Endothelial cells provide a niche for placental hematopoietic stem/progenitor cell expansion through broad transcriptomic modification☆

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Received 9 January 2013; received in revised form 17 July 2013; accepted 31 July 2013
Available online 9 August 2013

Abstract Umbilical cord blood (UCB) is an attractive source of hematopoietic stem cells (HSCs). However, the number of HSCs in UCB is limited, and attempts to amplify them in vitro remain inefficient. Several publications have documented amplification of hematopoietic stem/progenitor cells (HSPCs) on endothelial or mesenchymal cells, but the lack of homogeneity in culture conditions and HSC definition impairs direct comparison of these results. We investigated the ability of different feeder layers, mesenchymal progenitors (MPs) and endothelial cells (ECs), to amplify hematopoietic stem/progenitor cells. Placental derived HSPCs (defined as Lin⁻CD45⁻/dimCD34⁺CD38⁻CD90⁺) were maintained on confluent feeder layers and the number of cells and their marker expression were monitored over 21 days. Although both types of feeder layers supported hematopoietic expansion, only endothelial cells triggered amplification of Lin⁻CD45⁻/dimCD34⁺CD38⁻CD90⁺ cells, which peaked at 14 days. The amplified cells differentiated into all cell lineages, as attested by in vitro colony-forming assays, and were capable of engraftment and multi-lineage differentiation in sub-lethally irradiated mice. Mesenchymal progenitors promoted amplification of CD38⁺ cells, previously defined as precursors with more limited differentiation potential. A competitive assay demonstrated that hematopoietic stem/progenitor cells had a preference for interacting with endothelial cells in vitro. Cytokine and transcriptomic analysis of both feeder cell types identified differences in gene expression that correlated with propensity of ECs and MPs to support hematopoietic cell amplification and differentiation respectively. Finally, we used RNA sequencing of endothelial cells and HSPCs to uncover relevant networks illustrating the complex interaction between endothelial cells and HSPCs leading to stem/progenitor cell expansion.

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Author contributions: Christophe M. Raynaud: conception, design and conduct of experiments; data analysis; editing of the manuscript; Jason M. Butler: conception, design and analysis of in vivo analysis; Najeeb M. Halabi: genomic data analysis; Faizzan S. Ahmad: assistance in editing the manuscript; Ahmed Badreldeen: assistance with provision of tissue and cord blood samples; Shahin Rafii: assistance with design of the experiments and provision of the cell line; Arash Rafii: assistance with designing the experimental procedures; writing the manuscript.

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http://dx.doi.org/10.1016/j.scr.2013.07.010

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Introduction

There is an increasing need of HSC transplantation for treatment of hematological disorders. Bone marrow is the main source of hematopoietic stem cells (HSCs) (Ballen, 2005; Broxmeyer et al., 1989, 1990, 1991; Copelan, 2006). However, researchers have investigated other sources of HSCs to overcome the challenges of finding compatible donors and the invasiveness of the procedure. Umbilical cord blood derived HSCs have been extensively investigated and used in the clinical setting (Tse et al., 2008a, 2008b). However, single cord blood samples contain insufficient numbers of HSCs to effectively treat adults requiring bone marrow transplantation (Brunstein et al., 2007; Majhail et al., 2006).

To overcome these threshold limitations, researchers have investigated optimal methods for ex vivo HSC amplification. Schiedlmeyer et al. used the ectopic expression of HOXB4 to obtain significant HSC expansion (Schiedlmeyer et al., 2007). Several studies have investigated the use of multiple cytokines to increase HSC numbers in culture without being able to clearly define the optimal cocktail (Metcalf, 2008; Zhang and Lodish, 2008). Delaney et al. amplified HSCs on Jagged 1 coated plates, but the expanded HSCs failed to achieve long-term engraftment, suggesting amplification of hematopoietic progenitors (Delaney et al., 2010).

Recently, investigators have tried to define the HSC niche in vivo (Levesque et al., 2010) in order to use its signaling pathways to expand HSCs while maintaining their stemness. The presence of HSCs in the cord blood suggests that the placenta and its cellular elements might also contain a niche for HSCs. Indeed hematopoietic progenitors and long-term culture-initiating cells have been found in the human placenta between 8 and 17 weeks of gestation (Barcena et al., 2009; Chen et al., 2004). Several follow-up studies also described the role of placenta in amplification of HSCs up to term (Robin et al., 2009; Serikov et al., 2009; Zhang et al., 2004).

The close proximity of HSCs to cellular elements in the placenta and the bone marrow suggests a requirement for complex signaling networks and has motivated the development of cellular based HSC expansion platforms. Many studies describe the expansion of HSCs on mesenchymal progenitor (MP) or endothelial cell (EC) feeder layers (Andrade et al., 2010; Butler et al., 2010; Fei et al., 2007; Hayashi et al., 2009; Huang et al., 2007; Jang et al., 2006; Levesque et al., 2010; Li et al., 2006; Mendez-Ferrer et al., 2010; Robin et al., 2009; Sasaki et al., 2010; Walenda et al., 2010; Yildirim et al., 2005; Zhang et al., 2004). HUVECs (human umbilical vein endothelial cells) have been used as a model for an endothelial niche (Butler et al., 2010; Kobayashi et al., 2010; Li et al., 2006; Yildirim et al., 2005) while bone marrow- and placental-derived mesenchymal progenitors (BM-MPs and PL-MPs) have been used as a surrogate for a mesenchymal niche (Andrade et al., 2010; Hayashi et al., 2009; Macmillan et al., 2009; Mendez-Ferrer et al., 2010; Resnick et al., 2010; Walenda et al., 2010). For instance, Robin et al. demonstrated that placenta derived perivascular stromal cells expressing CD13, CD29, CD44 and CD105 could support hematopoiesis in the placenta (Robin et al., 2009). Similarly, Yildirim et al. published that HUVECs could expand cord blood CD34+ cells (Yildirim et al., 2005). The hypothesis underlying these experiments is that secreted factors and membrane bound elements of the feeder cells will provide the signaling cues allowing cell expansion with maintenance of stemness. However, due to large variations in the cytokines used, a standardized protocol for the expansion of clinical grade HSCs is yet to be devised. Furthermore, the use of fetal bovine serum (FBS) may also modify the effects of components produced or secreted by the feeder cells themselves, confounding attempts to identify the key feeder derived factors required for HSC expansion.

Recently, our group elucidated the role of the endothelium and secretion of angiocrine factors in the expansion of mouse and human HSCs (Butler et al., 2010a, 2010b; Kobayashi et al., 2010). We developed a model of endothelial cells with autonomous Akt-activation (HUVECs-E4ORF1, referred to as E4'ECs) that can survive in the absence of FBS and exhibit secretion of angiocrine factors. We demonstrated that the E4'EC platform supported the expansion of serially engraftable HSCs. Similarly, we have isolated and comprehensively characterized mesenchymal progenitors from the placenta which are also able to survive in serum free conditions (Raynaud et al., 2012). However, few studies to date have systematically compared the capacity of endothelial and mesenchymal progenitors to facilitate the expansion of UCB-derived HSPCs. Therefore, in this study we compare the abilities of our previously engineered E4'EC platform and mesenchymal progenitors to support hematopoietic stem/progenitor cell (HSPC) proliferation in a serum-free environment.

Methods

Cord/placental blood collection and fluorescence-activated cell sorting of HSPCs

Following approval from the Internal Review Board (HMC-IRB protocol 9109/09, Weill Cornell Medical College, Qatar), cord blood and placental tissue were collected from donors at the Women’s Hospital at Hamad Medical Corporation immediately after elective Cesarean section. Exclusion criteria were absence of labor, preterm rupture of membrane (chorioamnionitis), and the presence of known chromosomal abnormalities.

In this study to retrieve the cord blood and placental hematopoietic stem and progenitor cells (HSPCs), we carefully aspirated and milked the placenta through the cord after detachment of the placenta. To obtain sufficient numbers of HSPCs, two to three cord/placenta samples were pooled for each experiment. Each experiment was repeated three to six times, and error bars in figures represent variability in the analysis of biological replicates.

Mono-nucleated cells were isolated after centrifugation on Ficoll gradients. CD34+ cells were enriched using magnetic beads labeled with anti-CD34 monoclonal antibodies (Miltenyi Biotech, 130-046-702) and separated on an affinity column following the manufacturer’s instructions. Hematopoietic stem/progenitor cells defined as Lin CD45dim/CD34+CD38-CD90+ (Supplementary Fig. 1), were further purified by fluorescence-activated cell sorting (FACS). The following antibodies were used anti-CD34-APC-Cy7 (BD Biosciences, 343514), CD38-PerCP-Cy5.5 (BD Biosciences, 551400), CD90-AF700 (BD Biosciences, 550402), CD45-AmCyan (BD Biosciences, 553400).
339192), and Lin-FITC (BD Biosciences, 340546). The workflow of the isolation and co-culture is given in Supplementary Fig. 1.

In our study, the antibody anti CD45-AmCyan was used to sort and define hematopoietic cells. The clone 2D1 from BD Biosciences used in this publication was previously shown to recognize CD45RA, CD45RB and CD45RO isoforms of CD45 (Behm et al., 1992). Therefore our definition of hematopoietic stem cells might be more restrictive than those previously published (Majeti et al., 2007; Seita and Weissman, 2010). In recognition of this, we hereafter refer to populations purified on the basis of these markers as hematopoietic stem/progenitor cells (HSPCs).

**E4**+ECs and MPs and co-cultures

E4**+**ECs were obtained as previously described (Seandel et al., 2008). The model of Akt-activated HUVECs (HUVECs-E4ORF1 (referred here as E4**+**ECs)) can survive in the absence of FBS and exhibit increased secretion of angiocrine factors. As previously published, HUVECs transformed with E4ORF1 have not been subjected to sub-cloning and form, like their mesenchymal progenitor counterpart, multi-clonal and heterogeneous primary cells.

E4**+**ECs were expanded in M199 supplemented with 20% FBS, 20 μg/ml β-ECGF (Sigma, E1388), 20 U/ml heparin (Sigma, H4784), and 1% penicillin/streptomycin (Thermo Scientific, SV30079.01).

PL-MPs were isolated from placenta and characterized as described previously (Raynaud et al., 2012). Cells were cultured in MP media: DMEM low glucose with 20% FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin (Barlow et al., 2008).

Bone marrow mesenchymal progenitors (BMS) were purchased from StemCells Inc. (#MSC-001F, StemCells Inc.) and cultured in the same conditions as PL-MPs.

24 h prior to co-culture with HSPCs, E4**+**ECs and MPs were seeded at 90% confluence on 24 well plates in HSPC culture media: X-VIVO media (Lonza, 04-418Q) supplemented with 50 ng/ml rhSCF (PeproTech, 300-07), 20 ng/ml rhTPO (PeproTech, 300-18), 50 ng/ml rhFlt3 (PeproTech, 300-19), and 1% penicillin/streptomycin (hereafter referred to as STF media). Media were changed just before sorted hematopoietic cells were seeded on feeders. A total of 10,000 Lin−CD45dim−/CD34+CD38−CD90+ were directly sorted onto the feeder layer in STF media (described below). All co-culture experiments were done in similar media regardless of the feeder layer.

**Ex vivo expansion of HSPCs**

Hematopoietic cells, either alone or on a feeder layer, were expanded in STF media. Half of the media were changed every day for the subsequent 21 days. We analyzed the cell

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**Figure 1**  CD34+CD31− localize in close proximity of the endothelium in the placenta. A–H: Confocal microscopy imaging of placental cross-section. HSCs were defined by double staining CD34+CD31− (green arrows) CD34-FITC, CD31-PE, CD73-APC and DAPI were used. I–P: Transversal imaging of a placental vessel. HSCs are defined as CD34+CD31− (green arrows) and endothelial cells are defined as CD34+CD31+ (yellow arrows). P. Large non vascular areas containing placental mesenchymal progenitors defined as CD73+ (*).
population every 7 days by polyvariate flow cytometry to determine the total number of hematopoietic cells and their specific marker expression. Feeders were changed every 7 days as follows: Lin+ cells were depleted using a magnetic bead-based Lin depletion kit (Miltenyi Biotech, 130-092-211). The remaining negative fraction was seeded on a fresh monolayer of E4+ECs or MPs as previously described or without a feeder layer as a negative control (Supplementary Fig. 1).

**Conditioned media**

To prepare conditioned media, E4+ECs were plated as per co-culture method previously described with STF media. Daily, half of the media conditioned by E4+ECs were collected and centrifuged at 1500 rpm for 5 min to eliminate cell debris and then passed through a 0.2 μm filter before being used. These conditioned media were used to replace half of the media in hematopoietic cell cultures on a daily basis.

**CD34 enrichment and Lin depletion**

CD34 positive selection before sorting and Lin depletion were performed using, respectively, the CD34 MicroBead Kit (Miltenyi Biotech, 130-046-702) and the Lineage Cell Depletion Kit (Miltenyi Biotech, 130-092-211), according to the manufacturer’s instructions. Briefly, cells were washed in X-VIVO media and re-suspended at 10⁶ cells per 300 μl, and then mixed with 100 μl per 300 μl of FcR Blocking Reagent (Miltenyi Biotech, 130-059-901) and 100 μl of CD34/Lin beads per 10⁸ cells. Samples were then incubated for 30 min at 4 °C. After washing, magnetic separation was performed on LS columns (Miltenyi Biotech, 130-042-401).

**In vivo transplantation**

All mice experiments were approved by the Weill Cornell Medical College Institutional Review Board. Eight week old NOD.Cg-Prkdcsidll2rgtm1Wjl/SzJ (NSG) mice were sublethally irradiated (250-rads) and transplanted with 1 × 10⁵ CD34⁺ ex vivo expanded human cord blood cells. Engraftment was assessed at 3-weeks and at 12 weeks by assessing the number of human CD45+ cells in the peripheral blood. At 12 weeks, the femurs were removed and the absolute number of human hematopoietic stem/progenitor cells (HSPCs) and the percentage of lineage-specific human cells were determined. Post engraftment HSPC analyses were performed as previously published (Butler et al., 2012). The in vivo results are the average of 3 independent experiments; each experiment was performed with 5 mice in each cohort.

**In situ immuno-fluorescence**

Placenta specimen (5 × 5 × 5 mm) were embedded in OCT (Optimal Cutting Temperature) compound (Sakura, 4583) and snap-frozen in liquid nitrogen, before storage at −80 °C; 7–8 μm sections were prepared using an AS 620 SME cryotome (Thermo Shandon) and fixed in acetone. Non-specific sites were blocked for 30 min with blocking media: PBS supplemented with 0.3% bovine serum albumin, 0.5% FBS and FcR

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**Figure 1** (continued).
Blocking Reagent. Sections were then incubated with primary antibodies in blocking media for 1 h, washed twice in PBS containing 0.5% Tween 20 (Sigma-Aldrich, P5927), and counterstained with 4',6-diamidino-2-phenylindole (DAPI). Primary antibodies were mouse anti-human CD31-PE (BD Bioscience, 553373), CD34-FITC (BD Bioscience, 345801), CD73-APC (BD Bioscience, 560847) and VEGFR2-APC (BD Bioscience, 560070). Slides were mounted with the Fluoromount Kit (Molecular Probes, P36935). Sections were analyzed with a Zeiss confocal microscope, Laser Scanning Microscope 710 (Carl Zeiss), and images were analyzed using the Zen 2008 V5.0,0228 software (Carl Zeiss).

**Immuno-staining and FACS analysis**

For flow analysis of cell-surface antigens the antibodies described above were used (CD45-AmCyan, Lin-FITC, CD34-APC-Cy7, CD38-PerCP-Cy5.5, and CD90-AF700). Briefly, 10^6 mono-nucleated cells were blocked for 30 min on ice in PBS containing 5% FBS, 1%BSA, and 1%FcR Blocking Reagent. Next, the cell suspension was incubated with specific antibodies for 45 min on ice. After wash in PBS, cells were analyzed by FACS on a SORP (Special Order Research Products) FACS Aria II (BD Biosciences) as described below. Data were processed using the FACSDiva 6.3 software (BD Biosciences). Following the technical

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**Figure 2**  Hematopoietic stem/progenitor cell expansion and differentiation on different feeders.  A – Phase contrast imaging of placental sorted HSPCs on different feeders. 10,000 sorted cells are cultured without feeder, on a E4+EC or placental-MP feeder. Half of the media are changed every day, Lin depletion is performed every 7 days and HSPCs are seeded on a new feeder.  B – Chart of flow cytometry analysis of total hematopoietic cells at different time-points.  C – Chart representing flow cytometry analysis of Lin^- CD45^-dimCD34^+CD38^- CD90^+ on different feeders during the expansion protocol (N = 6; ** P < 0.01).  D – Phase contrast microscopy of colony forming unit (CFU) assays carried out on MethoCult for 15 days. HSPC differentiation in CFU-E, CFU-M, CFU-G and CFU-GM is depicted.  E – Charts representing the ratio of different CFUs for HSCs after isolation from placenta, and expanded without feeder, on E4+ECs or Pl-MPs.  F – Comparison of the CD34/CD38 profiles for Lin^- CD45^+ hematopoietic cells during the expansion process.  G – Dot plot representation of CD34/CD38 profile of Lin^- CD45^+ cells cultured without feeder on Pl-MPs or E4+ECs.  H – Charts comparing the relative ratio of CD38^- of Lin CD45^-CD34^+ hematopoietic cells at different time-points during expansion on different feeders.
recommendation of Perfetto et al., doublets were excluded by FSC-W × FSC-H and SSC-W × SSC-H analysis, single-stained channels were used for compensation, and fluorophore-minus-one (FMO) controls were used for gating. When possible, a total of 500,000 events were acquired per sample (Perfetto et al., 2004). Lin−CD45−/dimCD34+CD38−CD90+ cell sorting was performed using the same parameters used for analysis and a purity mask was applied for sorting. Analysis of a fraction of sorted cells revealed that the purity of Lin−CD45−/dimCD34+CD38−CD90+ cells was 99% (data not shown). eGFP-E4+ECs were also used for cell sorting experiments to preclude the possibility that detaching endothelial cells contributed to the analysis of hematopoietic co-cultures.

Colony-forming assay

For colony-forming assays, 10^4 hematopoietic cells were cultured in 35 mm dishes containing MethoCult 3434 (StemCell Technologies), following the manufacturer’s protocols. Colonies were counted on day 12.

Transcriptomic analysis

Following 3 days of culture in STF media, PL-MP and E4+EC RNA was isolated using TRizol reagent followed by additional purification using the RNAeasy extraction kit from Qiagen (Qiagen, 74106), with RNA yields that produced satisfactory microarray data. Two quality-control measures were carried out: (1) spectrophotometric analysis, and (2) size-fractionation using a microfluidics instrument (Agilent Technologies, G2938C). Samples of total RNA (200 ng) were analyzed on Affymetrix GeneChip Human Genome U133 Plus 2.0 Arrays. Data were analyzed using the Partek Software (Affymetrix, V6.09.1110-6). Class comparison between E4+ECs and PL-MPs (three biological replicates of each) was performed to identify gene-expression changes with significant expression differences (P < 0.05) and 2-fold

**Figure 2 (continued).**
increased or decreased expression. Partek Software gene ontology tools were used to determine enrichment of functional categories (Dennis et al., 2003). Raw data are available in ArrayExpress with the accession number E-MEXP-3725.

RNA sequencing and analysis

Lin^-CD45^-/dimCD34^+CD38^-CD90^+ cells sorted from cord blood or after 7 days of culture on E4+EC feeder were submitted to RNA isolation as previously described. An additional DNase1 (Roche, 04716728001) digestion step was performed to ensure that samples were not contaminated with genomic DNA. Duplicates of each sample were performed. Library preparation and paired end sequencing (50 bp length) were done at the Weill Cornell Qatar Genomics Core Facility using Illumina HiSeq 2000 sequencer. Short reads were aligned to the reference human genome (Hg19) using TopHat (with bowtie 1) (Trapnell et al., 2009). Gene annotations were from Ensembl packaged into iGenome (Illumina). Alignments were processed with easyRNASeq (Delhomme et al., 2012) using the geneModel method to obtain a count table for each sequence. Differential expression was analyzed with the edgeR package after filtering out genes with counts of less than 10 counts per million (cpm) across all categories (Robinson et al., 2010). Differential expression was considered significant at a Benjamin-Hochberg FDR of 0.05.

Ingenuity Pathway Analysis

We used Ingenuity Pathway Analysis software (Ingenuity Systems) to identify and analyze relevant pathways from the gene lists obtained from the comparison of E4^+ECs and PL-MPs or RNA sequencing. Networks were constructed by overlaying the genes in the gene list onto a global molecular network developed from information contained in the Ingenuity Knowledge Base using keywords such as "proliferation/differentiation of hematopoietic cells." Networks of the genes up- or down-regulated in PL-MPs compared to E4^+ECs were then algorithmically generated based on genes known connectivity. A network is a graphical representation of the molecular relationships between genes. In the networks, genes are represented as nodes, and a biological relationship between two nodes is represented as a line. All edges are supported by at least one reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Knowledge Base. P values for the enrichment of canonical pathways were then calculated based on the hypergeometric distribution and with the right-tailed Fisher’s exact t-test for 2 × 2 contingency tables. To select E4^+EC cytokines and membrane up-regulated genes, compared to PL-MPs, upregulated genes were sorted according to their sub-cellular localization in ingenuity and a selection of membrane or extracellular domain genes was done. We inter-connected genes up/down-regulated in Lin^-CD45^-/dimCD34^+CD38^-CD90^+ cells cultured on E4^+ECs compared to Lin^-CD45^-/dimCD34^+CD38^-CD90^+ cells isolated directly from the cord blood/placenta with genes upregulated in E4^+ECs compared to PL-MPs. To do so, both lists previously generated were uploaded in Ingenuity Pathway Analysis software and direct/indirect relationship was built between those two lists using Ingenuity Knowledge Base.

Results

CD34^+CD31^- cells localize in close proximity to endothelial cells in the placenta

The placenta and in particular its stromal cells have been described as a niche for HSCs (Barcena et al., 2009; Robin et al., 2009; Sasaki et al., 2010; Serikov et al., 2009; Zhang et
Recent studies in human and mouse models suggest that endothelial cells (ECs) might play an important role in the HSC niche (Butler et al., 2012; Butler et al., 2010; Kobayashi et al., 2010). As the placenta could be a source of HSCs, we decided to compare whether PL-MPs and/or ECs constitute a platform for the maintenance and expansion of human HSPCs. First, we performed confocal analysis of term placenta to attempt to localize hematopoietic progenitors/stem cells within the placental structure. The placenta is a highly vascularized structure with CD31+CD34+ ECs distributed among dense CD73+ MP-rich stromal regions (Supplementary Figs. 2A–G) (Raynaud et al., 2012; Serikov et al., 2009). We were able to locate CD31−CD34+ cells in close proximity to ECs (Figs. 1A–H), often within the lumen of the

**Figure 3** Human CD34+ hematopoietic cells co-cultured with E4+ECs give rise to multi-lineage engraftment. A — Percentage of engrafted of peripheral blood human CD45+ hematopoietic cells at 3 and 12 weeks post-transplant. B — Total number of engrafted phenotypically marked human hematopoietic stem and progenitor cells. C — Percentage of terminally differentiated mature cells per femur. Note that human hematopoietic cells co-cultured with E4+ECs have greater engraftment potential and give rise to a higher number of multi-lineage human hematopoietic progenitors.

**Figure 4** Effect of a dual niche on HSPC expansion. A–C. Phase contrast imaging of HSPCs at day 5 on a mixed feeder (A and B) (eGFP-E4+ECs/Pl-MPs at ratio 1/1), or on eGFP-E4+EC feeder alone (C). (*) Areas containing only PL-MP. Red arrows: expanding HSPC clusters. D — Charts representing flow cytometry analysis of total HSPCs on E4+ECs, PL-MP or mixed feeder (N = 6; ** P < 0.01). E, F — Phase contrast imaging of HSCs cultured on eGFP-E4+EC feeder without cytokines (TPO, SCF and Flt3) (E), or cultured without feeder, without cytokine (F). G — Charts representing flow cytometry analysis of total HSPCs with or without E4+EC feeder complemented or not with cytokines (N = 6; ** P < 0.01).
vessels or in direct contact with ECs. A close proximity of the ECs with CD73+ does not allow concluding whether CD34+CD31− cells interact with ECs or mesenchymal cells. Often CD34+CD31− are observed between CD34+CD31+ cells and CD73+ cells. Nevertheless, transverse sections of the vessels confirmed luminal location of CD34+CD31+ cells (Figs. 1 I–L). No CD34+CD31− could be located within non-vascular placental areas (Figs. 1M–P).

Lin−CD45−/dimCD34+CD38−CD90+ cells expand in co-culture with endothelial feeder

We compared the abilities of MPs and E4+ECs to sustain Lin−CD45−/dimCD34+CD38−CD90+ cell expansion. In order to avoid bias and address the role of the different cell types comprehensively and for clinical relevance, we used a serum-deprived system. The viability of neither cell type (MPs and E4+ECs) was compromised in serum-free X-VIVO media supplemented with the STF cytokine cocktail (data not shown). A serum free system is primordial as our goal was to evaluate the intrinsic capacity of mesenchymal progenitors compared to ECs to amplify hematopoietic stem/progenitor cells. We therefore used the previously established endothelial model E4+ECs (Seandel et al., 2008), demonstrating the capacity to survive in serum-free media unlike HUVECs, human placental arterial endothelial cells (HPAECs), or human placental venous endothelial cells (HPVECs). Furthermore, maintenance of those primordial ECs would have required stimulation with additional cytokines and serum complement, resulting in loss of their angiocrine properties or introduction of co-founding factors.

Phase-contrast microscopy and flow cytometry analysis, performed up to 21 days, demonstrated that sorted Lin−CD45−/dimCD34+CD38−CD90+ cells do not survive without feeder over 21 days, whereas a large number of cells persisted on E4+ECs or MPs; however, while after 21 days of culture, sorted Lin−CD45−/dimCD34+CD38−CD90+ formed grapes-like colonies on the E4+EC feeder, they were scattered on the MP feeder (Fig. 2A).

We quantified the different hematopoietic cell populations by FACS. The total number of hematopoietic cells (non-feeder cells) was similar on E4+EC or PL-MP feeders, and was significantly increased compared to the feeder-free condition (Fig. 2B); however, only E4+ECs were able to expand and maintain a Lin−CD45−/dimCD34+CD38−CD90+ cell population (expansion by 2- and 3.3-fold at days 7 and 14 respectively, P < 0.05); this number persisted up to day 21 (Fig. 2C). This is concordant with our previous results demonstrating the ability of E4+ECs to expand hematopoietic stem/progenitors cells that give rise to repopulating cells (Butler et al., 2012).

We performed methylcellulose colony-forming assay to evaluate the differentiation potential of the expanded hematopoietic cells. Cells amplified on PL-MPs or E4+ECs were able to form CFU-E, CFU-M, CFU-G, and CFU-GM (Fig. 2D). Culture on both types of feeder induced more differentiation toward CFU-M and CFU-G formation than fresh placental Lin−CD45−/dimCD34+CD38−CD90+ cells. CFU numbers for each condition are provided in Supplementary Fig. 3.

To understand the dynamics of cell expansion we analyzed further our total expanded hematopoietic population. Consistent with previous results, we observed that a significant proportion of the amplified hematopoietic cells were Lin−CD45−/dimCD34+CD38− (Butler et al., 2012; Robin et al., 2009).
Figure 5  Transcriptomic comparison of E4+ECs vs. PL-MPs. A–C — Ingenuity Pathway Analysis (IPA) of differentially expressed membrane and extracellular proteins/cytokines retrieved from transcriptomic analysis of E4+ECs vs. PL-MPs, involved in: hematopoietic cell quantity (A), hematopoietic cell proliferation (B) and hematopoietic cell differentiation (C) (gene lists provided by IPA software). D — Specific cytokine/protein expression profiles and fold differences between E4+ECs and PL-MPs. E–F — Supervised Ingenuity analysis of RNA sequences differentially expressed between Lin$^{-}$CD45$^{-}$CD34$^{-}$dimCD38$^{-}$CD90$^{+}$ cells either directly issued from cord blood or after 7 days of culture on E4+ECs involved in: proliferation of hematopoietic progenitor cells (E), quantity of hematopoietic progenitor cells (F). G–H — Interactome between endothelial overexpressed genes compared to PL-MPs and genes over (G) or under (H) expressed in Lin$^{-}$CD45$^{-}$dimCD34$^{+}$CD38$^{+}$CD90$^{+}$ cells cultured on E4+ECs compared to cells directly isolated from cord/placenta blood.
While E4+EC feeder amplified the CD34+CD38− population, most of the cells grown on PL-MPs differentiated into CD34+CD38− previously described as progenitors (Seita and Weissman, 2010) (Figs. 2 F–G). Indeed, after 7 days on PL-MPs almost 90% of CD34+ cells had acquired the expression of CD38 compared to less than 5% on E4+ECs (Fig. 2H). This difference remained significant at all-time points indicating the ability of E4+ECs to sustain Lin−CD45−/dimCD34+CD38−CD90+ phenotype. We noticed that E4+EC conditioned media were also able to expand the Lin−CD45−/dimCD34+CD38−CD90+. This is in line with our previous finding demonstrating the role of E4+EC-secreted angiocrine factors in the expansion of HSCs (Kobayashi et al., 2010). (Supplementary Fig. 4).

As our data on the inability of PL-MPs to amplify the Lin−CD45−/dimCD34+CD38−CD90+ cells are conflicting with previously published data we controlled for site specific effect by comparing the effect of PL-MPs to BMS (which are routinely described). No statistical differences could be observed between PL-MPs and BMS regarding their ability to expand the hematopoietic cell populations (Supplementary Fig. 5).

In vivo engraftment capacities of amplified hematopoietic cells

To control the engraftment capacity of amplified hematopoietic cells on E4+ECs we evaluated the capacity of the CD34+ cells amplified on E4+ECs or cultured without feeder to engraft in sub-lethally irradiated NSG mice. To facilitate comparison with our previous study, bloods of engrafted NSG mice were analyzed for expression of CD45, CD34 and AC133 (Butler et al., 2012). 3 and 12 weeks post engraftment, human CD45− cells could be detected in the peripheral blood of mouse injected with CD34+ cells amplified on E4+ECs or without feeder (Figs. 3A and B). In the femur of engrafted mice after 12 weeks we were able to detect human HSPCs (as previously published (Butler et al., 2012)) when CD34+ cells were amplified on E4+ECs or without feeder. Finally we could illustrate the ability of the HSPCs amplified on E4+ECs to reconstitute multiple lineages as we could detect both CD33+ myeloid and CD19+ lymphoid cell lineage (Fig. 3C). This suggested that E4+ECs amplified CD34+CD38− maintained their functional abilities. Interestingly the engraftment experiments were concordant with the CD34+CD38− profile obtained by in vitro expansion. Indeed in Fig. 2 we illustrated the persistence without expansion of a CD34+CD38− population in the cytokine alone (no feeder). To compare with a mesenchymal platform, our preliminary in vitro results demonstrated that there was no discernible difference between the marker profiles of hematopoietic cells grown on PL-MP and BMS feeders (Supplementary Fig. 4). In light of this, we chose to perform hematopoietic/BMS cell co-culture experiments using serum free minimal cytokine conditions prior to engraftment into NSG mice. However, we observed that hematopoietic progenitor cells cultured under these conditions did not retain significant repopulating activity; a result consistent with the loss of CD34+CD38− on PL-MP and BMS feeders (Fig. 2 and Supplementary Fig. 4). These findings suggest that culture medium might be an important contributor to the ability of co-culture platforms to support the maintenance of hematopoietic cells with repopulating activity.

A competitive assay confirmed the important role of E4+ECs over PL-MPs

Because ECs and MPs are in close contact in the placenta, our in vitro cell-culture conditions might hinder a cooperative effect of the two cell types. We therefore created a combined MP-EC feeder and studied its ability to expand hematopoietic cells. Surprisingly, after seeding, the hematopoietic cells exclusively located on the E4+ECs (Figs. 3A and B). After a week of culture, hematopoietic cells expanded within grape-like structures exclusively in E4+EC-rich areas (Fig. 3C). While the bi-cellular niche expanded Lin−CD45−/dimCD34+CD38+CD90− cells more effectively than the PL-MP feeder alone (~ 3 fold, P < 0.05), it was significantly less efficient than the E4+EC feeder alone.
(3.1-fold, P < 0.05). Once again, the presence of PL-MPs induced differentiation toward CD38+ cells (data not shown). We next assessed whether the endothelium might have an effect in the absence of the STF cytokine cocktail used in our expansion system. E4+ECs alone were able to maintain the Lin−CD45−/dim CD34+CD38−CD90+ cell population (Figs. 3E–G), albeit without inducing an expansion in cell numbers.

Distinctive characteristics of the PL-MP and E4+EC platforms

Our serum-free feeder based model provides a robust tool for understanding the cellular factors required for expansion of HSPCs. We characterized the cytokine secretion of both cell types cultured in STF-containing media. We found that the PL-MPs secreted significantly more GROα (CXCL1) (~2-fold), IL6 (~8-fold), IL8 (~2-fold), and CCL5 (not detected in E4+EC secretion). E4+ECs secreted more MCP-1 (CCL2) (~20-fold) and MIF (~1.2-fold) (Supplementary Fig. 6). Overall, cytokines secreted by MPs include those implicated in progenitor differentiation (Emadi et al., 2005), while the cytokines produced by the E4+ECs are often associated with chemo-attraction (Abangan et al., 2010).

We also characterized the differences between the two different feeders by performing whole-genome transcriptomic analysis. 5142 genes were differentially expressed between the two cell types (≥2-fold, FDR > 0.05) (Supplementary Table 1). We confirmed the cytokine-array data with the transcriptomic data; CXCL1 (48 fold), IL6 (10 fold), IL8 (200 fold), and CCL5 (275 fold) were overexpressed in PL-MPs compared to E4+ECs.

We next performed Ingenuity Pathway Analysis (IPA) of the genes differentially expressed between PL-MPs and E4+ECs, using keywords related to quantity, proliferation, or differentiation of hematopoietic cells. PL-MPs expressed more molecules responsible for differentiation of hematopoietic cells than E4+ECs (Figs. 3A–C). We also compared the expression of previously described HSCs amplifying angiocrine factors (Kobayashi et al., 2010) between the two feeders (Fig. 4D and Supplementary Table 2). Interestingly while the PL-MPs expressed a majority of the predicted angiocrine factors responsible for HSC self-renewal they had very low expression of Notch ligands (Jagged 1 (9 fold), Jagged 2 (5 fold), and DLL1 (12 fold)), previously described as critical for HSC amplification (Kobayashi et al., 2010).

RNA sequencing analysis

To monitor the possible modifications triggered by HSPC amplification on E4+EC feeder, RNA sequencing was performed on Lin−CD45−/dimCD34+CD38−CD90+ cells either before or after 7 days of culture on E4+ECs. A PCA representation of normalized results is shown in Supplementary Fig. 7. This analysis demonstrated differences between freshly isolated Lin−CD45−/dimCD34+CD38−CD90+ cells and the same population that had been cultured on E4+ECs. Indeed, 1430 RNA sequences were differentially expressed between Lin−CD45−/dimCD34+CD38−CD90+ cells isolated from blood and cultured cells on E4+ECs (Supplementary Table 3).
Supervised ingenuity analysis indicated that these 1430 RNA sequences included genes involved in proliferation of hematopoietic progenitor cells. In particular, three critical transcription factors are up-regulated in Lin\(^-\)CD45\(^-\)/dimCD34\(^+\)CD38\(^-\)CD90\(^+\) cell culture on E4\(^+\)ECs: MYC, CEBPA and CDK2 (Fig. 4E). Similarly, MYC and CEBPA are central transcription factors when analyzing genes involved in the quantity of hematopoietic progenitor cells (Fig. 4F). We went further and decided to correlate those results with the previously described genes over expressed in E4\(^+\)ECs compared to PL-MPs and build the interactome between endothelial and Lin\(^-\)CD45\(^-\)/dimCD34\(^+\)CD38\(^-\)CD90\(^+\) cells. Genes overexpressed in E4\(^+\)ECs localized in the extracellular space or at the plasma membrane were selected and direct/indirect correlation was analyzed with up or down-regulated genes in cells cultured on E4\(^+\)ECs compared to PL-MPs (Fig. 5A). The main most central protein TGFB1 was previously proposed as a component leading to hibernation of HSCs in the bone marrow, thus inhibiting differentiation.

Lin\(^-\)CD45\(^-\)/dimCD34\(^+\)CD38\(^-\)CD90\(^+\) cells’ up-regulated central components were MYC, CDK2, CEBPA, BIRC5, MYBL2, ACTB, GRB2, MMP2 and ANXA2 (Fig. 4G). It is interesting to find as a major component of the interactome the 3 transcription factors previously described as involved in proliferation of hematopoietic stem/progenitor cells. Lin\(^-\)CD45\(^-\)/dimCD34\(^+\)CD38\(^-\)CD90\(^+\) cells’ downregulated central interactome components were PIK3R1, ETS1, CXCR4, NFE2L2, TAB2, ERG, GATA2, HIF1A, NFKBIA, PTK2B, SMAD7, FOXO1, FYN and IGF1R (Fig. 4H).

**Conclusion and discussion**

Understanding normal cell–cell interactions and signaling pathways regulating HSC self-renewal and differentiation leads to a framework to study the development and expansion of hematopoietic stem cells. Here we demonstrated that endothelial cells are able to expand cord blood/placental CD38\(^-\) hematopoietic stem/progenitor cells capable of engraftment and replenishment of sub-lethally irradiated mice, while mesenchymal progenitors induce expansion of CD38\(^+\) cells.

Many studies described the role of placental MPs in UCB derived hematopoietic cell expansion. In competition assays using PL-MP and E4\(^+\)EC feeders together, we show a clear preference of hematopoietic cells for interacting with endothelial cells indicating either the role of membrane bound factors or stronger chemo-attraction by endothelial cells than PL-MPs. Our transcriptomic analysis demonstrated many differences between the two feeders. The main difference in terms of angiocrine factor was the over-expression of Notch...
ligands by E4+ECs. However transcriptomic and cytokine array analysis suggested that the observed functional differences are not based on a single molecule, but most likely rely on the fine-tuning of factors involved in regulating stemness, expansion, and differentiation. RNA sequencing analysis demonstrated that cells amplified on E4+EC feeder layers retained expression of genes involved in 'cell cycle', hematopoietic progenitor cell 'quantity' and 'development'. The broad interactome we were able to build is concordant with recently published data on the ability of feeder cells (mesenchymal progenitors) to expand pancreatic progenitors via a pattern of signaling cues rather than single genes (Sneddon et al., 2012) (Fig. 5).

Our bi-cellular niche showed that the ability of ECs to expand hematopoietic stem/progenitor cells might partially be linked to cell adhesion and membrane bound signaling. Indeed, in confocal analysis, CD34+CD31- cells were always seen in close proximity to the vessels. Hematopoietic cells formed grape-like colonies on E4+ECs, while they were scattered on the PL-MPs, suggesting different dynamics of interaction with the two different types of feeder cells. Finally, in the competition assay we were surprised to see all hematopoietic cells adhering to endothelial cells and expanding on the endothelial network. This suggested that the placental MPs lacked the appropriate membrane signaling for chemo-atraction and/or adherence of hematopoietic cells.

These observations are concordant with the knowledge of the HSC niche. For example, it is well described that in the relationship between the stem cell and their niche elements, cell to cell position translates into functionality (Barbier et al., 2012; Vied et al., 2012). Similarly, it is believed that in the case of neuronal stem cells, asymmetric division and maintenance of stemness occur close to the niche (Falcao et al., 2012; Winkler et al., 2010). In mouse bone marrow (Ding et al., 2012) or in the human placenta (Serikov et al., 2009), HSCs reside in a perivascular niche where ECs and perivascular stromal cells play functionally important roles. Both our cell types express the key niche component SCF (KIT-LG) indispensable for HSC expansion (Ding et al., 2012). The role of the endothelium in HSC expansion has been clearly established by our group and confirmed by others. Morrison's group not only confirmed the role of the endothelium as being essential, but also demonstrated that after knock-out of Nestin+ MPs, HSCs still expanded in the endothelial niche (Ding et al., 2012). Consistent with these findings, we could not demonstrate that placental MPs expanded Lin-CD45-CD34-CD38-CD90- cells in our serum-free, reduced cytokine culture conditions.

In this report we directly compared two cell types to provide a hematopoietic stem/progenitor cell niche in a human system. Many authors have reported the ability of MSCs to expand the HSCs or CD34+ cells. However our flow cytometric studies demonstrated that the majority of the CD34+ cells amplified in our conditions were actually CD38+ progenitors. We sought to compare our EC system with a mesenchymal platform that could be more easily standardized. As such, MPs were cultured under serum free minimal cytokine conditions that incorporated rhSCF, rhTPO and rhFLT3 as growth factors. Unlike previous reports in which serum containing medium was used (Andrade et al., 2010; Fei et al., 2007; Hayashi et al., 2009; Huang et al., 2007; Levesque et al., 2010; Mendez-Ferrer et al., 2010; Robin et al., 2009; Sasaki et al., 2010; Walenda et al., 2010; Zhang et al., 2004), we found that MPs cultured under our conditions were unable to support the expansion of hematopoietic cells with repopulating activity. This result highlights the potentially critical role of culture conditions in revealing the ability of MPs to amplify HSPCs.

The transcriptomic comparison of PL-MPs and E4+ECs showed that MPs express relatively higher levels of cytokines previously reported as responsible for HSC expansion (such as ANG1 and IGFBP3), but also higher levels of cytokines reported to trigger chemo-attraction, differentiation and mobilization of HSCs (such as CXCL1, IL6, IL8, CCL5, CSF1, and CXCL12 (Bernad et al., 1994; Ding et al., 2012; Ergen et al., 2012; Pruijt et al., 2002)). By contrast, ECs expressed higher levels of factors previously described as essential for HSC expansion, such as Notch ligands (Jagged 1, Jagged 2, and DLL1) or DHH from the hedgehog family of transcription factors (Butler et al., 2010; Delaney et al., 2010; Pajcini et al., 2011).

We performed RNA sequencing analysis of expanded Lin−CD45−/dimCD34−CD38−CD90− cells in comparison to cord blood/placental Lin−CD45−/dimCD34−CD38−CD90− cells. In our culture settings, Lin−CD45−/dimCD34−CD38−CD90− cells cultured on E4+ECs up-regulated essential genes for cell cycle regulation: MYC and CDK2. Iriuchishima et al. described that under hypoxic conditions HSC quiescence and stemness are MYC dependent (Iriuchishima et al., 2011). CDK2 has been shown to be a MYC cofactor leading to the regulation of stemness and self-renewal (Hydbring and Larsson, 2010). Similarly, CEBPA (1), BIRC5 (2), MYBL2 (3,4) and GRB2 (5) which were also upregulated in HSPCs co-cultured with E4+ECs, have been previously demonstrated to contribute to HSC amplification. We found as central components of the interactome, down-regulation of genes involved in HSC differentiation like: ETS1 (6), NFkBIA (De Molfetta et al., 2010), IGF1R (14) and PTK2B (7), or downregulation of NFE2L2 and GATA2 necessary for HSC amplification (8, 11). Downregulation of these genes in our E4+EC co-culture system may explain the maintenance of hematopoietic cells stem/progenitor activity.

By building the interactome between the transcriptomes of E4+ECs and hematopoietic cells, we identified components such as TGFβ1 (over expressed in E4+ECs compared to PL-MPs) that might be a major factor interacting with genes upregulated and/or downregulated in hematopoietic cells. TGFβ1 seems therefore to play a central role in the hematopoietic cell expansion in our culture settings. The understanding of the precise mechanism underlying TGFβ1's central role is beyond the scope of this work. Though, we can point out the known indirect interaction of TGFβ1 with up-regulated genes involved in proliferation of hematopoietic cells like BIRC5, CDK2, MYC, CEBPA and PIM1. Similarly, TGFβ1 interacts with genes down-regulated during the differentiation of hematopoietic cells like: NFkBIA, FYN, PTPRC, FOSL1, IRF1 and SOX4. These results raise the hypothesis that TGFβ1 may play multiple complementary roles in hematopoietic stem/progenitor cell amplification on E4+ECs. TGFβ1 may therefore represent a key target to optimize HSC in vitro amplification.

Future studies should focus on identifying the essential factors of the endothelial niche responsible for the maintenance and expansion of hematopoietic stem/progenitor cells and enabling widespread use in clinical applications. A mixed
strategy based on a cellular platform, with fine molecular targeting strategy, will represent an optimal system for developing clinically relevant applications.

Supplementary data to this article can be found online at [http://dx.doi.org/10.1016/j.scr.2013.07.010](http://dx.doi.org/10.1016/j.scr.2013.07.010).

**Conflict of interest**

The authors declare no competing financial or personal interests.

**Acknowledgments**

This work was funded by Qatar Foundation NPRP Grant 08-663-3-140 and NPRP Grant 09-1099-3-279; Qatar Foundation UREP Grant 06-116-1-023; and a Qatar Foundation Qatar Technology Transfer Grant. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the Qatar National Research Fund.

**References**


Yildirim, S., Boehmler, A.M., Kanz, L., Mohle, R., 2005. Expansion of cord blood CD34+ hematopoietic progenitor cells in coculture with autologous umbilical vein endothelial cells (HUVEC) is superior to cytokine-supplemented liquid culture. [Comparative Study Research Support, Non-U.S. Gov’t]. Bone Marrow Transplant 36 (1), 71–79. http://dx.doi.org/10.1038/sj.bmt.1705001.
