

CD34+ CD45- Cells Isolated from Human Blood Have Molecular and Functional Characteristics of Vascular Endothelial Progenitors

Yajuan Jiang¹, Alexander M. Tsankov²,
Alexander Meissner² and David W. O'Neill¹

¹PCT, a Caladrius Company, Allendale, NJ, USA

²Harvard University, Cambridge, MA, USA



HARVARD
UNIVERSITY



Background

Rare CD45- cells have been described in human blood, bone marrow and umbilical cord blood that lack blood cell lineage markers (Lin-) but express antigens found on hematopoietic progenitors (CD34 and/or CD133). There is no consensus regarding the identity of this Lin- CD45- CD34 and/or CD133+ (CD34/133+) population, or whether it may be a heterogeneous mixture of cells with different functions. There is evidence that at least some of these cells have stem or progenitor activity, with pluripotent (Zuba-Surma 2009, Havens 2014) and vascular endothelial progenitor (Yoder 2012) activities described. However, the identity, phenotype and gene expression patterns of such putative stem or progenitor cells remain a matter of debate.

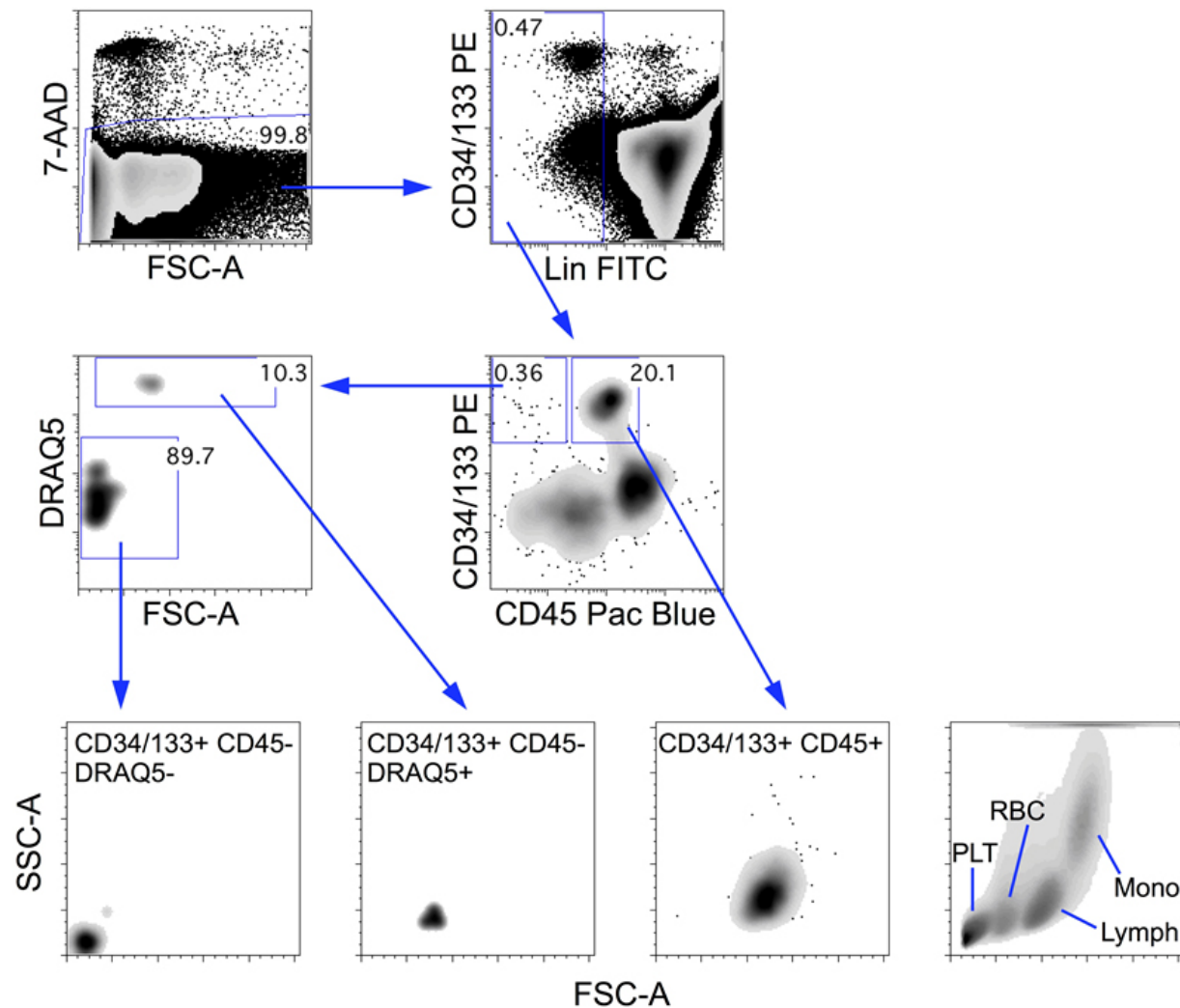
To better characterize the Lin- CD45- CD34/133+ cell population in human blood, we purified these cells using elutriation, immunomagnetic selection and FACS sorting from both adult peripheral blood (G-CSF mobilized leukapheresis) and umbilical cord blood, analyzing cell fractions for physical characteristics (size, density, morphology), cell surface phenotype (multicolor flow cytometry) and molecular evidence of heterogeneity (single cell gene expression analysis).

Results

Characterization of Lin- CD45- CD34/133+ cells from G-CSF mobilized peripheral blood by multicolor flow cytometry. Initial experiments used 5-color staining with antibodies to blood cell lineage markers (FITC-conjugated cocktail for CD2, CD3, CD4, CD7, CD8, CD10, CD11b, CD14, CD19, CD20, CD41, CD56 and CD235a), CD45 (APC-conjugated), CD34 and CD133 (both PE-conjugated, to including both CD34 and/or CD133+ cells in the same gate), and the dyes 7-AAD (membrane impermeable, stains the nuclei of dead cells) and DRAQ5 (membrane permeable, and stains nucleic acids in both live and dead cells), which were used to distinguish live nucleated cells from dead cells, extracellular vesicles and debris.

We observed that 10% or fewer of the Lin- CD45- CD34/133+ events stained brightly with DRAQ5, indicating the presence of a nucleus, with the remaining Lin- CD45- CD34/133+ events staining DRAQ5 negative. These DRAQ5- events displayed FSC-SSC characteristics similar to platelets, suggesting a size of 2 to 3 microns. DRAQ5+ events were larger but still small compared to blood leukocytes, and were approximately the size of a red blood cell (**Fig. 1**).

Fig. 1. 5-color flow cytometry of G-CSF mobilized peripheral blood shows two populations of Lin- CD45- CD34/133+ events. Cells were stained using a cocktail of anti-CD34 PE and CD133 PE antibodies so that cells that express either CD34 or CD133 and cells that co-express both markers would be included in the same gate. Two populations of Lin- CD45- CD34/133+ events were shown by DRAQ5 (nucleic acid) staining (center row, left panel). DRAQ5- events are very small (size of platelets, bottom row). DRAQ5+ events are approximately the size of red blood cells, whereas CD45+ hematopoietic progenitor cells are considerably larger (comparable to a large lymphocyte). Bottom right panel is a SSC-FSC plot of partially lysed human blood, with platelet (PLT), red cell (RBC), lymphocyte (Lymph) and monocyte (Mono) populations shown.



Physical characterization studies. Elutriation of G-CSF mobilized peripheral blood (**Fig. 2**) also was consistent with two populations of Lin- CD45- CD34/133+ events: DRAQ5+ objects that elutriate at intermediate counterflow rates (70 ml/min, similar to small cells such as lymphocytes or RBCs), and DRAQ5- objects that elutriated at very low counterflow rates (35 ml/min, similar to objects the size of platelets).

Differential centrifugation experiments (**Fig. 3**) also showed 2 populations of Lin- CD45- CD34/133+ objects: a light DRAQ5- population (does not pellet at 400 x g, 10 min) and a denser DRAQ5+ population (pellets at 100 x g, 10 min).

Lin- CD34/133+ objects were purified by elutriation, CD34/133 immunomagnetic selection (Miltenyi Biotec) and 4-color FACS sorting (MoFlo XDP cell sorter, with no DRAQ5, which is toxic to cells) and then analyzed by light microscopy of cytospin preparations (**Fig. 4**). CD45- CD34/133+ objects that elutriated at 70 ml/min were small nucleated cells, considerably smaller than CD45+ CD34/133+ hematopoietic progenitors (**Fig. 4C**). CD45- CD34/133+ objects that elutriated at 35 ml/min had the appearance of small extracellular vesicles, approximately the size of platelets (2 to 3 microns in diameter). Electron microscopy (**Fig. 4C**, lower right, inset) showed these objects to be morphologically different from platelets, which have characteristic large granules.

Fig. 2. Elutriation of G-CSF mobilized peripheral blood shows two populations of Lin- CD45- CD34/133+ objects.

(A) Experimental design. Elutriation (which separates cells by size) into 3 fractions (small, intermediate and large) was followed by 5-color flow cytometry analysis. (B) Average cell counts in elutriation fractions (n=15 experiments). Of the CD45- CD34/133+ objects, the DRAQ5- population purifies at low counterflow (35 ml/min), whereas the DRAQ5+ population purifies at intermediate counterflow (70 ml/min). Most CD45+ blood leukocytes purify in the rotor-off fraction (>70 ml/min). (C) DRAQ5 staining shows >99% of Lin- CD45- CD34/133+ objects in elutriation fraction 35 are DRAQ5-, whereas >75% of the Lin- CD45- CD34/133+ objects in elutriation fraction 70 are DRAQ5+.

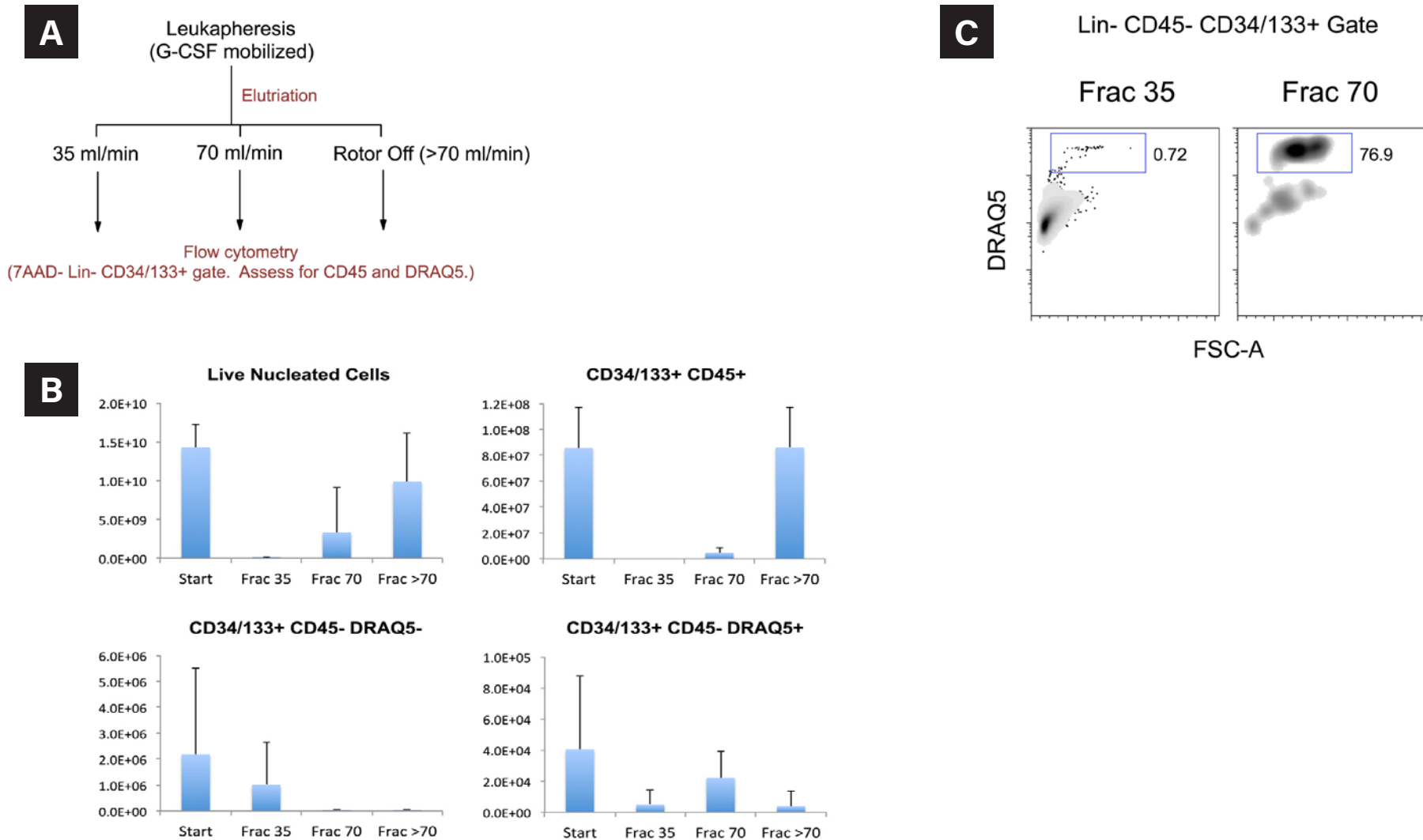


Fig. 3. Differential centrifugation of G-CSF mobilized peripheral blood shows two populations (dense DRAQ5+ and light DRAQ5-) of CD45- CD34/133+ objects. (A) Experimental design. Cells were centrifuged at 400 x g for 10 min and separated into supernatant and pellet fractions. Pellets were re-suspended in buffer and centrifuged at 100 x g for 10 min. Pellet and supernatant fractions were again taken. All fractions were analyzed by 5-color flow cytometry. (B) Without DRAQ5 staining, the vast majority of Lin- CD45- CD34/133+ objects do not pellet at 400 x g. When DRAQ5+ objects only are assessed, almost all Lin- CD45- CD34/133+ objects pellet at 100 x g.

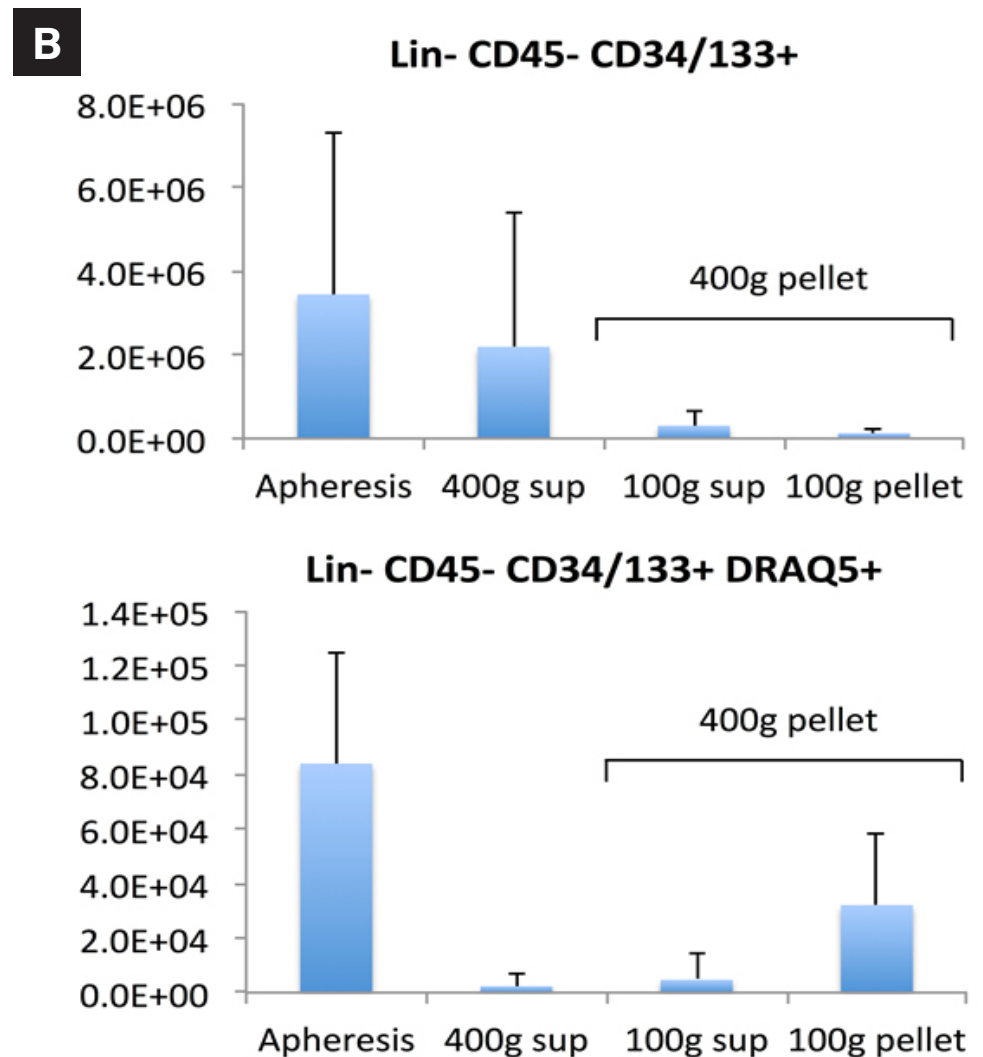
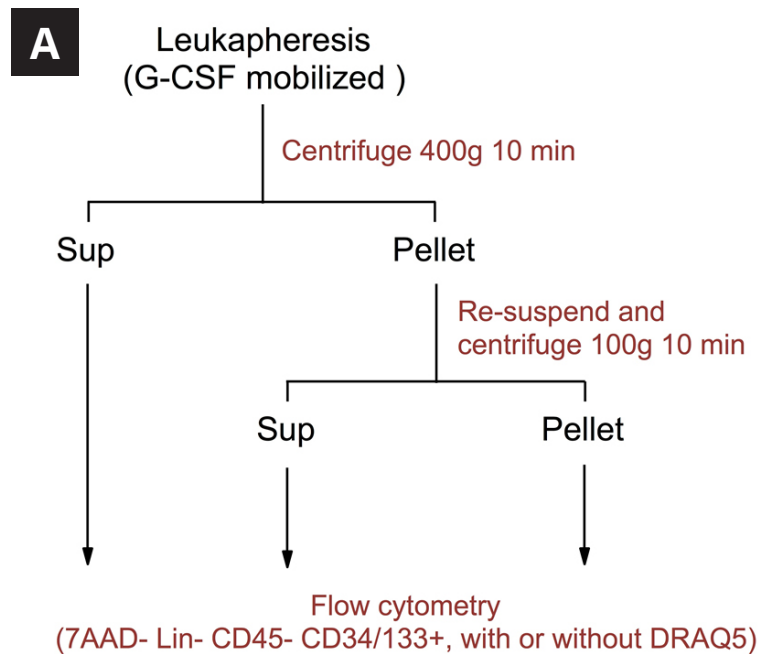
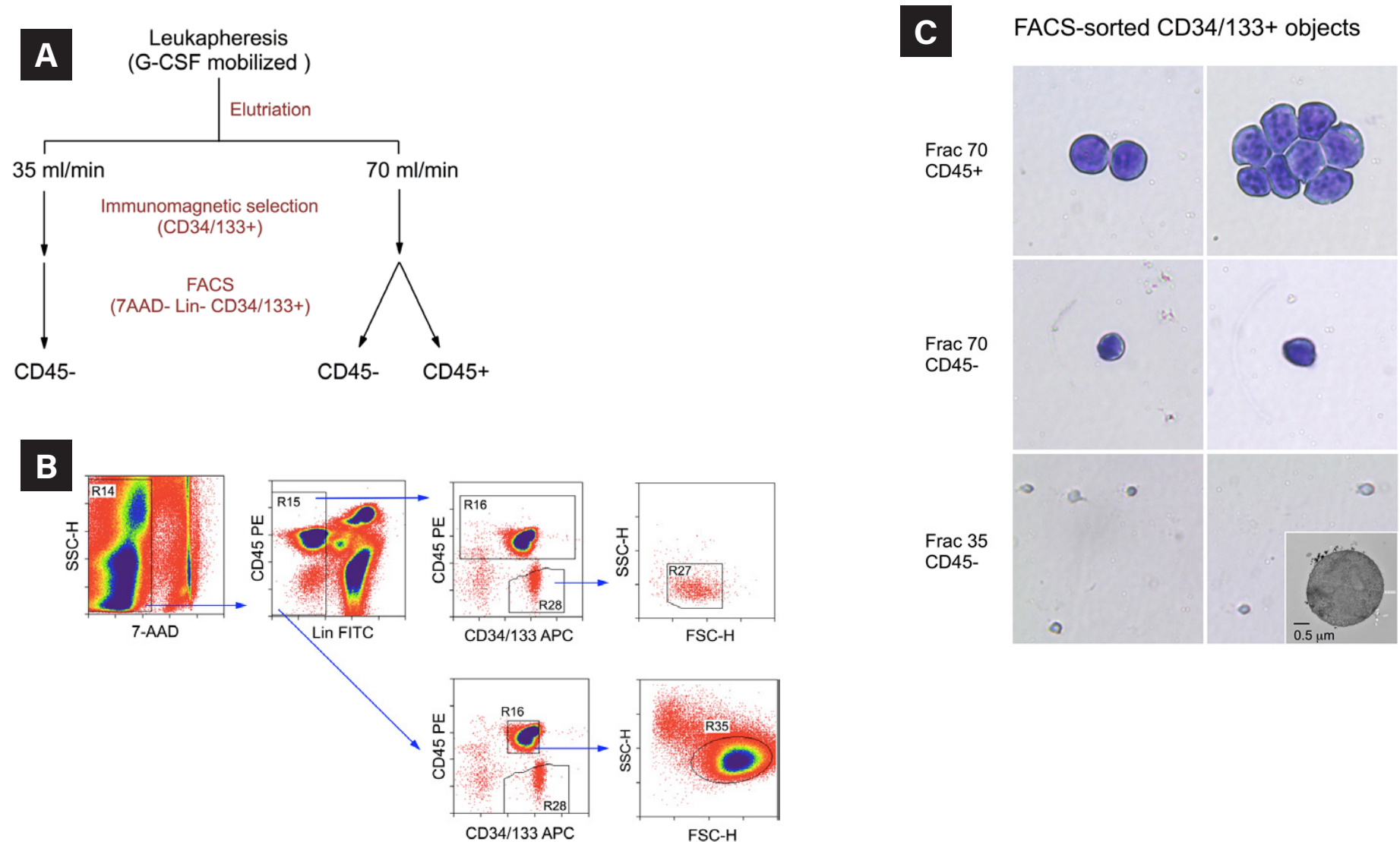
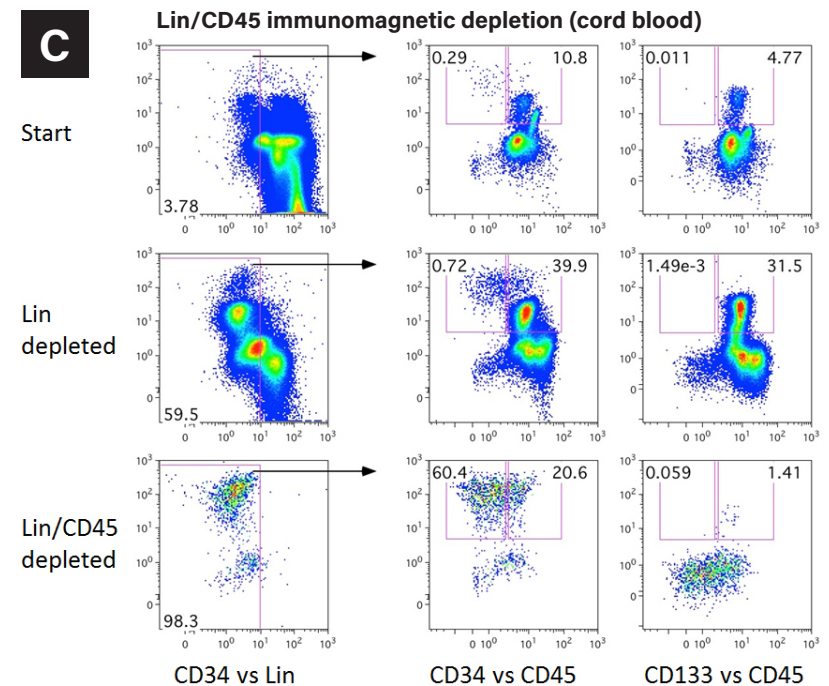
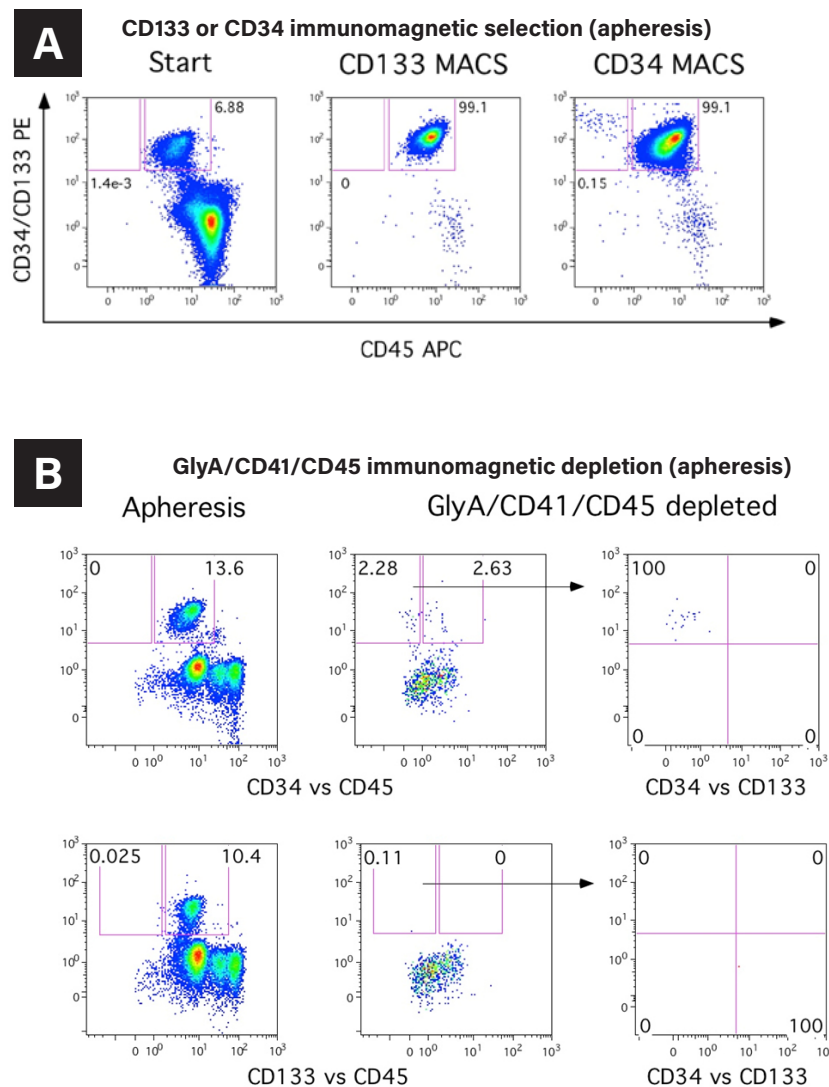


Fig. 4. Morphology of FACS-sorted Lin- CD34/133+ objects from G-CSF mobilized peripheral blood. (A) Experimental design. Lin- CD34/133+ objects were purified by elutriation, CD34/133 immunomagnetic selection and 4-color FACS sorting. (B) Representative FACS gating and acquisition data for sort of 70 ml/min elutriation fraction. (C) Cytospin preparations of Lin- CD34/133+ objects after FACS sorting (1,000x magnification). Inset in lower right panel is an electron micrograph of a Lin- CD45- CD34/133+ object from the 35 ml/min elutriation fraction. Lin- CD45- CD34/133+ objects from elutriation fraction 70 are small nucleated cells. Lin- CD45- CD34/133+ objects from elutriation fraction 35 are very small (1-3 micron) extracellular vesicles.



Extended flow cytometry phenotyping. 6-color flow cytometry (**Fig. 5**) using CD34 and CD133 antibodies in different fluorescences (CD34 VioBlue and CD133 PE) showed that almost all of the nucleated Lin⁻ CD45⁻ CD34/133⁺ cells in G-CSF-mobilized peripheral blood express CD34 but not CD133. This Lin⁻ CD45⁻ CD34⁺ CD133⁻ nucleated cell population has a frequency of approximately 1 in 300,000 blood mononuclear cells. Lin⁻ CD45⁻ CD133⁺ cells were variably seen and extremely rare (1 in 10 million PBMC). Although difficult to characterize, they appear to lack expression of CD34 (Lin⁻ CD45⁻ CD34⁻ CD133⁺). Similar vesicle and cell populations were found in umbilical cord blood, at somewhat higher frequencies than in G-CSF mobilized peripheral blood.

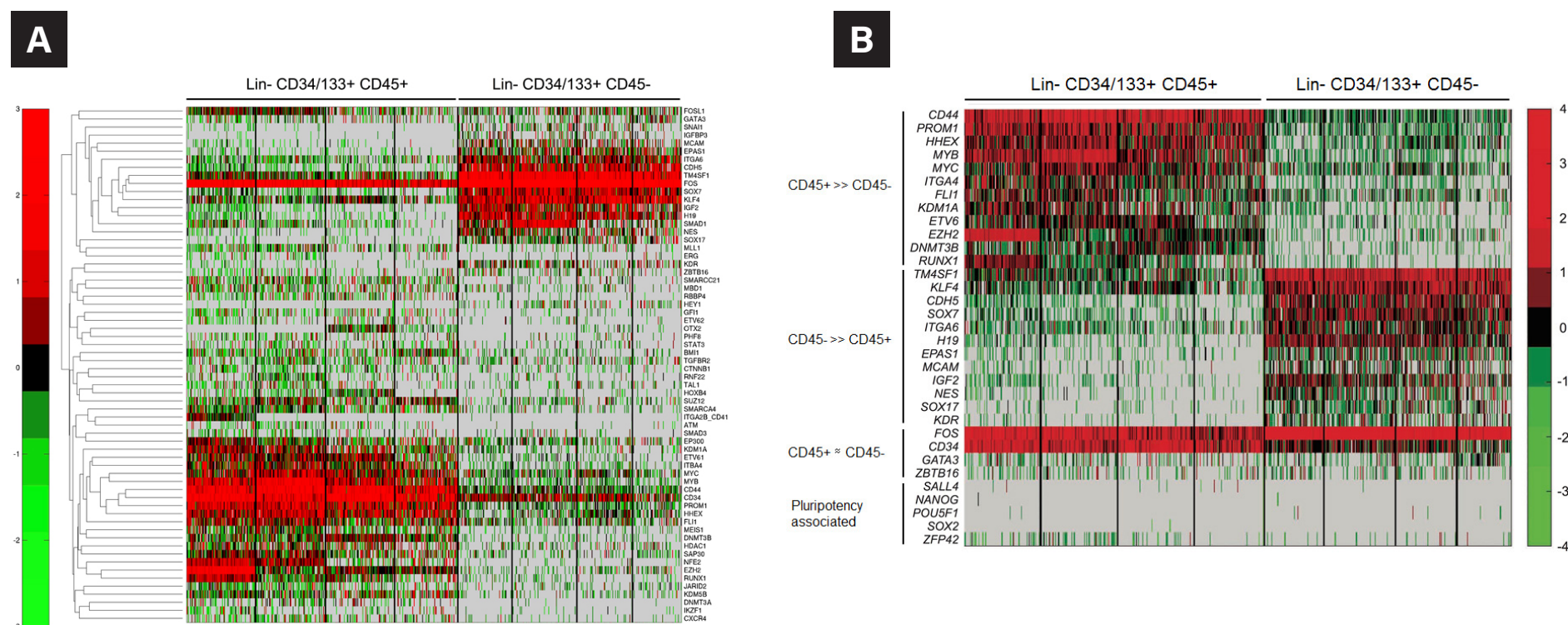
Fig 5. CD133 expression is largely restricted to CD45+ cells, with CD45-CD133+ cells at the limit of detection in human G-CSF mobilized peripheral blood and umbilical cord blood. (A) Immunomagnetic selection with anti-CD133 beads does not enrich for CD45- CD34/133+ cells. (B) Immunomagnetic depletion of RBCs, platelets and CD45+ cells enriches for Lin- CD45- CD34+ CD133- cells and very rare Lin- CD45- CD34- CD133+ cells. (C) Enrichment of Lin- CD45- CD34+ cells, but not Lin- CD45- CD133+ cells from human umbilical cord blood by Lin and CD45 immunomagnetic depletion.



Single cell gene expression analysis of Lin- CD34/133+ cells. To better characterize the Lin- CD45- CD34/133+ cell population, single Lin- CD45- CD34/133+ cells from G-CSF mobilized peripheral blood were enriched by elutriation and CD34/133 immunomagnetic selection and then sorted (0.5 cells per well) into wells of 384-well plates for targeted RNA sequencing (Cellular Research Precise™ assay) using a panel of 150 primers for mesodermal, pluripotent and hematopoietic-associated RNAs (**Fig. 6A**). Lin- CD45+ CD34/133+ hematopoietic progenitors were sorted as controls. Wells contained 5 µl of 1/100x New England BioLabs Phusion® HF buffer in nuclease-free water. RNA was processed for targeted RNA sequencing using the Cellular Research Precise™ Assay kit. Approximately 400 cells were individually analyzed for both populations.

The two Lin- CD34/133+ cell populations had very distinct gene expression patterns, with CD45+ cells having RNAs consistent with hematopoietic progenitors (CD44, CD133, HHEX, MYB, MYC, CD49d, FLI1) and CD45- cells having RNAs associated with the vascular endothelial lineage (TM4SF1, KLF4, CDH5, SOX7, SOX17, KDR, CD49f, H19, IGF2, CD146, Nestin, **Fig. 6B**). No clear evidence of heterogeneity was seen within either cell population. CD133 (PROM1) expression was clearly evident only in the CD45+ cell population. Aside from KLF4 (in the CD45- population) and MYC (in the CD45+ population), no clear expression of pluripotency-associated genes was seen (**Fig. 6B**).

Fig. 6. Cellular Research Precise™ Assay based single cell gene expression measurements. (A) Normalized gene expression level heat map for genes with measurable expression (out of a panel of 150 selected genes). (B) Heat map for a selected set of genes ordered from top to bottom as follows: higher expression in CD45+ than in CD45-; higher in CD45- than in CD45+; comparable expression in both and largely negative expression of representative pluripotency-associated transcription factors. Samples in the panels are from left to right derived from four 96 well plates each, Lin- CD34/133+ CD45+ and Lin- CD34/133+ CD45-, respectively. Scale represents normalized expression in terms normalized UMI (unique molecular identifier) counts.



Functional assays on sorted cells. To assess whether Lin⁻ CD34⁺ CD45⁻ cells have endothelial progenitor activity, we FACS sorted umbilical cord blood into CD45⁻CD34⁻, CD45⁺CD34⁻, CD45⁻CD34⁺ and CD45⁺CD34⁺ fractions for analysis using a well established bioassay [Endothelial Colony Forming Cell, or ECFC assay (Ingram 2004), **Fig. 7**]. Enrichment of ECFC activity (compared to the level of activity observed in cord blood mononuclear cell preparations) was observed only in the CD45⁻CD34⁺ fraction (**Fig. 7D**). Cells expanded from the ECFC colonies had both phenotypic (expression of vWF, KDR, CD31, CD144 and CD146) and functional (capillary-like structures in Matrigel, Ac-LDL uptake) characteristics of endothelial cells (**Fig. 8**).

Fig. 7. FACS sorting of human umbilical cord blood into 4 populations based on CD34 and CD45 expression, followed by Endothelial Colony-Forming Cell (ECFC) Assay. FACS-sorted cells (A and B) were seeded into EGM-2 complete medium (containing VEGF, EGF, FGF and R3-IGF-1) in wells of 6-well plates pre-coated with collagen. As a control, cord blood mononuclear cells were seeded at 50 million cells per well. ECFC colonies, which form a well-circumscribed cobblestone monolayer (C), begin to appear after 5 to 22 days, after which cells become confluent and can continue to be passaged. (D) ECFC activity enriched only in the CD34+CD45- fraction.

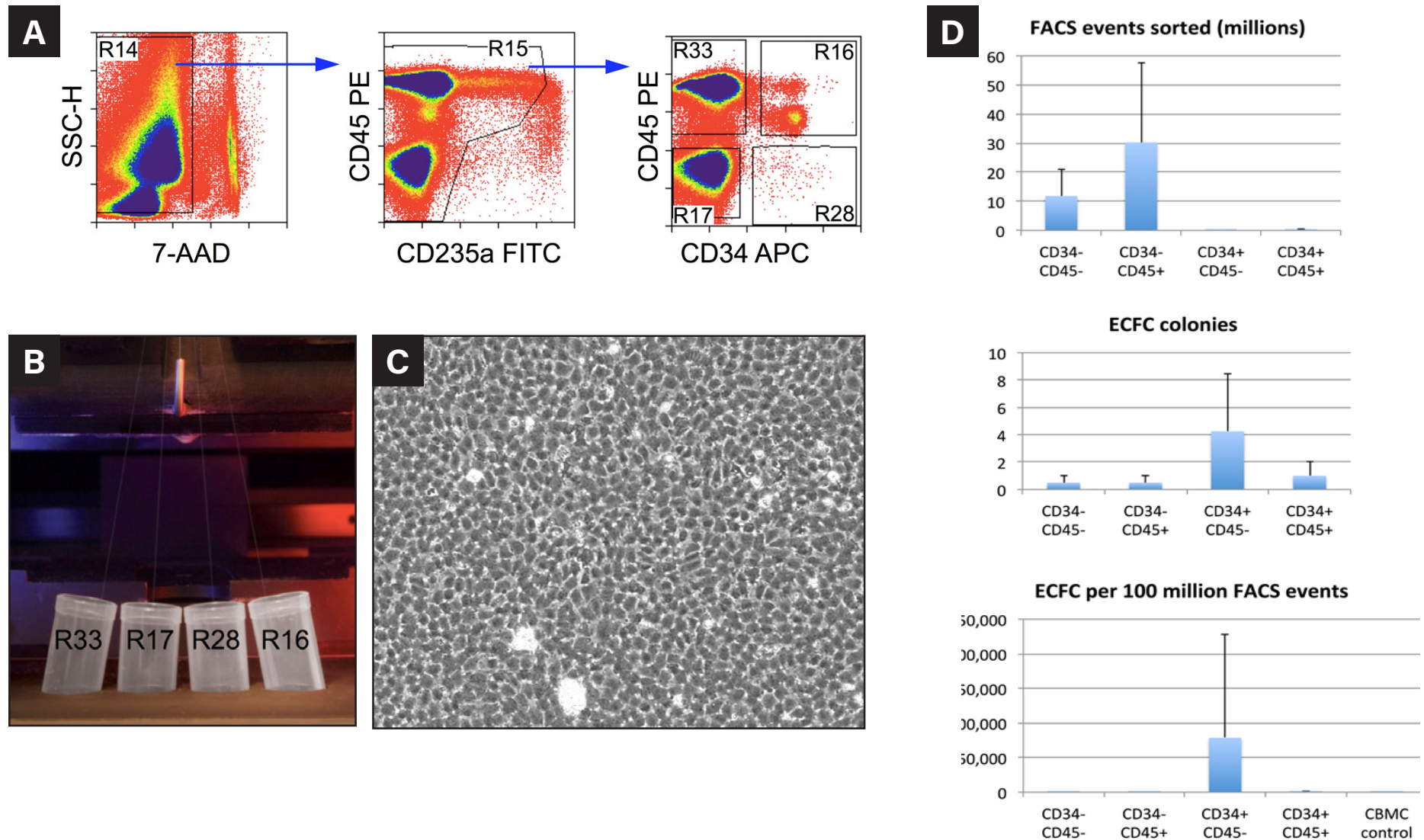
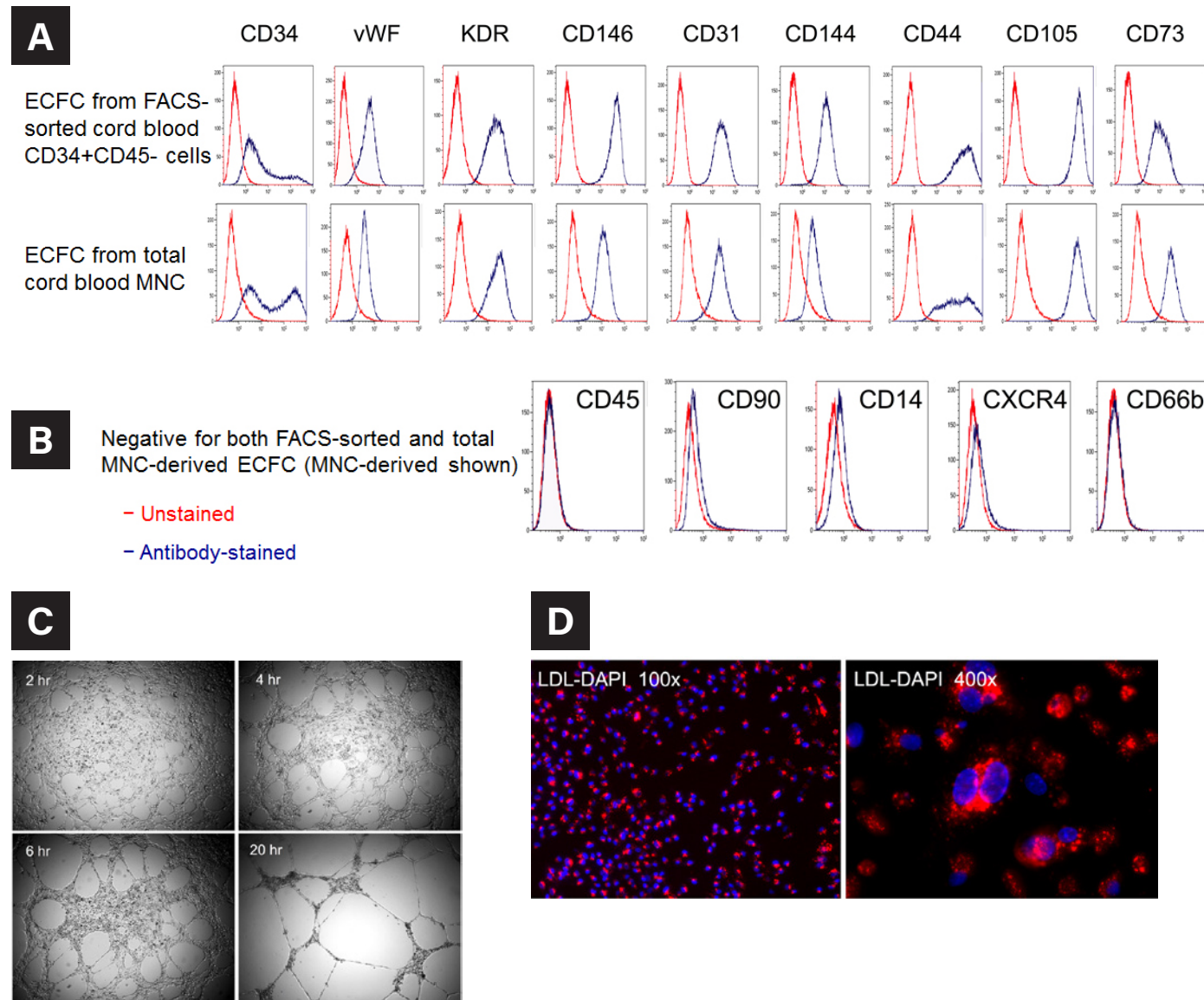


Fig. 8. Endothelial cell phenotype and function of expanded ECFC from sorted CD34+CD45- cells. (A and B) Cell surface phenotype of ECFC derived from FACS-sorted CD45-CD34+ cells compared to ECFC from total cord blood MNC. Both populations show comparable expression of multiple endothelial lineage-associated markers. (C and D) Endothelial function in vitro of expanded ECFC from FACS-sorted cord blood CD34+CD45- cells. (C) Capillary-like structures in Matrigel (40x magnification). (D) Dil-Ac-LDL uptake.



Conclusions

- **Over 90% of Lin- CD45- CD34/133+ objects in human blood are extracellular vesicles and not cells. All or almost all of these objects are CD34+ CD133-. They are most likely of vascular origin. Their function, if any, is not known.**
- **Less than 10% of Lin- CD45- CD34/133+ objects in human blood are live nucleated cells. These cells are rare (typically less than 1 in 100,000 blood mononuclear cells in mobilized peripheral blood, and less than 1 in 10,000 cord blood mononuclear cells) and are relatively small - about the size of a red blood cell. Almost all of these cells are CD34+ CD133-.**
- **Lin- CD45- CD133+ CD34- cells appear to exist but are at the limit of detection (perhaps less than 1 in 10 million blood mononuclear cells) and therefore difficult to characterize.**
- **By single cell gene expression analysis, Lin- CD45- CD34+ cells have a signature associated with the vascular endothelial lineage. There does not appear to be significant heterogeneity in this population. The cells do not express CD133 or, other than KLF4, markers associated with pluripotency, although a rare subtype in this population cannot be ruled out.**
- **Co-expression of CD34 and CD133 is only observed in cells of the hematopoietic lineage.**
- **Endothelial progenitor activity in human cord blood, as measured by ECFC (Endothelial Colony Forming Cell) assay of FACS sorted cells, is greatly enriched in the CD45- CD34+ cell population.**

References

Alvarez-Gonzales, C., et al. PloSOne (2013).

Asahara, T., et al. Science (1997).

Danova-Alt, R. et al. PloSOne (2012).

Havens, AM. et al. Stem Cells Dev (2014).

Ingram, DA. et al. Blood (2004).

Mund, JA. et al. Arterioscler Thromb Vasc Biol (2012).

Yoder, MC. Cold Spring Harb Perspect Med (2012).

Zuba-Surma, EK. et al. Eur J Hematol (2009).

This work was supported by Caladrius Biosciences.