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Running title: Hematopoietic cell support by perivascular cells
Key Point

- Perivascular cells maintain HSPCs ex vivo

Abstract

Hematopoietic stem and progenitor cells (HSPCs) emerge and develop adjacent to blood vessel walls in the yolk sac, aorta-gonad-mesonephros region, embryonic liver, and fetal bone marrow (FBM). In adult mouse BM, perivascular cells shape a “niche” for HSPCs. Mesenchymal stem/stromal cells (MSCs), which support hematopoiesis in culture, are themselves derived in part from perivascular cells. In order to define their direct role in hematopoiesis, we tested the ability of purified human CD146+ perivascular cells, as compared to unfractionated MSCs and CD146- cells, to sustain human HSPCs in co-culture. CD146+ perivascular cells support the long-term persistence, through cell-to-cell contact and at least partly via Notch activation, of human myelo-lymphoid HSPCs able to engraft primary and secondary immunodeficient mice. Conversely, unfractionated MSCs and CD146- cells induce differentiation and compromise \textit{ex vivo} maintenance of HSPCs. Moreover, CD146+ perivascular cells express, natively and in culture, molecular markers of the vascular hematopoietic niche. Unexpectedly, this dramatic, previously undocumented ability to support hematopoietic stem cells is present in CD146+ perivascular cells extracted from the non-hematopoietic adipose tissue.
**Introduction**

Blood and vasculature are indispensable to embryonic development, and are thus the first differentiated tissues produced in life. Incipient human hematopoiesis adapts to the rudimentary anatomy of the embryo and proceeds first in the yolk sac, then transiently in the placenta and liver before being stabilized in fetal bone marrow (FBM). Definitive hematopoietic stem and progenitor cells (HSPCs) first emerge in the aorta-gonad-mesonephros region of the embryo.\(^1\) Therefore, several organs of distinct germ line origins, structures and eventual roles converge functionally to produce blood cells during development. What remains remarkably constant through pre- and postnatal life is the physical association of incipient hematopoietic cells with blood vessels. In the yolk sac, erythroid cells emerge within intravascular blood islands.\(^2\) It is now also well accepted that, from fish to humans, specialized blood-forming endothelial cells present in the dorsal aorta and possibly other organs supply the embryo with hematopoietic cells,\(^3\)-\(^7\) an ontogenic transition that has been modelled in human embryonic stem cells.\(^8\) In addition to this direct developmental affiliation between embryonic endothelial cells and HSPCs, there is evidence that vascular cells nurture blood cells in pre- and postnatal life. The cellular and molecular mechanisms involved in this support can be analyzed in co-cultures of stromal and hematopoietic cells.\(^9\)-\(^11\) For instance, cultured endothelial cells use angiocrine factors to regulate HSPC differentiation or self-renewal.\(^12\)-\(^14\) Mesenchymal stem/stromal cells (MSCs), the multi-lineage mesodermal progenitors spontaneously selected in long-term cultures of unfractionated cells from bone marrow and other tissues\(^15\)-\(^18\) can also, to some extent, sustain hematopoiesis *in vitro*.\(^19\)-\(^24\) However, the relevance of this support to physiologic blood cell production *in vivo* has been unknown.
since MSCs have long eluded prospective identification.25 Similarities between MSCs and pericytes, which ensheath capillaries and microvessels in all organs, have been described.26-28 In an experimental approach combining stringent cell purification by flow cytometry and differentiation in culture and in vivo, we have demonstrated that human CD146+ perivascular cells represent ubiquitous ancestors of MSCs.29

Although hematopoietic stem cells were originally detected in the endosteal regions of the bone marrow,30 recent findings have suggested the existence of a distinct, perivascular niche for HSPCs.31-34 Perivascular reticular cells expressing CXCL12 were found to play a role in murine HSC maintenance.35 In a seminal study by Mendez-Ferrer et al., the function and identity of perivascular niche cells were further defined. The authors showed the existence in murine bone marrow of perivascular nestin+ MSCs associated with HSCs. Ablation of nestin+ MSCs led to a significant reduction in the number and homing ability of HSCs.36 The direct role for perivascular cells in hematopoiesis regulation was confirmed in a recent study by Ding et al.37 Selective shut-off of c-kit ligand expression in leptin receptor (Lep-R) positive cells surrounding murine bone marrow blood vessels significantly reduced the frequency of long-term reconstituting hematopoietic stem cells.37

In the present study we demonstrate that CD146+ perivascular cells express in vivo nestin, CXCL-12 and Lep-R in human FBM as well as in adult adipose tissue. We also report for the first time that human CD146+ perivascular cells are a subset of MSCs able to directly support the ex vivo maintenance of human HSPCs. We further demonstrate that cultured CD146+ perivascular support HSPC through cell-to-cell contact and activation of Notch signalling. Conversely, conventional unfractionated MSCs or the
CD146- subset of MSCs favour differentiation at the expense of stemness. CD146+
perivascular cells can therefore be considered as the *bona fide* human equivalents of the
hematopoietic perivascular niche components recently described in the mouse.

**Methods**

**Isolation of human primary stromal cells**

Human stromal cells were derived from human lipoaspirate specimens (n=4) and fetal
bone marrow (FBM) (n=2) as previously described. Lipoaspirates were obtained as
discarded specimens without identifiable information, therefore no IRB approval was
required. Fetal bones (16-18 weeks of pregnancy) were obtained from Novogenix.
Hundred mL of lipoaspirate were incubated at 37°C for 30 min with digestion solution
composed by RPMI 1640 (Cellgro), 3.5% BSA (Sigma) and 1mg/ml collagenase type II
(Sigma). Adipocytes were discarded after centrifugation while the pellet was resuspended
and incubated in red blood cell lysis (eBioscience) to obtain the stromal vascular fraction
(SVF). Fetal bones were split open to flush the bone marrow cavity. The bones were
placed in digestion solution for 30 min at 37°C. Mononuclear cells (MNCs) were isolated
using Ficoll-Paque (GE Healthcare). Hematopoietic cells were excluded by magnetic
immunodepletion of CD45+ cells as per manufacturer’s instructions (Miltenyi). An
aliquot of SVF or CD45-depleted MNCs was plated in tissue culture treated flask for the
expansion of conventional MSCs. Another aliquot of SVF or CD45-depleted MNCs
was processed for FACS sorting. Cells were incubated with the following antibodies:
CD45-APC-cy7 (BD Biosciences), CD34-APC (BD Biosciences), and CD146-FITC
(AbD Serotec). The viability dye DAPI (Sigma) was added before sorting, on a
FACS Aria III (BD Biosciences), DAPI-CD45-CD34-CD146+ perivascular cells or
DAPI-CD45-CD34+CD146- cells, as previously described. In some experiments, CD146- cells were purified from cultured MSCs.

For the animal studies, an ACUC protocol (ARC#2008-175-11) was approved for the injection of human cells into immunodeficient mice and for the analysis of engraftment of transplanted cells.

**Isolation of human CD34+ cells from cord blood**

Umbilical cord blood (CB) was collected from normal deliveries without individually identifiable information, therefore no IRB approval was required. MNCs were isolated by density gradient centrifugation using Ficoll-Paque (GE Healthcare). Enrichment of CD34+ cells was then performed using the magnetic-activated cell sorting (MACS) system (Miltenyi Biotec) as per manufacturer’s instructions.

**Immunophenotype analysis of stromal cells**

Cultured MSCs, CD146+ and CD146- cells (between passages 3 and 10) were analyzed on a LSR II flow cytometer (Becton Dickinson). Cells were stained with monoclonal antibodies: CD146-FITC (AbD Serotec), CD31-APC (Biolegend), CD44-PE, CD73-PEcy7, CD105-PE, CD90-APC, CD45-FITC (all from BD Biosciences) and. Unstained samples were used as negative controls. Data were analysed using FlowJo software (Tree Star).

**Mesodermal lineage differentiation assays**

The ability of cells to differentiate into mesodermal lineages was tested in osteogenic or adipogenic differentiation medium (Hyclone). After 3 weeks of culture in differentiation
conditions, cells were stained with Alizarin red or Oil red O (Sigma) for the detection of mineral deposits or lipids as previously described.²⁹

**Quantitative RT-PCR**

Five hundred thousand cultured cells were processed for RNA extraction using a Qiagen micro kit (Qiagen). Omniscript reverse transcriptase kit was used to make cDNA, which was subjected to qPCR using Sybr green probe based gene expression analysis (Applied Biosystems) for two housekeeping genes, TBP and GAPDH, and the target genes CD146, nestin, α-SMA, and NG2. A 7500 real time PCR system was used (ABI). Data were analyzed using the comparative C(T) method.

**Western blotting**

Cells were lysed in denaturing cell extraction buffer (Invitrogen) containing protease inhibitor tablets (Roche). Proteins were then separated by SDS-PAGE and analyzed using the XCell II™ Western blot system (Invitrogen). Rat anti-human Jagged-1 (Abcam, 1:50) and monoclonal mouse anti-β actin (Sigma, 1:5000) antibodies were used. Donkey anti-rat HRP and donkey anti-mouse HRP (Jackson, Immunoresearch Inc., 1:5000) were used as secondary antibodies. The blots were developed using ECL Plus Western Blotting Substrate (Pierce).

**Co-culture of stromal cells and CB CD34+ cells**

Cultured stromal cells (between passages 3 and 8) were irradiated (20Gy) and plated on 96 multi-well plates at 1.5x10⁴ cells/well. Twenty-four hours later, CB CD34+ cells (5-7x10⁴/well) were plated on top of the stromal layer. Stroma-free cultures were performed
seeding CB CD34+ cells on recombinant retronectin (RN, Lonza) coated wells. Co-
cultures were performed in RPMI 1640, 5% FBS, 1x pen/strep. No supplemental
cytokines were ever added. Cells were harvested after 1, 2, 4 and 6 weeks. Co-cultures in
the absence of cell-to-cell contact were performed in 96 multiwell transwell plates
(Corning). For the inhibition of Notch, 10μM DAPT (Sigma) or 10μg/ml of anti-human
Notch 1 neutralizing antibody (Biolegend) were added to each well every 48 hours. An
equal volume of DMSO (Sigma), or an equal concentration of mouse unrelated IgG
(Biolegend) were added to wells as negative controls for DAPT and anti-Notch1 antibody
respectively.

**Flow cytometric analysis of cultured CB CD34+ cells**

After 1, 2, 4, and 6 weeks of co-culture, cells were harvested and stained with the
following antibodies: CD45-APC-cy7, CD34-PE-cy7, CD14-APC, CD10-APC, CD33-
PE, CD19-FITC (all from BD Biosciences). Dead cells were identified with propidium
iodide (PI) (BD Biosciences).

**Colony forming unit assay**

After 1, 2, 4 and 6 weeks of co-culture cells were harvested and 2.5x10^3 cells were plated
in methylcellulose (Methocult GF H4435, Stem Cell Technologies). Colonies, here
reported as the sum of the progeny of granulo-macrophage (CFU-GM), erythroid (BFU-
E) and mixed (CFU-GEMM) colony forming units, were scored after 14 days.
In vivo repopulation assay

CB CD34+ cells were co-cultured with MSCs or CD146+ cells for two weeks in RPMI 1640, 5% FBS, 1x pen/strep. An equal number of CD45+ cells ($10^5$) obtained from the co-cultures was intra-tibially injected in sub-lethally irradiated (250cGy), 6-8 week old NSG mice (Jackson Laboratories). Mice were sacrificed 6 weeks post-transplantation. Engraftment of human hematopoietic cells was evaluated by FACS analysis after staining with anti-human specific monoclonal antibodies: CD45-APC-cy7, HLA (A/B/C)-PE, CD34-PE-cy-7, CD19-FITC, CD14-APC, CD15-APC, CD33-APC (all from BD Biosciences). For secondary transplantation, bone marrows from 2 engrafted mice were pooled and intra-tibially injected into a secondary host (n=4). Engraftment was evaluated 4 weeks post-transplantation.

Immunocyto- and immunohistochemistry

For immunofluorescence analysis, human adipose tissue frozen sections, cells cultured in chamber slides (Millipore) or cytospun on microscope slides were fixed with cold methanol/acetone (1:1) for 5 min at RT prior to incubation with blocking solution (PBS 5% donkey serum) for 1 hr at RT. Overnight incubation at 4°C was performed with unconjugated primary antibodies: mouse anti-human CD146 (BD Biosciences), mouse anti-human CD45 (eBioscience), rat anti-human Jagged-1, rabbit anti-human N1ICD, mouse anti-human nestin, rabbit anti-human CXCL-12, rabbit anti-human leptin receptor, rabbit anti-human CD146 (all from Abcam). Tissue sections or cells were incubated for 2 hrs at RT with FITC conjugated mouse anti-human von Willebrand factor (US Biological). Tissue sections or cells were incubated for 1 hr at RT with the following
conjugated antibodies: donkey anti-rabbit-Alexa 488, donkey anti-rabbit-Alexa 647, donkey anti-rat-Alexa 594 or donkey anti-mouse-Alexa 594 (all from Jackson Immunoresearch Inc.). For immunohistochemistry on human fetal bone marrow, fetal bones (16-18 weeks of pregnancy) were fixed in 4% paraformaldehyde (Sigma-Aldrich). Fixed tissues were embedded in paraffin and sections were stained with the same antibodies against nestin, CXCL-12, Lep-R and CD146. Secondary horseradish peroxidase (HRP) conjugated IMPRESS anti-rabbit and anti-mouse antibodies and 3, 3'-diaminobenzidine (DAB, Vector Labs) were used for revelation. As negative controls, tissue sections or cells were incubated only with secondary antibodies. Images were acquired on an Axiovision (software version 4.8) microscope (Carl Zeiss, Germany) equipped with ApoTome.2 modules for Axio Imager.2 and Axio Observer, with 10x, 20x, 40x and 63x (1.4 NA) objectives.

**Statistical analysis**

Mean and standard deviations were used to summarize continuous variables. Bivariate cross-sectional comparisons of continuous variables were performed using paired t-tests. Continuous outcomes such as total numbers of CD45+ and CD34+ cells, frequency of CD34+Lin- cells and CFUs were collected over time. The experimental design involved two within-experiment factors, MSCs and pericytes and time (week 1,2,4,6), which corresponded to a strip-plot design. Mixed model approach was used. Within the mixed model framework, we performed hypothesis testing for the comparison of MSCs and pericytes at different time points. Pearson’s correlation (r) was reported to assess the linear correlation between CD34+lin- cells and CFUs. For the qPCR data, ΔCT values were calculated for each marker. A randomized block design model was fitted on ΔCT
values. Donors were treated as random effects while stromal cells groups were treated as fixed effects. For all statistical investigations, tests for significance were two-tailed. To account for type-I error inflation due to multiple comparisons, p-values were adjusted by Bonferroni correction. Fisher exact test was performed to compare engraftment and not engraftment ability. Statistically significant threshold of p-value was set at 0.05. Statistical analyses were carried out using SAS version 9.2 (SAS institute, 2008).

Results

**Human CD146+ perivascular cells express nestin, CXCL-12 and Lep-R in hematopoietic and non-hematopoietic tissues.**

Recent studies have described murine perivascular cells as key players for the maintenance of HSPCs. Perivascular niche cells, displaying MSC features, have been identified based on the expression of CXCL-12\textsuperscript{34}, nestin\textsuperscript{36}, and Lep-R\textsuperscript{37}. We have previously demonstrated that pericytes, surrounding microvessels and capillaries, can be detected in multiple human tissues on expression of CD146.\textsuperscript{29} Consistent with our previous findings, immunohistochemistry performed on human fetal bone marrow (FBM) revealed the presence of CD146-expressing perivascular cells (Figure 1a). Nestin, CXCL12 and Lep-R, markers of the perivascular niche previously described in murine studies, were also expressed in human perivascular cells in FBM (Figure 1b-d). We further investigated the expression of the same stromal cell markers in human adult adipose tissue, considered as an abundant source of MSCs and recently suggested to also be a reservoir of HSCs.\textsuperscript{39} Nestin, CXCL-12 and Lep-R were all expressed in cells immediately adjacent to von Willebrand factor (vWF) positive endothelial cells (Figure
Multi-color immunofluorescence showed that CD146+ pericytes, surrounding microvessels and capillaries, co-express nestin, CXCL-12 and Lep-R (Figure 1h-s). Thus human CD146+ perivascular cells express *in situ* markers previously identified in murine studies to mark the perivascular hematopoietic niche.

**Purified and *ex vivo* expanded CD146+ perivascular cells maintain expression of markers of the perivascular niche**

We then analyzed the expression of the perivascular niche markers in purified and *ex vivo* expanded CD146+ perivascular cells as compared to unfractionated MSCs and CD146- cells. MSCs were conventionally derived from the adipose tissue stromal vascular fraction (SVF) by plastic adherence, while CD146+ perivascular cells and CD146- cells were purified by FACS sorting as previously described (Figure 2a).²⁹,³⁸ CD146+ perivascular cells demonstrated expression of cell surface markers typical of unfractionated cultured MSC, such as CD44, CD105, CD73 and CD90 and did not express the hematopoietic and endothelial cell markers CD45 and CD31 (Supplemental Figure S1a,b). Also similar to unfractionated MSC, cultured CD146+ cells were able to differentiate into osteoblasts and adipocytes in culture (Supplemental Figure S1c-f). CD146+ perivascular cells retained uniform CD146 expression in culture, as did a small fraction of MSCs, while CD146- cells remained negative for CD146 expression in culture (Figure 2b). Quantitative RT-PCR analysis of established cultures confirmed that CD146+ cells expressed higher levels of the perivascular cell markers CD146, α-SMA, NG2 and nestin than did either unfractionated MSCs or CD146- cells derived from fat (Figure 3a) or FBM (Figure 3b). Furthermore, immunocytochemistry demonstrated that cultured CD146+ perivascular cells isolated from fat or FBM express higher levels of
nestin and CXCL-12 than CD146- cells do. No significant difference in the expression of leptin receptor (Lep-R) was observed between cultured CD146+ and CD146- cells (Figure 3c-n).

**CD146+ perivascular cells support hematopoietic stem and progenitor cells (HSPCs) ex vivo**

The ability of distinct stromal cells to support HSPCs ex vivo was assessed by co-culturing cord blood-derived CD34+ cells (CB CD34+) in direct contact with either CD146+ perivascular cells, unfractionated MSCs, or CD146- cells all obtained from both lipoaspirate specimens and fetal bone marrow. These cultures were performed in basal medium with a low concentration of serum (5%) and in the absence of any supplemental cytokines, so that the specific effect of each stromal cell subset could be assessed with minimal influence of exogenous factors. In the absence of any stromal cells or cytokines, hematopoietic cells cultured on retronectin (RN) died within the first two weeks, whereas CD45+ cells survived for up to 6 weeks in the presence of either MSCs or CD146+ perivascular cells (Figure 4a). The total number of CD45+ cells recovered from CD146+ cell co-cultures remained significantly higher at any time of culture when compared to MSC co-cultures (Figure 4a). A similar pattern was observed for the total number of CD34+ cells (Figure 4b). CD34 expression identifies human hematopoietic cells without discriminating between HSCs and lineage-committed progenitors. The most immature progenitors present in co-cultures were further defined as CD34+Lin- cells based on expression of CD34 and lack of the early myeloid cell marker CD33 and lymphoid cell markers CD10 and CD19. CD146+ cell co-cultures contained a significantly higher frequency and number of CD34+Lin- cells at all time points (Figure 4c,d). Consistent
with these findings, culture in the presence of MSCs resulted in accelerated differentiation of CB CD34+ cells into CD14+ myeloid cells and CD10/CD19+ lymphoid cells, relative to co-culture with CD146+ cells (Figure 4e,f). The increased frequency of myeloid and lymphoid cells was counterbalanced by the lower numbers of CD45+ cells in MSC co-cultures, hence no significant difference in the absolute numbers of myeloid or lymphoid cells was observed (Figure 4e,f). Furthermore, the number of clonogenic cells detected after 1, 2, 4, and 6 weeks was significantly higher when CB CD34+ cells were co-cultured with CD146+ perivascular cells compared to MSCs (Figure 5a).

CD146+ perivascular cells isolated from either FBM or adipose tissue sustained significantly more CD34+Lin- cells and CFUs from CB CD34+ cells than CD146-stromal cells did (Supplemental Figure 2a-d), thus confirming that within the heterogeneous MSC population the ability to support HSPCs is confined to the subset of CD146+ perivascular cells, regardless of the tissue of origin.

**CD146+ perivascular cells maintain human HSPCs with repopulating ability and self-renewal potential**

We next investigated whether co-culture with MSCs or CD146+ perivascular cells retains functional HSPCs. Sub-lethally irradiated NOD/SCID/IL-2 receptor γ-chain null (NSG) mice were injected with hematopoietic cells co-cultured with CD146+ perivascular cells or MSCs for 2 weeks in low serum concentration without added cytokines. Strikingly, all mice transplanted with hematopoietic cells co-cultured with perivascular cells exhibited human hematopoietic cell engraftment 6 weeks post-transplantation, whereas no engraftment was observed in any of the mice transplanted with hematopoietic cells co-
cultured with MSCs (n=11 mice per group, n=3 individual experiments) (Fisher exact test p<0.0001) (Figure 5b,c). Human CD34+ progenitors, CD19+ lymphoid cells and CD14+ myeloid cells were detected in the chimeric mice (Figure 5d). Human CD45+HLA+ cells were not only detected in the medullary site of injection, but also in the contra-lateral tibia, thus suggesting that HSPCs co-cultured with CD146+ perivascular cells maintained the ability to migrate and home to distant sites after initial engraftment (Figure 5e). To assess the self-renewal potential of HSPCs cultured in the presence of CD146+ perivascular cells, bone marrow from chimeric mice was transplanted into secondary NSG mouse hosts. Lymphoid and myeloid engraftment of human cells was still detectable in secondary hosts (Figure 5f-i), demonstrating that the CD146+ cell fraction of MSCs is uniquely able to sustain human HSPCs with multi-lineage repopulating capacity and self-renewal ability.

**Contact with CD146+ cells is required for HSPC maintenance**

In addition to the phenotypic and functional differences described above, a different morphology and spatial distributions was observed between hematopoietic cells co-cultured with MSCs or CD146+ perivascular cells. When CD146+ cells were used as a feeder layer, hematopoietic cells appeared small, rounded and clustered (Supplemental Figure S3a). In the presence of MSCs, hematopoietic cells were larger, less uniform in size and scattered throughout the cultures, consistent with more vigorous hematopoietic differentiation (Supplemental Figure S3c). Immunocytochemical analysis confirmed the presence of clusters of CD34+ cells in contact with underlying CD146+ cells in perivascular cell co-cultures but not in MSC co-cultures (Supplemental Figure S3b,d). Based on these observations, we next investigated the role of cell-to-cell contact on
HSPC maintenance. When direct contact between CD146+ cells and CB CD34+ cells was prevented in a transwell culture system, the total number of CD45+ cells was dramatically reduced after 1 week of co-culture (Supplemental Figure S3e). In these non-contact conditions, hematopoietic cells were barely detectable after two weeks and the limited number of cells recovered did not allow us to perform further immunophenotypic or functional analyses.

**CD146+ perivascular cells express Notch ligands and activate Notch in hematopoietic cells**

Transwell culture experiments suggested that CD146+ perivascular cells sustain hematopoietic cells through cell-to-cell contact rather than by secretion of soluble factors. As Notch signaling is one of the key pathways through which the microenvironment affects growth and differentiation of HSPCs during development,40 we investigated whether CD146+ perivascular cells sustain HSPCs through the activation of Notch. Immunocytochemistry revealed that all cultured CD146+ perivascular cells express high levels of the Notch ligand Jagged-1. In contrast, only rare MSCs expressed Jagged-1, the majority of which also expressed CD146 (Figure 6a). Western blot analysis detected Jagged-1 expression at high levels in CD146+ perivascular cells compared to unfractionated MSCs (Figure 6b). Expression of other Notch ligands (Jagged-2, DLL-1 and DLL-4) was also detected by qPCR in MSCs, albeit at a lower level compared to CD146+ perivascular cells (Figure 6c).

We used an antibody recognizing an epitope exclusively exposed after Notch 1 receptor cleavage (NICD), to measure the frequency of hematopoietic cells activating Notch in the
presence of CD146+ cells or MSCs (Supplemental Figure S4a-b). As expected, Notch activation was not observed when direct contact between CD146+ cells and hematopoietic cells was inhibited in transwell co-cultures (Supplemental Figure S4c-d). MSCs, which express all four Notch ligands tested, were able to activate Notch1 in approximately 50% of hematopoietic cells and progenitors (Figure 6d-e). The percentage of NICD+CD45+ cells was significantly higher in CD146+ cell co-cultures than in co-cultures with total MSCs or CD146- cells, regardless of the tissue of origin (FBM or fat) (Figure 6d). Furthermore, Notch activation was significantly stronger in CD34+ progenitors co-cultured with CD146+ cells compared to those co-cultured with MSCs or CD146- cells (Figure 6e).

**Notch inhibition in CD146+ cell/HSPC co-cultures reduces progenitor cell numbers and stimulates B-cell differentiation**

To further assess the functional role of Notch activation in HSPCs, CB CD34+ cells and CD146+ perivascular cells were co-cultured in the presence of the gamma-secretase inhibitor DAPT. Notch inhibition resulted in significantly reduced total number of CD45+ cells, CD34+Lin- cells and CFUs compared to control co-cultures performed in the presence of the DMSO solvent alone (Figure 7a-c). A significantly higher frequency of propidium iodied (PI) positive dead cells was measured after Notch inhibition (Figure 7d). Of note, the frequency of PI+ cells was not increased when CD146+ perivascular cells or CB CD34+ cells were treated separately with DAPT in the absence of supplemental cytokines, thus excluding non-specific cytotoxicity from DAPT (Supplemental Figure S5a). Notch inhibition also resulted in a dramatic increase in B-cell differentiation (Figure 7e). A comparable decrease in output of total CD34+Lin- cells and
increase in B-lymphoid cells (Supplemental Figure S5b-d) was also observed when co-cultures were performed in the presence of an antibody to specifically block the Notch-1 receptor. However, the effect was less pronounced when compared to DAPT treatment. To explain this difference we determined the levels of Notch inhibition following DAPT or anti-Notch-1 blocking antibody treatment. While Notch activation was totally abrogated by DAPT, low-level activation was still detected in a few cells after antibody treatment, confirming that the latter treatment is less efficient than chemical inactivation of Notch (Supplemental Figure S4e-g).

Altogether, these results show that CD146+ perivascular cells are a subset of MSCs able to support HSPC and regulate lineage commitment in vitro through cell-to-cell interaction and partially through Notch activation.

Discussion

Blood formation in Vertebrates is an opportunistic phenomenon that does not take place exclusively in specialized, hematopoiesis restricted sites such as the bone marrow, thymus, spleen and avian bursa of Fabricius. Blood cells are also produced transiently in organs assuming other functions, such as the yolk sac, placenta, allantois and embryonic AGM and liver. Moreover extramedullary hematopoiesis can be resumed in pathologic conditions of the adult. Such anatomic diversity in blood-forming ability implies that developmentally and structurally distinct cellular environments can sustain hematopoiesis. Different blood-forming tissues may therefore share stromal cell subsets involved in blood formation. While hematopoietic stem and progenitor cells have been characterized in detail and purified to homogeneity, the identity and function of the
stromal cells involved in hematopoiesis have remained largely unknown. Although stroma-dependent hematopoiesis has been recapitulated in vitro for more than three decades using primary stromal cells or stromal cell lines, the nature of the stromal cells involved has been elusive. As an obstacle to characterization, native stromal cells involved in supporting hematopoiesis are infrequent: Wineman et al. found that only a rare subpopulation of clonal fetal liver stromal cells is able to maintain HSPCs.

Mesenchymal stem/stromal cells are cultured, multipotent adherent cells that can support hematopoiesis. We hypothesized that MSCs contain distinct subsets of cells with different roles in the regulation of HSPCs. Based on recent descriptions of i) a key contribution of murine perivascular cells to the medullary hematopoietic “niche”, and ii) a pericyte ancestry for human MSCs, we directly addressed whether cultured human perivascular cells can sustain human HSPCs. Conventionally derived, heterogeneous MSCs and CD146+CD34-CD45- perivascular cells can be obtained from virtually all human vascularised tissues. In the present study we derived MSCs and CD146+ perivascular cells from fetal bone marrow (FBM) and human adipose tissue, which is commonly used as a convenient and abundant source of MSCs. Interestingly, the sustained presence of hematopoietic cells within adipose tissue has been recently reported.

CD146+ perivascular cells expressing nestin, CXCL12 and Lep-R were found in situ in the hematopoietic FBM as well as in adipose tissue. Sorted CD146+ perivascular cells homogeneously expressed in culture CD146 and higher levels of nestin, CXCL-12 and Jagged-1 compared to unfractionated MSCs or to CD146- cells. CD146+ perivascular cells therefore appear to represent the human counterpart of the CAR cells or nestin+
cells recently described in the mouse. A similar cell population has been documented in human bone marrow, where CD146+ perivascular cells expressing CXCL-12 and Jagged-1 can clonally recapitulate an ectopic hematopoietic microenvironment when implanted into mice. Human bone marrow reticular stromal cells, including CD146+nestin+VCAM+ cells, regulate HSPC homing through the secretion of CXCL-12. Pericyte-like cells from the human placenta have been also suggested to support hematopoietic cells in culture. However, direct evidence for the ability of prospectively purified human perivascular cells to sustain primitive hematopoietic cells in long-term culture has not been provided. Several studies have investigated the ability of MSCs to maintain HSPCs in co-culture systems, but these have routinely used cytokine supplementation either by direct addition or through transgene expression in MSCs. In most cases the decisive assays, primary and secondary transplantations of co-cultured hematopoietic cells into immunodeficient mice, have not been used to document the maintenance of primitive self-renewing stem cells. Most importantly, the identity of the specific subset of MSCs directly involved in the interaction with HSPCs is still unknown. In the present study, culture of CD34+ cells with MSCs or CD146+ perivascular cells without the addition of exogenous cytokines allowed us to define the intrinsic properties of these stromal populations in terms of hematopoietic cell support. Remarkably, unfractionated MSCs and purified CD146+ perivascular cells derived from the same specimen exhibited profound differences in the ability to sustain HSPCs. Both stromal cell populations improved the survival of hematopoietic cells compared to stroma-free, cytokine-free cultures. However, the total number of recovered HSPCs was consistently and significantly higher in CD146+ cell co-cultures. Furthermore, only
CD146+ perivascular cells sustained primitive HSPCs able to establish multi-lineage hematopoiesis in immunodeficient mice. Conversely, MSCs promoted rapid HSPC differentiation with consequent loss of engraftment ability. Our results also demonstrate that cell-to-cell contact between HSPCs and CD146+ perivascular cells plays a key role in HSPCs maintenance *in vitro*. We show that CD146+ cells express Notch ligands and that Notch inhibition in co-cultures results in decreased numbers of HSPCs and increased B-cell development, as previously described.\textsuperscript{49} Regulation of HSPCs by perivascular cells is very likely to be a multifaceted process, Notch signaling being only one of the mechanisms involved in HSPC maintenance. Although our results show an increase in PI+ dead cells in the whole co-culture following Notch inhibition, suggesting a role for Notch in supporting cell survival, additional studies will be required to determine whether Notch activation prevents specifically apoptosis/death in HSPCs.

De Toni et al. recently described the clinical-grade expansion of adipose MSCs able to support hematopoietic reconstitution in immunodeficient mice when co-injected with fresh CB CD34+ cells.\textsuperscript{50} The authors showed higher frequency of human CD45+ cells 3 weeks post-transplantation in mice that received CD34+ cells and MSCs as compared to mice injected with CD34+ cells alone. This difference was no longer observed 11 weeks post-transplantation, suggesting that fat MSCs support short-term progenitors. The lack of secondary transplantation assay did not allow the authors to establish whether fat MSCs can support maintenance of HSPC self-renewal. In our study we demonstrate that unfractionated MSCs failed to *ex vivo* support HSPCs with reconstituting ability even in primary recipients. Conversely, we demonstrate for the first time that the CD146+ subset of MSCs was uniquely able to maintain self-renewing HSPCs after 2 weeks in culture.
without growth factors, as showed by reconstitution of primary and secondary hosts. The identification of CD146+ perivascular cells as a defined and specific subset of human stromal cells able to sustain HSPCs may therefore have a critical impact on future clinical applications based on *ex vivo* expansion and genetic manipulation of HSPCs. Butler et al. recently described a cellular platform for the expansion of human HSPCs based on the co-culture with transformed endothelial cells in the presence of defined growth factors. In the present work co-cultures were performed using non-transformed stromal cells in the complete absence of added growth factors. Having identified CD146+ perivascular cells as the specific subset of MSCs involved in HSPC maintenance, further studies aimed to define optimal conditions to promote HSPC expansion will be needed.

For the first time to our knowledge, we document the direct role of an anatomically and phenotypically defined subset of human stromal cells – the CD146+ perivascular cells – in maintaining cultured hematopoietic stem and progenitor cells. A fraction of native and all cultured pericytes express α-SMA, therefore these findings also support a myofibroblastic identity for human hematopoietic stromal cells. Besides a functional ability to support hematopoietic cells following dissociation, purification and culture, human CD146+ perivascular cells from non-hematopoietic tissues share a similar phenotype with the perivascular niche cells recently described in murine bone marrow. Perivascular cells are ubiquitous and may therefore represent the key stem cell support shared by all blood-forming organs. It remains to be determined if and how this ability to sustain hematopoietic stem cells is repressed *in situ* in non-hematopoietic tissues, and may be reactivated in pathologic conditions, as in the course of extramedullary hematopoiesis or leukaemic dissemination.
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Authorship Contribution

M.C. designed and performed research, analyzed and interpreted data and wrote the manuscript; C.P., A.S., W.W., D.E. and C.J.C. performed research; X.W. performed statistical analysis; E.M and L.L. analyzed and interpreted data and contributed to writing the manuscript; G.M.C and B.P. designed research, analyzed and interpreted data and wrote the manuscript.
Conflict-of-interest disclosure

The authors declare no competing financial interests.

References:

Figure Legends

**Figure 1. In situ expression of hematopoietic niche markers by human perivascular cells:** a-d) Immunohistochemistry performed on paraffin embedded sections of 17 week old human fetal bone marrow. Pericytes surrounding microvessels express CD146 (a), nestin (b), CXCL-12 (c) and leptin receptor (Lep-R) (d) (magnification 63x). e-s) Immunohistochemistry performed on human adipose tissue cryosections. e-g) von Willebrand factor (vWF) positive endothelial cells (green) are surrounded by perivascular cells expressing nestin (e), CXCL-12 (f) and Lep-R (g). h-s) Triple staining immunohistochemistry performed on human adipose tissue cryosections shows co-expression of CD146 with nestin (k-m), CXCL12 (n-p) and LepR (q-s). Single staining with anti-vWF antibody followed by incubation with conjugated IgG controls revealed the lack of autofluorescence (h-j) (magnification 40x).

**Figure 2. Isolation and culture of MSCs and stromal subsets from lipoaspirate.** a) Stromal vascular fraction (SVF) was obtained from human lipoaspirate specimens (n=4 donors). An aliquot of SVF was directly seeded in tissue culture plates for the isolation of conventional MSCs by plastic adherence. Another aliquot of SVF was processed for FACS sorting of DAPI-CD45-CD34-CD146+ perivascular cells and DAPI-CD45-CD34+CD146- cells. b) FACS analysis of cultured fat-derived MSCs, CD146+ perivascular cells and CD146- cells. After 9 passages in culture, MSCs retain a low percentage of CD146+ cells, while purified CD146+ perivascular cells and CD146- cells retain a stable phenotype homogeneously positive and negative for CD146, respectively.
Figure 3. Cultured CD146+ perivascular cells express markers of hematopoietic perivascular niche cells: a,b) *ex vivo* expanded CD146+ perivascular cells purified from fat and fetal bone marrow (FBM) similarly express higher levels of mRNA of perivascular cell markers when compared to MSCs and CD146- cells (n=2 donors for each tissue). c-n) Fat and FBM derived CD146+ perivascular cells similarly and almost exclusively express nestin (c-f) and CXCL-12 (g-j) in culture compared to CD146- cells. No difference in Lep-R expression was observed between CD146+ and CD146- cells from either fat and FBM (k-n) (magnification 20x).

Figure 4. CD146+ perivascular cells promote *ex vivo* maintenance of undifferentiated HSPCs: a) In the absence of cytokines and stromal cell feeder layer (No feeder), CD45+ hematopoietic cells cultured in retronectin (RN) treated wells rapidly died within the first two weeks of culture. At any time of culture, the total number of CD45+ cells recovered from CD146+ cell co-cultures was significantly higher when compared to MSC co-cultures (n=at least 5 independent experiments for each time point, each experiment was performed in triplicate, ** p<0.01; *** p<0.001). b) A similar pattern was observed for the total number of CD34+ cells (n=at least 5 independent experiments for each time point, each experiment was performed in triplicate, *** p<0.001). c) Representative FACS analysis after 2 weeks of co-culture of CB CD34+ cells with MSCs or CD146+ cell co-cultures. After gating on CD45+CD10-CD19- cells, CD34+33- cells were defined as CD34+Lin- cells (black box) d) The absolute number of CD34+Lin- cells was significantly higher in CD146+ cell co-cultures, compared to MSC co-cultures, at any time of culture (n=at least 5 independent experiments for each time point, each experiment was performed in triplicate, ** p<0.01; *** p<0.001). e-f) Co-
culture of CB CD34+ cells with MSCs lead to significantly higher frequency of CD14+ myeloid cells after 2 weeks (e) (40.24 ± 2.723% vs. 26.67 ± 2.075%. n=10 independent experiments, each experiment was performed in triplicate, *** p<0.0001) and higher frequency of CD10+/CD19+ lymphoid progenitors or mature cells after 4 weeks of co-culture (f) (5.155 ± 1.918% vs. 0.9541 ± 0.2564%. n=8 independent experiments, each experiment was performed in triplicate, * p<0.05). No difference in the absolute numbers of myeloid and lymphoid cells was observed between CD146+ cell and MSC co-cultures. All data are presented as mean+/− SEM.

Figure 5. CD146+ perivascular cells but not MSCs sustain functional HSPCs with engraftment potential and self-renewal ability. a) Colony-forming unit assay (CFU) revealed significantly higher number of CFUs in CD146+ cell co-cultures after 1, 2, 4 and 6 weeks of co-culture as compared to MSC co-cultures (n=3 independent experiments, each experiment was performed in triplicate, * p<0.05; *** p<0.001). b) Representative flow cytometry analysis for the detection of human CD45+HLA+ cells in bone marrow of NSG mice 6 weeks post transplantation with PBS, or with the same number of CD45+ cells (10^5) harvested after 2 weeks of CB CD34+ cell co-culture with MSCs or CD146+ cells. c) All mice injected with CD45+ cells obtained from CD146+ cell co-cultures showed human engraftment whereas no engraftment was ever detected (ND) in mice that received MSC co-cultures (n=3 independent experiments. n=11 mice per group) (*** p<0.0001). d) Frequency of CD34+ progenitors, CD19+ lymphoid and CD14+ myeloid cells within the CD45+HLA+ population of cells in the bone marrow of chimeric mice. e) Human CD45+HLA+ hematopoietic cells were also detected six weeks post transplantation in the contra-lateral tibia of mice injected with HSPCs co-cultured
with CD146+ perivascular cells. f-i) Representative flow cytometry analysis of secondary host bone marrow. f) Bone marrow from primary hosts transplanted with MSC co-culture was injected in secondary hosts as a negative control. g) Human engraftment was observed 4 weeks after secondary transplantation of bone marrow from chimeric mice transplanted with CD146+ cell co-culture (n=3 engrafted mice out of 4). h) Both CD19+ lymphoid and CD33/CD14/CD15+ myeloid cells were detectable within the human CD45+ engrafted hematopoietic cells in secondary hosts. i) Quantification of the level of chimerism in secondary mice. All data are presented as mean +/-SEM.

**Figure 6. CD146+ perivascular cells induce Notch activation in hematopoietic cells:**

a) Immunocytochemical staining for Jagged-1 (JAG1, red), CD146 (green) and nuclei (DAPI, blue) performed on fat-derived CD146+ perivascular cells and MSCs (magnification 20x). White arrows indicate clusters of cells within the MSC that co-express JAG1 and CD146. b) Western blot analysis showing significantly higher expression of Jagged-1 in CD146+ perivascular cells compared to MSCs derived from fat. c) qPCR analysis revealed that fat-derived MSCs and CD146+ perivascular cells express multiple Notch ligands. d,e) Quantification of CD45+ (d) and CD34+ (e) hematopoietic and progenitor cells with activated Notch pathway (CD45+NICD+) after one week of co-culture with fat or FBM derived CD146+ perivascular cells, MSCs and CD146- cells. (n=3 independent experiments, n=40 random fields analyzed, ***p<0.0001). Data are presented as mean +/- SEM.

**Figure 7. Notch inhibition affects survival and B-cell differentiation of HSPCs.**

Inhibition of Notch was achieved by addition of 10μM DAPT to CD146+ perivascular cells and CB CD34+ cell co-culture every other day. Vehicle (DMSO) was added to
control co-cultures. a-b) Total number of CD45+ cells and CD34+Lin- cells was significantly reduced after two weeks of co-culture with DAPT (5.03 ± 0.54x10^4 vs. 3.02 ± 0.37x10^4 CD45+ cells, n=4 independent experiments, each experiment was performed in triplicate, ** p<0.01; 1.5 ± 0.16 x10^4 vs. 0.82 ± 0.12x10^4 CD34+Lin- cells, n=4 independent experiments, each experiment was performed in triplicate, ** p<0.01). c) Similarly, the total number of CFUs was significantly reduced after 4 weeks of co-culture with DAPT (478.3 ± 112.4 vs. 191.0 ± 43.28, n=3 independent experiments, each experiment was performed in triplicate, * p<0.05). d) Flow cytometry viability analysis revealed a significantly higher frequency of PI+ dead cells in co-culture performed in the presence of DAPT (13.08 ± 1.13% vs. 19.94 ± 1.31, n=4 independent experiments, each experiment was performed in triplicate, *** p<0.0001). e) Notch inhibition also significantly increased B-cell development (0.13 ± 0.04x10^3 vs. 1.72 ± 0.55x10^3 of lymphoid cells, n=3 individual experiments, each experiment performed in triplicate, ** p<0.01). Data are presented as mean +/- SEM.
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