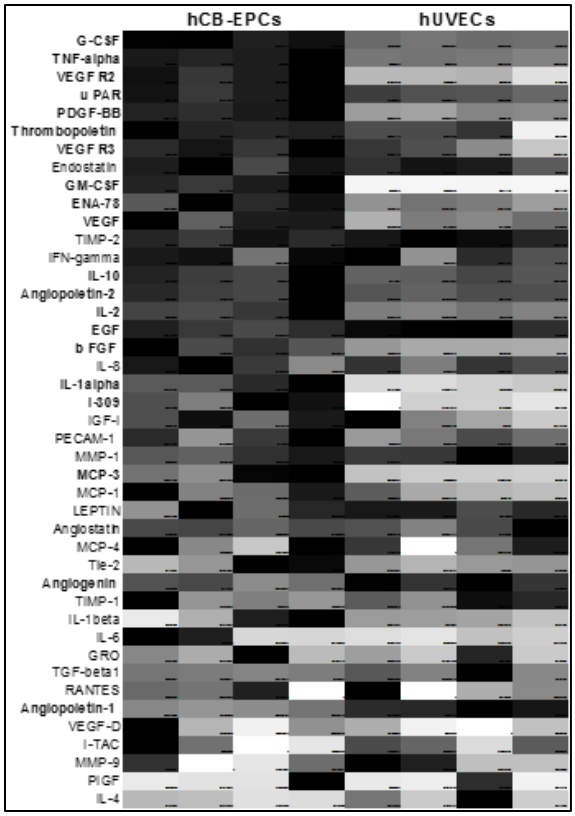

Figure 4. CELLvo™ hCB-EPCs secrete higher levels of pro-angiogenic cytokines. The pro-angiogenic paracrine profile of CELLvo™ hCB-EPCs and hUVECs was measured using the RayBiotech Human Angiogenic Cytokine Array. Normalized cytokine concentrations from conditioned media are visualized in the heat map. Higher concentrations are shown using darker colors. Cytokine names in bold exhibited expression differences that were statistically significant ($p<.05$) from hUVECs.

Discussion

A great deal of work has been done with hUVECs and other mature endothelial cell types to understand angiogenesis and to develop potential therapies for the treatment of ischemic tissues. Recent work describing CELLvo™ hCB-EPCs as a novel population of cells that may be less mature than hUVECs, more pro-angiogenic, and have greater intrinsic vessel formation capacity is highly promising [4]. These cells may be more therapeutically useful in the treatment of ischemic tissues and may be useful in understanding how mature endothelial cell types are derived in vivo. It has even been suggested that a small fraction of these cells in more mature endothelial cell populations may be largely responsible for desirable functional attributes.

Though a growing excitement surrounds these cells, it has been difficult to isolate large quantities for research as they are relatively rare in cord blood, and extremely rare in adult peripheral blood. StemBioSys® methods make it possible to efficiently isolate hCB-EPCs from cord blood making them more available to researchers. This innovation makes it practical for research labs to shift away from hUVECs and hAECs to cells that are potentially much more potent. Here, we offer evidence supporting previous claims that StemBioSys® CELLvo™ hCB-EPCs are distinct from hUVECs and other commonly used endothelial cell types, and that they differ in terms of clonal expansion capability and angiogenic potential.

References

1. Ingram, D. A. *et al.* Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. *Blood* **104,** 2752–60 (2004).
2. Joo, H. *et al.* Human endothelial colony forming cells from adult peripheral blood have enhanced sprouting angiogenic potential through up-regulating VEGFR2 signaling. *Int J Cardiol* **197,** 33–43 (2015).
3. Marinkovic, M. *et al.* One size does not fit all: developing a cell-specific niche for in vitro study of cell behavior. *Matrix biology: journal of the International Society for Matrix Biology* **54-55,**426–441 (2016).
4. Critser, P. J. & Yoder, M. C. Endothelial colony-forming cell role in neoangiogenesis and tissue repair. *Curr Opin Organ Transplant* **15,** 68–72 (2010).

Information about ordering:

SUPPLIER No.	DESCRIPTION
EPC-500-000	Vial of 500,000 CELLvo™ hCB-EPCs
EPC-500-6WP	Vial of 500,000 EPC + sleeve of five CELLvo™ Matrix 6 well plates
EPC-500-T75	Vial of 500,000 EPC + sleeve of five CELLvo™ Matrix T75s
EPC-500-T150	Vial of 500,000 EPC + sleeve of five CELLvo™ Matrix T150s