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Expansion of human umbilical cord blood hematopoietic progenitors with cord vein pericytes

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Abstract: The vascular niche is a site rich in blood vessels, whereas endothelial cells, pericytes, and smooth muscle cells create a microenvironment that recruits mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs), which is important for stem cell mobilization, proliferation, and differentiation. In this study, CD146 + pericytes were purified and enriched from the human umbilical cord vein. In order to define their direct role in hematopoiesis, we tested the CD146 + pericytes as compared with osteoblasts derived from umbilical cord blood (UCB) MSCs to sustain human UCB hematopoietic progenitor cells in noncontact coculture settings or in culture media previously conditioned (CM) by these cells. The growth of UCB cells was the greatest in pericyte cocultures (2.8-fold vs. the control). The increased growth in pericyte and pericyte CM cultures was largely the result of increased frequency of CD34 + and CD38 + hematopoietic progenitors, CD34 + CD41 + megakaryocyte progenitors, and CD235 + erythroblasts. A total of 29 factors were found to be secreted by pericytes higher than by osteoblasts. The most secreted growth factor by pericytes was vascular endothelial growth factor (1.3-fold). We demonstrate for the first time that human CD146 + perivascular cell coculture and CM are able to directly support the ex vivo maintenance of human hematopoietic progenitor cells.

Key words: Pericyte, hematopoietic stem cells, conditioned medium, umbilical cord, umbilical cord blood

1. Introduction

Hematopoietic stem cells (HSCs) are maintained in specialized microenvironments in tissues known as "niches", in which supporting cells secrete factors that promote stem cell maintenance (Ding et al., 2012). The identification of cellular compartments of the HSC niche has recently been the subject of many studies (Bianco, 2011; Morrison et al., 2014). In bone marrow (BM), there is broad discussion of the possible presence of two niches able to maintain and regulate HSCs, which are the endosteal and vascular niches (Lilly et al., 2011). Previous reports suggested that N-cadherin + osteoblasts could be proposed to promote HSC quiescence through direct contact and secretion of some cytokines and extracellular matrix proteins (Bromberg et al., 2012; Greenbaum et al., 2012). Other studies found that most BM HSCs are localized near sinusoidal vessels where endothelial cells and perivascular stromal cells maintain them (Kunisaki et al., 2013). Pericytes are a perivascular stromal cell population embedded on the capillary wall in a shared basement membrane with the endothelium (Winkler et al., 2014). They are similar to mesenchymal stem cells (MSCs) and can be obtained from several organs (Gökçinar-Yagci et al., 2016). They are most commonly characterized by observing the positive expression of perivascular markers (CD146, PDGFR-β, NG2, α-SMA, and MAP1B) and MSC markers (CD44, CD73, CD90, and CD105) (Gökçinar-Yagci et al., 2015). They have been shown to improve heart function and to form skeletal muscle, dental tissues, cartilage, and bone. They may be involved in tissue regeneration as niche cells for specialized stem cells for hematopoiesis (Birbrair et al., 2015). Corselli et al. reported that CD146 + perivascular cells are a subset of MSCs able to directly support the ex vivo maintenance of human hematopoietic progenitor cells (HPCs) (Corselli et al., 2013). Kunisaki et al. mentioned that quiescent HSCs are specifically associated with small caliber arterioles, which are predominantly distributed in the endosteal BM. Moreover, their new imaging analyses suggested that physical ablation of the arteriolar niche causes HSC localization to sinusoidal niches, where HSCs are switched to nonquiescent status (Kunisaki et al., 2014). It was previously shown that arterioles are associated with both quiescent NG2 + niche cells and HSCs, suggesting that the vessel itself may be a critical gatekeeper of stem cell quiescence in the BM (Kunisaki et al., 2013). A culture

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system that closely recapitulates marrow physiology is essential to study the niche-mediated regulation of HSC fate (Sharma et al., 2012).

The umbilical cord (UC) contains one vein and two arteries buried within Wharton's jelly. Unlike the BM, the umbilical vein carries oxygenated, nutrient-rich blood from the placenta to the fetus and umbilical arteries carry deoxygenated, nutrient-depleted blood from the fetus to the placenta (Wang et al., 2010). Umbilical cord blood (UCB) can be viewed as a promising source of stem cells for research and clinical applications (Dumont et al., 2014; Horwitz et al., 2015; Zhu et al., 2015). It is necessary to develop ex vivo culture systems to scale up the expansion of HSCs and progenitors. Human UC perivascular cells have been considered as an alternative source of mesenchymal progenitors for ex vivo expansion of HPCs. Ex vivo expansion of UCB HSCs provides a means to raise the dose of transplantable progenitors to reduce graft failure and to transiently sustain early engraftment (Dumont et al., 2014). Coculture of UCB mononuclear cells (MNCs) or HPC-enriched CD34 + cells with MSCs has been shown to support and improve the expansion of HSCs (Schattner et al., 1996; Kawano et al., 2003; McNiece et al., 2004; da Silva et al., 2005). Consistent with this, we previously showed that irradiated BM MSCs in optimized megakaryocyte progenitor cocktail (OMPC) increase the growth of UCB cells independently of contact, that irradiation induced the differentiation of BM MSCs into osteogenic-like cells, and that osteoblasts derived in vitro from the same MSC lines could recapitulate the growth effects (Çelebi et al., 2011).

In this study, CD146 + perivascular cells were purified and enriched from the UC vein. In order to define their direct role in hematopoiesis, we tested the CD146 + pericytes as compared with osteoblasts derived from UCB MSCs to sustain human UCB HPCs in noncontact coculture settings or in culture media previously conditioned by these cells. We demonstrate for the first time that human CD146 + pericyte coculture and conditioned medium (CM) are able to directly support the ex vivo maintenance of human HPCs.

2. Materials and methods

2.1. Isolation, culture, and characterization of human vein pericytes

In this study, UC and UCB samples were obtained after the birth of healthy babies born at the Hacettepe University Faculty of Medicine Obstetrics and Gynecology Clinic. For the use of these noninvasive samples, the Noninterventional Clinical Research Ethics Committee approved the study protocol (GO 13 / 376 - 14).

Umbilical cord samples were cut into pieces of 3–4 cm in length and then UC fragments and veins were washed with phosphate-buffered saline (PBS, Sigma-Aldrich, St. Louis, MO, USA). Wharton's jelly tissue around the veins and arteries was removed with the help of a sterile lancet. Each umbilical vein piece was sutured at both ends with sterile surgical sutures. Sutured vein pieces were placed into collagenase solution (1 mg/mL, Sigma) and incubated at 37 °C for 16 h. After centrifugation, the pellet was dissolved in 50 mL of PBS, and then it was filtered with membrane filters of 100 µm and 40 µm (BD Biosciences, Franklin Lake, NJ, USA). Cell suspension passed through the membrane filters was centrifuged at 1500 rpm for 5 min. The pellet was cultured in T-75 flasks with Pericyte Growth Medium (PGM, Promocell, Heidelberg, Germany). Cultures were incubated at 37 °C in a 5% CO_2 incubator until confluence. CD146 + pericytes were enriched (70 ± 8.0%) by positive selection according to the manufacturer's instructions (n = 6, Miltenyi Biotec, Bisley, UK). Pericytes were cultured in PGM until confluence. At 80%–85% confluence, adherent cells were trypsinized with TrypLE solution (GIBCO, Invitrogen, Burlington, ON, Canada), and cell viability was checked by trypan blue dye exclusion.

The cell surface markers of passage 3 pericytes were examined by flow cytometry (FACSAria, BD Biosciences). Cells were incubated in the dark with the following antihuman antibodies: CD146 - phycoerythrin (PE), CD31 - fluorescein isothiocyanate (FITC), and CD105 - allophycocyanin (APC). All antibodies were obtained from Becton Dickinson Pharmingen (Mississauga, ON, Canada). Analyses were performed by using BD FACSDiva Analysis Software v6.1.2 (BD Biosciences). Moreover, CD146 + pericytes (passage 3) were prepared for antibody staining by fixing them for 20 min at 37 °C with 3.7% formaldehyde. They were permeabilized by incubation with 0.2% Triton/PBS for 5 min at 37 °C and preblocked for 30 min at 37 °C with 10% rabbit serum/PBS. Immunofluorescence staining was performed by an overnight incubation at 4 °C with primary antihuman antibody MAP1B (Abcam, Cambridge, UK). Cells were then washed five times with 0.05% Tween/PBS. Following blockage for 30 min at 37 °C with 10% rabbit serum/PBS, secondary antibody staining and nuclear staining were performed for 1 h at 37 °C with secondary antibody Alexa Fluor 488 conjugate (Invitrogen), DAPI (4',6-diamidino-2-phenylindole, AppliChem, Darmstadt, Germany), and phalloidin 555 for actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The fluorescent signal was visualized with an Olympus FV1000 confocal microscope (Olympus, Tokyo, Japan). The image was processed using FV10-ASW software (Olympus).

2.2. Isolation of human MSCs and osteogenic differentiation

Human MNCs obtained from whole CB cells were cultured in α-minimal essential medium (GIBCO,

Invitrogen) supplemented with 20% fetal bovine serum (FBS, Invitrogen), 100 U/mL penicillin, 100 µL/mL streptomycin, and 1% L-glutamine (Sigma) and incubated at 37 °C in a humidified incubator (5% CO_2). On day 3, cells in suspension were discarded and fresh medium was added. At 80%–85% confluence, adherent MSC cells were trypsinized with TrypLE solution (GIBCO, Invitrogen), and cell viability was checked by trypan blue dye exclusion. A total of four MSCs were generated from independent samples. Osteogenic differentiation was induced using DMEM (Invitrogen) supplemented with 10% FBS, 10^{-7} M dexamethasone, 0.2 mM ascorbic acid 2-phosphate, and 10 mM glycerol 2-phosphate for 7 days. The mineralization capacity of cells was evaluated by Alizarin Red S staining. All chemicals were purchase from Sigma-Aldrich.

2.3. Isolation of CD34+ hematopoietic UCB cells

Human UCB MNCs and CD34 + cell enrichment was carried out as previously described (Çelebi et al., 2012). The cells were separated over a Ficoll-Paque Plus density gradient (1.077 g/mL; GE Healthcare Life Sciences**,** Buckinghamshire, UK). Fresh or thawed CB samples were pooled before manipulation (4 or 5 samples). MNC concentration was adjusted to 2×10^7 cells/mL, and CD34 + cells were enriched by negative selection using the Easy Sep Cell Separation System and the Human Progenitor Enrichment Cocktail (STEMCELL Technologies, Vancouver, BC, Canada). Purity of CD34 +-enriched cells was confirmed by flow cytometry (purity 75.0 ± 5.0%; Supplementary Figure 1).

2.4. Production of pericyte and osteoblast conditioned medium

Confluent pericyte or MSC-derived osteoblast culture plates were rinsed with PBS solution and then incubated for 7 days with the serum-free medium used for hematopoietic cell cultures (Iscove's modified Dulbecco's medium (IMDM, Invitrogen) supplemented with 20% serum substitute solution (bovine serum albumin/insulin/transferrin (BIT, STEMCELL Technologies), 40 µg/mL low density lipoprotein (LDL, Sigma), and 5×10^{-5} M 2-mercaptoethanol (Sigma)). Cells were removed from the CM by centrifugation. The CM was aliquoted and stored at –20 °C until use.

2.5. Coculture conditions

CD34 + cells were grown in serum-free medium; IMDM (Invitrogen) supplemented with 20% serum substitute solution (BIT, Stem Cell Technologies), 40 µg/mL LDL (Sigma), 5×10^{-5} M 2-mercaptoethanol (Sigma), and different combinations of human cytokines (TPO, SCF, and Flt-3 ligand) were purchased from PeproTech (Rocky Hill, NJ, USA). In the expansion phase, 2×10^4 CB CD34 + cells/mL were placed in cultures for 7 days of culture with the OMPC (TPO 35 ng/mL, stem cell factor (SCF) 10 ng/mL, FL 11 ng/mL).

Pericytes and osteoblasts were subcultured in 24-well plates (2×10^4 cells/mL) for 3 days. On day 0, cells were rinsed with PBS and UCB CD34 + cells (2×10^4 cells/mL) were then plated on top of the feeder layers in transwell inserts (0.4-µm microporous filter, Corning Life Sciences, Lowell, MA, USA) in OMPC-medium or UCB CD34 + cells (2×10^4 cells/ mL) were cultivated in pericyte or osteoblast CM with OMPC-medium. CD34 + cells in OMPC-medium were used as a control. All cultures (n = 4) were done in duplicate and were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. Viable nucleated cells on day 7 were counted with a hemocytometer and 0.4% trypan blue.

2.6. Flow cytometry analysis of hematopoietic cells

CD34-enriched UCB cells grown for 7 days were phenotyped by flow cytometry as previously described (Çelebi et al., 2012). The antibodies used were anti-CD235a -conjugated to phycoerythrin (PE), anti-CD41a (GPIIb) - allophycocyanin (APC), anti-CD34 fluorescein isothiocyanate (FITC), and anti-CD38 conjugated to phycoerythrin (PE) corresponding to control antibodies (mouse isotype APC, PE and FITC controls). All antibodies were purchased from Becton Dickinson Pharmingen.

2.7. Progenitor assays

Human myeloid clonogenic hematopoietic progenitor assays (CFU-C) were performed using the MethoCult SF H4436 according to the manufacturer's instructions (STEMCELL Technologies).

2.8. Cytokine array

Detection of cytokines, chemokines, and growth factors released in CM was done using the human Cytokine Array C5 (AAH-CYT-5-4) following the manufacturer's instructions (RayBiotech, Norcross, GA, USA). Densitometry analyses were done using the Fluor Chem FC3 System imaging apparatus (ProteinSimple Co., Santa Clara, CA, USA) with AlphaView Software (ProteinSimple). All values were normalized with the mean intensity of the positive and negative controls.

2.9. Statistical analysis

The data were analyzed with SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was evaluated based on the Student paired t-test and $P < 0.05$ was considered significant.

3. Results

3.1. Morphology of umbilical cord vein pericytes and pericyte-specific marker expression

Pericytes exhibited a rather flat, spindle-shaped morphology, and cells grew to near confluence within 12 days (Figure 1A). Immunofluorescence staining demonstrated that MAP1B was invariably expressed in pericytes (Figure 1B). Flow cytometry results revealed that

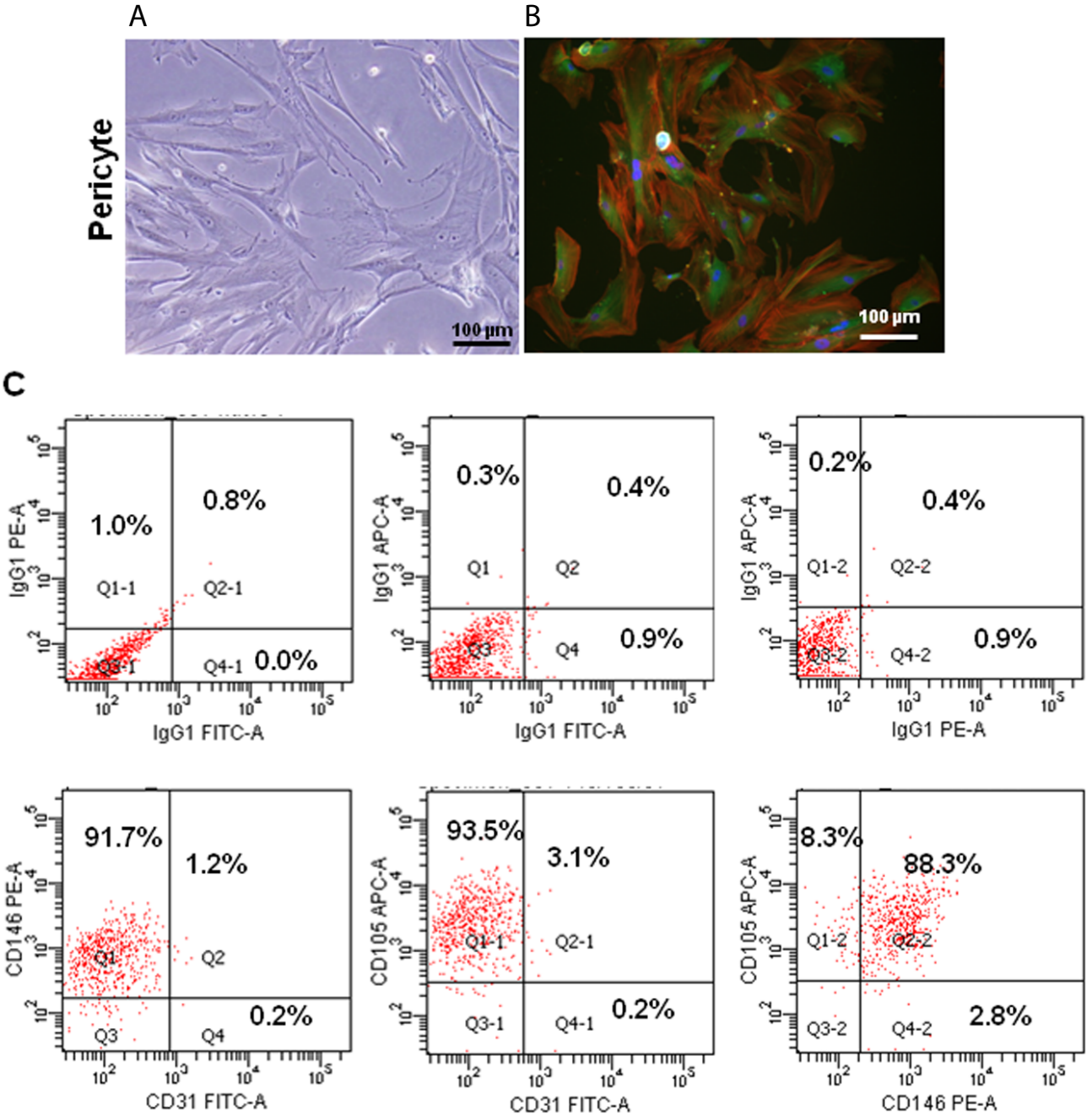

Figure 1. Morphology and characterization of human umbilical cord vein pericytes. A) Phase-contrast microphotographs showing human umbilical vein pericytes, scale bar = 100 µm. B) Representative immunofluorescence images of the pericyte stained with anti-MAP1B (green), actin-phalloidin (red), and nucleus-DAPI (blue), scale bar = 100 µm. C) Representative FACS analysis of pericytes. Cells highly expressed CD105 and CD146 (>90.0%) and lacked CD31 (<3.0%) markers.

cells highly expressed CD105 and CD146 (>90.0%) and lacked CD31 (<3.0%) markers (Figure 1C).

3.2. Pericytes are superior to osteoblasts to enhance the expansion of hematopoietic progenitors

We set out to determine whether human cord vein pericytes or osteoblasts derived from the UCB MSCs (Supplementary Figure 2) differed in their capacity to support without contact (transwell insert) the expansion of UCB hematopoietic progenitors. In parallel, we also tested whether media conditioned with either one could recapitulate the beneficial effects of coculture. All UCB CD34 +-enriched cell cultures were carried out with the

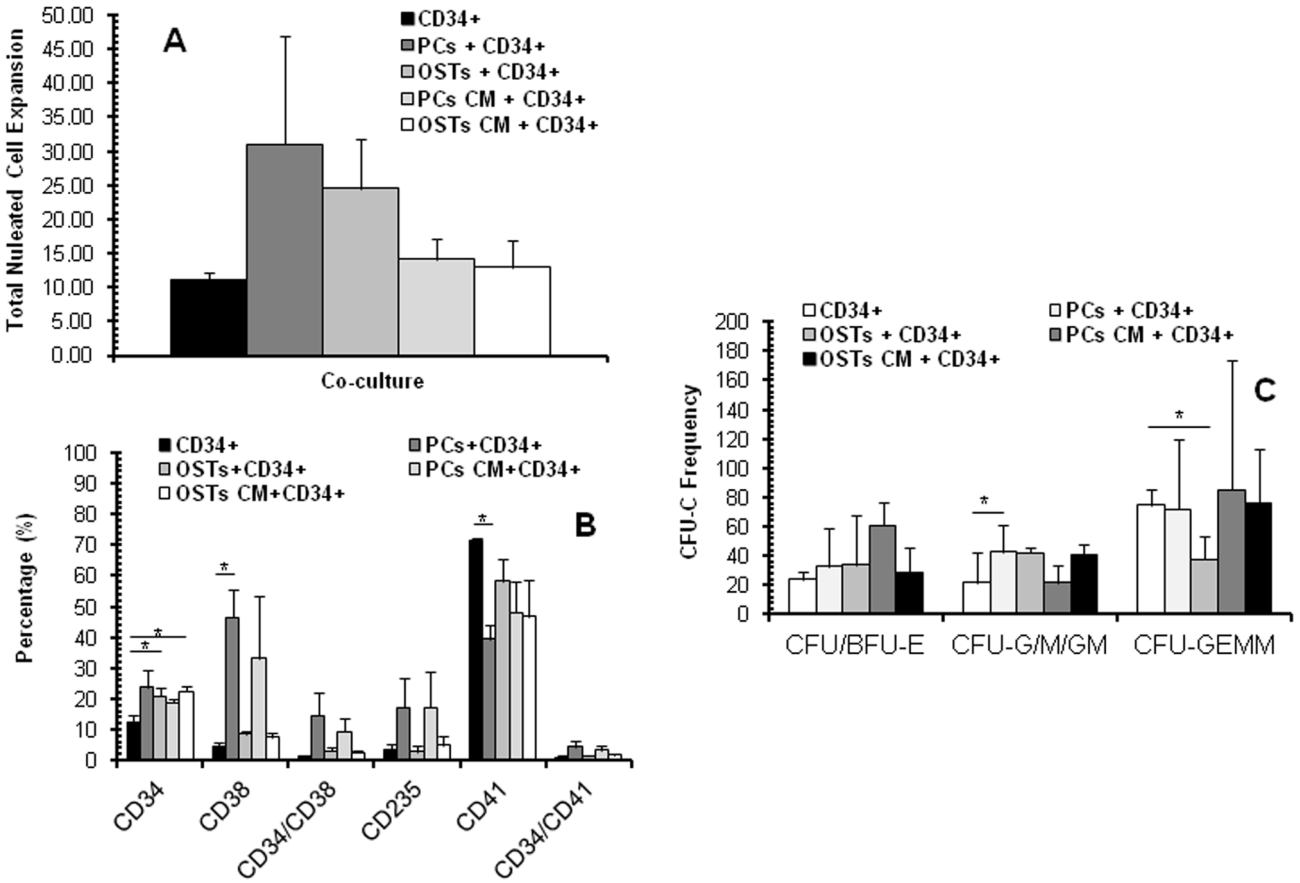

Figure 2. Expansion and differentiation of umbilical cord blood CD34 + cells in cocultures and medium conditioned with pericyte and osteoblast. CD34 + cells were grown in conditioned media (CM) or in coculture for 7 days at 37 °C with the cytokine cocktail OMPC. Cocultures were done with pericytes (PCs + CD34 +) or MSC-derived osteoblasts (OSTs + CD34 +) without contact. Control (CD34 +) cultures are shown for each setting. A) Total nucleated cell expansion per seeded cells. B) Frequencies of the cells in cultures at day 7. The following marker recognize these cell population: CD34 +, immature cells; CD38 +, mature progenitor cells; CD38 + CD34 +, more mature progenitor cells; CD34 + CD41 +, early committed megakaryocytes; CD41 +, megakaryocytes; CD235 +, erythroids. C) Frequencies of myeloid progenitors (CFU, colony forming unit; BFU-E, burst-forming erythroids; CFU-E, CFU erythroids; CFU-G/M/GM, CFU-granulocyte/monocytes/granulomonocytes; CFU-GEMM, CFU-granulocytes-erythroids-monocytes-megakaryocytes (mixed colony)) at day 7 of culture. Mean ± SEM of four independent experiments is presented. Significant differences were determined by paired multigroup ANOVA test; *: $P < 0.05$.

same serum-free medium supplemented with the cytokine cocktail OMPC over a period of 7 days at 37 °C.

The growth of UCB cells was the greatest in pericyte cocultures, increased by 2.8-fold ($P > 0.05$) versus the control (Figure 2A). Cell frequency also tended to be greater in the cocultures with osteoblasts versus the control being increased 2.2-fold ($P > 0.05$, Figure 2A), while it was modestly promoted in pericyte CM and osteoblast CM. The frequencies of CD34 + cells in pericyte- and osteoblast-based cultures were higher than those seen in the CD34 +-alone control (23.9 ± 5.7% and 18.8 ± 1.2% in pericyte coculture and pericyte CM, $P > 0.05$; 20.8 ± 2.7% and 22.6 ± 1.7% in osteoblast coculture and osteoblast CM vs. 12.5 ± 2.3% in control, $P < 0.05$, Figure 2B). The increased growth in pericyte and pericyte CM cultures was largely the result of increased frequency of CD38 + cells (46.5 ± 9.3% and 33.3 ± 20.1%, respectively), CD34 + CD41 + megakaryocyte progenitors (4.3 ± 2.1% and 3.2 ± 1.7%, respectively), and CD235 + erythroblasts (17.3 ± 9.3% and 17.1 ± 11.6%, respectively, Figure 2B). In contrast, the percentage of CD41 + megakaryocyte-committed cells was significantly reduced, an effect partially lost in all cocultures and CM cultures (pericyte cocultures: 39.3 ± 4.6%; osteoblast cocultures: 58.3 ± 7.2%; pericyte CM: 48.1 ± 10.3%; osteoblast CM: 46.9 ± 11.8%) versus the control (71.7 ± 0.8%, Figure 2B).

To address the impacts of the cocultures and CM on HPC frequencies, we measured the total number of myeloid progenitors by colony assays (Figure 2C). Myeloid colonies were scored based on their morphology (BFU-E: burst-forming erythroids, CFU-E: CFU erythroids, CFU-G/M/GM: CFU granulocytes/monocytes/granulomonocytes, CFU-GEMM: CFU-granulocytes-erythroids-monocytes-megakaryocytes (mixed colony)). The net CFU-C expansion was greater in pericyte and pericyte CM cultures than the control (2.5-fold and 1.8-fold; data not shown). The frequency of erythroid progenitors (CFU-E/BFU-E) was greater in all culture conditions than the control (Figure 2C) and the frequency of granulomonocytic colonies (CFU-G/M/GM) was significantly greater (2.0-fold, $P < 0.05$) in the pericyte cocultures than that seen in the control (Figure 2C). However, enhanced CFU-GEMM progenitor frequencies were observed in the control versus osteoblast cocultures (2.0-fold, $P < 0.05$, Figure 2C). Taken together, these results demonstrate that pericyte coculture and pericyte CM can be advantageous for the expansion of hematopoietic cells ex vivo.

3.3. Comparisons of soluble factors present in pericyte and osteoblast conditioned medium

Finally, we set out to determine whether we could identify soluble factors released by pericytes that could recapitulate the improved expansion of the CB cells. The pattern of 80 cytokines/chemokines/growth factors released by the pericyte was compared to osteoblasts from four independent donors by cytokine array (RayBiotech, AAH-CYT-5-8). A total of 29 factors were found to be secreted by pericytes at higher rates than by osteoblasts. The most secreted growth factor by pericytes was VEGF as compared to osteoblasts (1.3-fold, Figure 3).

4. Discussion

The vascular niche is a site rich in blood vessels, whereas endothelial cells, pericytes, and smooth muscle cells create a microenvironment that recruits MSCs and HSCs, which is important for stem cell mobilization, proliferation, and differentiation (Ribatti et al., 2015). In the BM vascular niche, continuous exchange between the sinusoidal (mobilizable and proliferative) and the arterial (quiescent and dormant) niches contributes to maintain a tightly controlled balance between proliferation and dormancy in HSCs (Kunisaki et al., 2014). Himburg et al. and Kobayashi et al. suggested that soluble factors produced by BM perivascular cells may be responsible for HSC self-renewal and maintenance in vivo, but the detailed mechanisms remain unknown (Himburg et al., 2010; Kobayashi et al., 2010; He et al., 2014). Perivascular cells, namely pericytes, which are in close association with endothelial cells, have been shown to possess stem cell-like qualities and have thus been hypothesized to be the in vivo counterparts of MSCs (Diaz-Flores et al., 2009). Crisan et al. mentioned that human pericytes were negative for hematopoietic and endothelial cell markers (CD45 and CD31/CD34, respectively) and positive for CD146 (Crisan et al., 2008). Additional markers commonly used to define pericytes include PDGFRβ, NG2, desmin, MAP1B, and α-SMA (Crisan et al., 2008). It was reported by Crisan et al. as well that pericytes located on venules were NG2 -/α - SMA + and on arterioles NG2 +/α - SMA + (Crisan et al., 2012). In the present study, we derived CD146 + perivascular cells from the UC vein after enzymatic dissociation and magnetic separation. Our flow cytometric and immunofluorescent analyses showed that CD146 + cells expressed pericyte marker MAPB1 and they were positive for pericyte/MSC markers CD146 and CD105. In addition, we previously reported that CD34 expressions of pericytes are very low as are MSCs (3.0 ± 0.6 vs. 1.2 ± 0.2) (Gökçinar-Yagci et al., 2015).

Pericytes have been shown to play a role in niche maintenance for hematopoietic stem cells in the BM (Kunisaki et al., 2013). It has been also mentioned that CD146 + cells were uniquely able to maintain self-renewing HPCs after 2 weeks in culture without growth factors (Corselli et al., 2013). On the other hand, quiescent HSCs are specifically associated with small caliber arterioles, which are predominantly distributed in the endosteal BM (Kunisaki et al., 2014). Arterioles are associated with both quiescent NG2 + niche cells and HSCs, suggesting that the vessel itself may be a critical gatekeeper of stem cell quiescence in the BM (Kunisaki et al., 2013). Unlike the BM, the umbilical vein carries oxygenated, nutrient-rich blood from the placenta to the fetus and umbilical arteries carry deoxygenated, nutrient-depleted blood from the fetus to the placenta (Wang et al., 2010). In this context, the question remains whether HSCs that are in close contact with umbilical vein cells are functionally similar to those located near arterioles in the BM. In order to define their direct role in hematopoiesis, we tested the CD146 + perivascular cells as compared with osteoblasts derived from UCB MSCs to sustain human UCB HPCs in noncontact coculture settings or in culture media previously conditioned by these cells. As distinct from Corselli et al., who mentioned that the supportive effect of CD146 + pericytes required cell-to-cell contact (Notch ligand Jagged-1, abundantly expressed at the surface of these pericytes, interacting with its receptor Notch1 expressed on HSCs (Corselli et al., 2013)), we found the greatest HPC expansion with pericyte cocultures (2.8-fold vs. the control) without contact and we obtained a high frequency of CD34 +, CD38 +, CD34 + CD41 +, and CD235 + cells with pericyte cocultures and pericyte CM. In this work, we tried to answer the question addressed by Levesque of whether CD146 + pericytes could support the actual ex vivo expansion of human reconstituting HSCs by

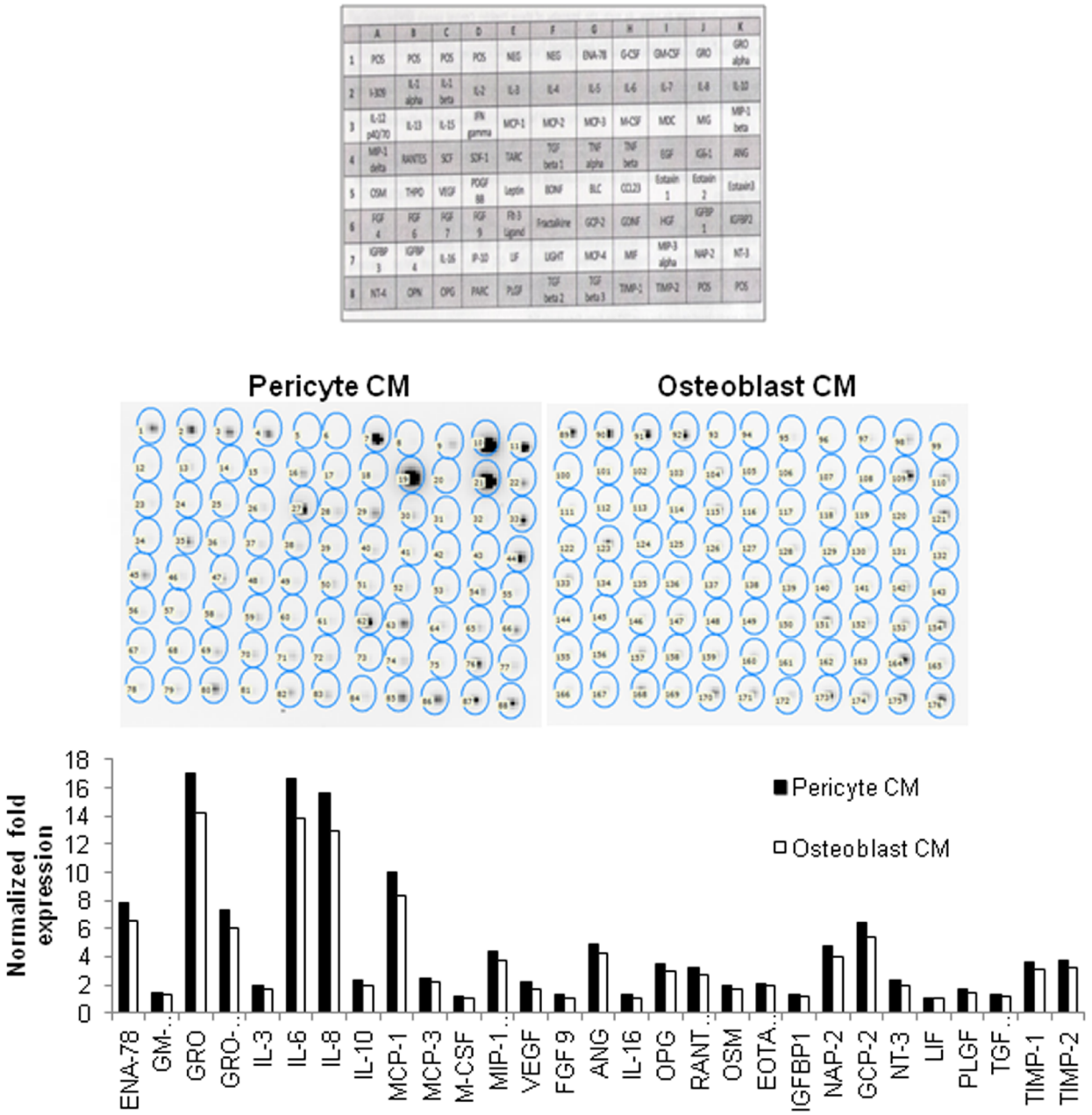

Figure 3. Soluble factors in pericyte and osteoblast conditioned media. Expression level presented as arbitrary units (AU). Normalized fold increase in pericyte CM vs. osteoblast CM (n = 4).

quantifying the content in reconstituting HSCs before and after coculture (Levesque, 2013).

We previously demonstrated that contact is not required to promote the expansion of hematopoietic stem/progenitor cells (HSPCs) ex vivo and the media conditioned with BM MSC-derived osteoblasts improves engraftment of the expanded HSPCs, clearly establishing that soluble factors released by osteoblasts have major modulatory activities on HSPCs (Dumont et al., 2014). It is known that pericytes interact with endothelial cells to support vasculature growth, maintain vessel integrity, and promote resistance of endothelial cells to antiangiogenesis therapies. They can protect endothelial cells from VEGF withdrawal by activating alternative proangiogenic pathways (Potente et al., 2011). It was shown that pericytes release VEGF, which acts as a chemotactic agent and survival factor for proliferating endothelium (Darland et al., 2003; Hall, 2006). Identification of the soluble factors

responsible for the rise in HPC expansion would certainly represent a major breakthrough. We set out to determine whether we could identify soluble factors released by pericytes that could recapitulate the improved expansion of the UCB cells. A total of 29 factors were found to be secreted by pericytes at higher levels than osteoblasts. The most secreted growth factor by pericytes compared to osteoblasts was VEGF. This production may indirectly affect HPC expansion and differentiation.

In conclusion, this study first demonstrates that soluble factors released by pericytes and UCB MSC-derived osteoblasts have important modulatory impacts on the growth and differentiation of UCB CD34 + cells.

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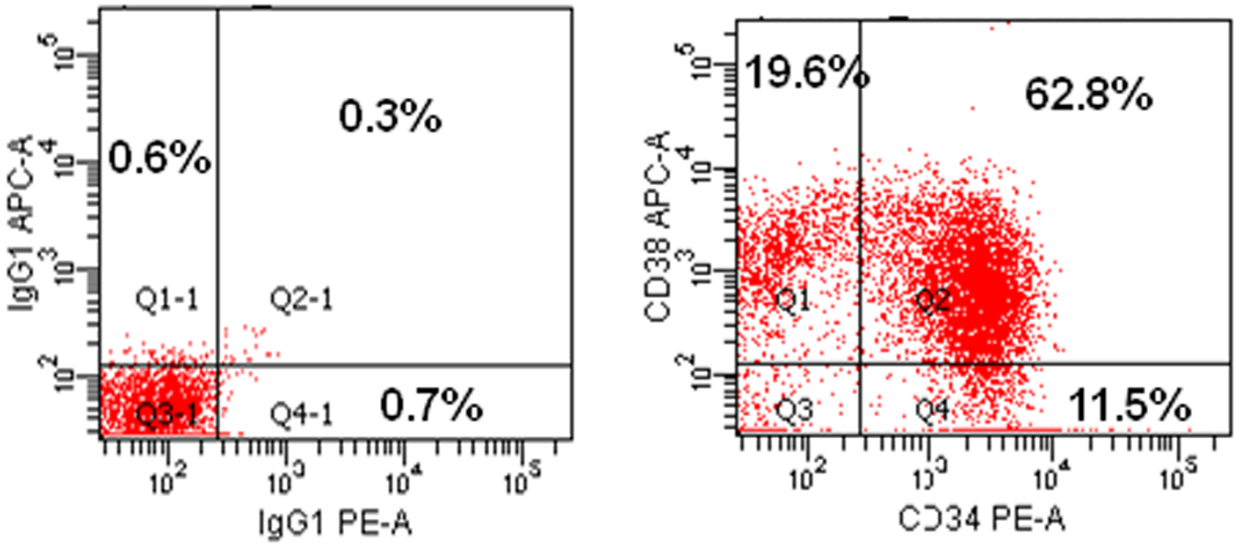
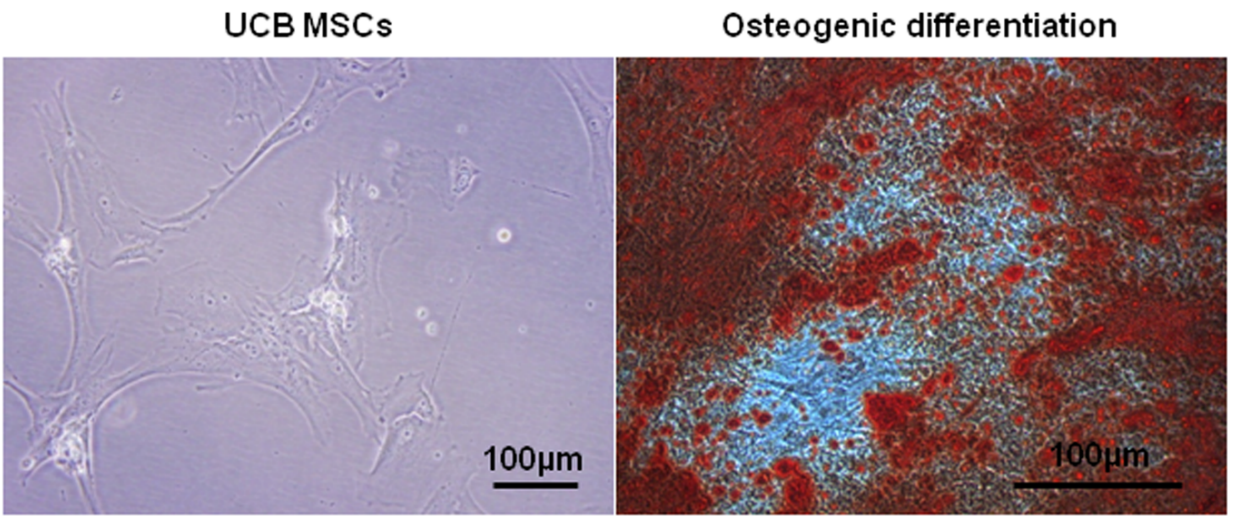


Supplementary Figure 1. Representative flow cytometry analysis of CD34 and CD38 expression by UCB CD34 + cells after purification at day 0.



Supplementary Figure 2. Osteogenic differentiation of human umbilical cord blood mesenchymal stem cells. Phase-contrast microphotographs showing human UCB MSCs at passage 2 and Alizarin Red staining of cells at day 7 of culture, scale bar = 100 µm.