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A comparison of haematopoietic stem cells from umbilical cord blood and peripheral blood for platelet production in a microfluidic device

Katrijn R. Six,[1,2] Géraldine Sicot,[3] Rosalie Devloo,[1] Hendrik B. Feys,[1,2] Dominique Baruch[3,4] & Veerle Compernolle[1,2,5]

[1]*Transfusion Research Center, Belgian Red Cross-Flanders, Ghent, Belgium*
[2]*Faculty of Medicine and Health Sciences, Ghent University, Ghent, Belgium*
[3]*PlatOD, Paris, France*
[4]*INSERM, UMR_S1140, University Paris Descartes, Sorbonne Paris Cité, Paris, France*
[5]*Blood Services, Belgian Red Cross-Flanders, Mechelen, Belgium*

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Background and objectives Several sources of haematopoietic stem cells have been used for static culture of megakaryocytes to produce platelets *in vitro*. This study compares and characterizes platelets produced in shear flow using precursor cells from either umbilical (UCB) or adult peripheral blood (PB).

Materials and methods The efficiency of platelet production of the cultured cells was studied after perfusion in custom-built von Willebrand factor-coated microfluidic flow chambers. Platelet receptor expression and morphology were investigated by flow cytometry and microscopy, respectively.

Results Proliferation of stem cells isolated out of UCB was significantly higher ($P < 0{\cdot}0001$) compared to PB. Differentiation of these cells towards megakaryocytes was significantly lower from PB compared to UCB where the fraction of CD42b/CD41 double positive events was 44 ± 9% *versus* 76 ± 11%, respectively ($P < 0{\cdot}0001$). However, *in vitro* platelet production under hydrodynamic conditions was more efficient with 7·4 platelet-like particles per input cell from PB compared to 4·2 from UCB ($P = 0{\cdot}02$). The percentage of events positive for CD42b, CD41 and CD61 was comparable between both stem cell sources. The mean number of receptors per platelet from UCB and PB was similar to that on blood bank platelets with on average 28 000 CD42b, 57 000 CD61 and 5500 CD49b receptors. Microscopy revealed platelets appearing similar to blood bank platelets in morphology, size and actin cytoskeleton, alongside smaller fragments and source megakaryocytes.

Conclusion This characterization study suggests that platelets produced *in vitro* under flow either from UCB or from PB share receptor expression and morphology with donor platelets stored in the blood bank.

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Key words: haematopoiesis, megakaryopoiesis, platelets, stem cells, tissue engineering.

Introduction

Transfusion of platelet concentrates (PC) can be a life-saving treatment for patients suffering from thrombocytopenia [1]. Platelet inventory management is a major challenge because donor attendance is unpredictable and

Correspondence: Hendrik B. Feys, Transfusion Research Center, Belgian Red Cross-Flanders, Ottergemsesteenweg 413, 9000 Ghent, Belgium
E-mail: hendrik.feys@rodekruis.be

the demand for PC is increasing, in addition to the limited shelf life of PC [2, 3]. This has stimulated researchers to investigate *in vitro* production of platelets as an alternative to donor-derived PC. The platelet moreover offers an ideal model cell for tissue engineering with limited risks of carcinogenesis because platelets are anucleate and thus do not replicate and the final product could be irradiated using low intensity gamma irradiation before transfusion. [4, 5].

Platelets are produced by megakaryocytes (MK) that reside outside the blood vessel but protrude cellular extensions that release (pro)platelets in circulation [6, 7]. When this physiological production process can be mimicked *in vitro*, an attractive alternative for the current platelet blood banking chain is offered. The ideal unlimited cell source for MK is a self-renewable embryonic [8] or induced pluripotent stem cell [9, 10] or immortalized or chemically defined MK cell line [5, 11]. However, research into the platelet production process currently still requires isolated primary haematopoietic stem cells (HSC) until limitations of the current self-renewable cell sources are overcome. Umbilical cord blood (UCB) is often used as HSC source [12–16] but requires specialized access and is thus not always available to research teams. Leuko-depletion filters as a waste product of whole blood donations therefore offer an attractive alternative source [17]. But even simpler, buffy coats from routine whole peripheral blood donations (PB) are available daily in many blood banks in Europe and may be source material for stem cell isolation [18] and *in vitro* platelet production on a research scale [19].

In this study, the efficiency and quality of *in vitro* platelet production in a microfluidic flow chamber chip coated with von Willebrand factor (VWF) [20] was evaluated for cells derived from UCB compared to PB. The platelets were studied by differential interference contrast (DIC) and fluorescent microscopy and flow cytometry. Data were compared to standard blood bank platelets (bPLT).

Materials and methods

Isolation of $CD34^+$ haematopoietic stem cells

Human HSC were obtained from UCB or from buffy coats isolated from PB. The research protocols were approved by ethical committees of Ghent University Hospital (2016/0448) and Antwerp University Hospital (B300201420404). Isolation of HSC from UCB was by selection of $CD34^+$ cells using an immunomagnetic cell-sorting system (QuadroMACS, Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Briefly, red blood cell lysis was induced with 2% dextran (w/v) before performing density-gradient centrifugation using ficoll (970 g – 20 min – room temperature (RT)). The mononucleated cell layer was aspirated and washed. Total nucleated cell number was determined in a Malassez haemocytometer. The nucleated cells were resuspended to 10^8 cells in 300 μl phosphate-buffered saline (PBS) (1·5 mM KH_2PO_4, 137 mM NaCl, 2·7 mM KCl and 8 mM Na_2HPO_4 at pH 7·4) supplemented with 2 mM ethylenediaminetetraactic acid (EDTA) and 0·5% bovine serum albumin (BSA) (w/v) before addition of 100 μl anti-CD34 labelled magnetic beads and 100 μl Fc-receptor blocking agent per 10^8 cells. Cells were incubated for 90 min at 4°C during constant agitation before removal of unbound cells by centrifugation (300 g – 10 min – RT) and resuspension in PBS with EDTA and BSA. Bound $CD34^+$ cells were collected and counted in the Malassez haemocytometer. After isolation, the cells were cryopreserved at $0{\cdot}5–1{\cdot}0 \times 10^6$ cells per mL in fetal bovine serum and 10% (v/v) dimethylsulphoxide at –80°C (≥24 h) before transfer to our liquid nitrogen (–196°C) long time storage facility. Three buffy coats prepared from voluntary whole blood donations by the Belgian Red Cross-Flanders Blood Service were pooled prior to isolation of HSC according to the protocol described previously. Whole blood donations contain approximately 14% (v/v) citrate-phosphate-dextrose-acid as anticoagulant. After storage overnight at RT, whole blood donations were centrifuged (4556 g – 13 min – RT). Automated separators were used for separation into red blood cell concentrate, plasma and a buffy coat. Excess buffy coats not used for platelet concentrate preparation were used in this work. The number of magnetic beads used per total number of nucleated cells was lower, and additional washing steps were used compared to isolation from UCB to compensate for the low ratio HSC *versus* total nucleated cells in PB [21].

Cell culture towards megakaryocytes

Cryopreserved HSC were thawed in a 37°C water bath before addition of 10 ml prewarmed HP01 medium (Macopharma, Tourcoing, France) and centrifugation (300 g – 10 min – RT). HSC were resuspended in HP01 supplemented with 10 nM thrombopoietin peptide (TPO, Sigma-Aldrich, Saint Louis, MO), 25 ng/ml stem cell factor (SCF, Miltenyi Biotech) and 2 ng/ml interleukin 3 (IL3, Miltenyi Biotech) and were seeded at 300 000 cells/ml for 6 days of expansion. On day 7 (D7), cells were split at 500 000 cells/ml in HP01 containing 50 nM TPO and 2 ng/ml SCF for differentiation towards MK. All cells were harvested on D12 and subjected to BSA gradient centrifugation for removal of small particles [22].

Definition of platelets and cultured cells

In this manuscript, the term (input) *cells* is used for particles with a diameter ≥ 7 μm counted in a Malassez haemocytometer. Particles ≤ 7 μm are called *platelet-like particles* (PLP). Size of particles was measured using the analytical software tools for microscopy (Zen 2, Blue edition, Carl Zeiss). The same term (PLP) is also used for flow cytometry events appearing inside a polygon gate set as a contour around bPLT as source material [23] (Figs S1a and b). This polygon gate is further referred to as the *morphology gate*. Particles within the morphology gate that stain positive for both surface receptor CD42b and CD41 (see below) were then defined as *platelets* (Fig S1c).

Standard blood bank platelets

Platelets from PC were used as reference material to compare with *in vitro* platelet production and are defined as *standard blood bank platelets*. The PC were prepared according to standard blood banking methods as described previously [24, 25], in this case by pooling of six buffy coats. A final 65% (v/v) of additive solution (SSP+, Macopharma, Tourcoing) was targeted.

Phenotyping by flow cytometry

Differentiation of cultured cells was determined by flow cytometry (Attune, Life Technologies, Carlsbad, CA) on D0, D4, D7, D10, D11 and D12. The haematopoietic precursor lineage was determined with an antibody against CD34 (phycoerythrin (PE)-anti-CD34, BD, Biosciences, Erembodegem, Belgium). Determination of MK differentiation was with fluorescein (FITC)-labelled anti-CD42b (Life Technologies) and PE-Cy7-labelled anti-CD41 (BD Biosciences). Cells and antibodies were incubated for 10 min in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered saline (HBS) (10 mM HEPES, 155 mM NaCl and 1 mM $MgSO_4$, pH 7·4) at RT [26]. Expression of CD42b (GPIbα), CD41 (GPIIb) and CD61 (GPIIIa) on PLP was detected using FITC-labelled anti-CD42b, PE-Cy7-labelled anti-CD41 and allophycocyanin (APC)-labelled anti-CD61 (Life Technologies).The median number of CD42b, CD41 and CD49b (GPIa) receptors per PLP was quantified using the GP screen kit (Biocytex, Marseille, France). Threshold gates were set including 0·5% of 10 000 events incubated with corresponding isotype antibody negative controls.

In vitro platelet production

Microfluidic flow chamber chips were manufactured from polydimethylsiloxane as described before [27]. One chip (length = 17·3 cm) consisted of 16 parallel channels composed of a structured network of pillars. The radius of one pillar is 15 μm, and the distance between neighbouring pillars, from centre to centre, is 85 μm. The pillars are oriented so that the angle between the pillar array and the flow direction of the channel is 10°. Prior to cell perfusion, the chip was coated overnight with 40 μg/ml purified VWF (Wilfactin, LFB Biotechnologies, France). The perfusion experiment was performed according to Blin *et al.* [27]. In brief, the harvested cell suspension on D12 of culture was placed on an orbital shaker and was connected to the perfusion chamber inlet and outlet by tubing. A peristaltic pump (Watson-Marlow, Falmouth, UK) created continuous perfusion of the cell suspension at a wall shear rate of 1800 s^{-1} for two hours during which platelets were formed. Adhering cells were visualized by phase contrast microscopy at 100× magnification (Axio observer A1, Carl Zeiss). After perfusion, the suspension was centrifuged at 110 g for 20 min to remove MK. Next, the supernatant was centrifuged at 1240 **g** for 15 min to pellet the platelets.

Differential interference contrast microscopy

The morphology of PLP was determined by DIC microscopy (Axio observer A1, Carl Zeiss). Cell suspensions were resuspended in Tyrode's washing buffer (135 mM NaCl, 2·7 mM KCl, 12 mM $NaHCO_3$, 430 μM NaH_2PO_4, 1 mM $MgCl_2$, 5 mM HEPES with 0·1% (w/v) D-glucose and 0·35% (w/v) BSA) at pH 7·4 before fixation with 2% (w/v) paraformaldehyde. Fixed cells were centrifuged at 2000 **g** for 20 min onto glass coverslips, coated with 0·1% (w/v) poly-L-lysine (Sigma Aldrich, Saint Louis). These coverslips were then mounted on glass carriers. A 100× oil immersion objective (numerical aperture = 1·4) was used to a final magnification of 1000×. To control for potential side-effects downstream of platelet production caused by centrifugations, bPLT were subjected to the same centrifugation protocol prior to preparation for DIC imaging.

Cytoskeleton staining

Actin was visualized on PLP from UCB and on bPLT by Alexa Fluor (AF) 405 labelled-phalloidin (Invitrogen, Carlsbad, CA). Coverslips were prepared as described under differential interference contrast microscopy. Cells were permeabilized with 0·5% (v/v) triton X-100 (Sigma Aldrich, Saint Louis) followed by staining with 165 μM AF405~phalloidin and mounting onto glass carriers.

Statistical analysis

Results are reported as mean with standard deviation (SD). Comparison between stem cell sources was by two-

tailed *t*-test (with Welch's correction for unequal variances), by one-way ANOVA for comparison of mean number of receptors per PLP (Tukey's correction for multiple comparison) or with two-way ANOVA (Sidak's correction for multiple comparison) for comparison of both HSC sources at different time points of culture. Correlation was by Spearman's algorithm. Statistical analyses was performed with Prism version 7.04 (GraphPad Software Inc., La Jolla, CA).

Results

Haematopoietic stem cell isolation, proliferation and differentiation towards megakaryocytes

Table 1 shows that the mean number of nucleated cells in PB was at least 4-fold higher in comparison to UCB. However, the mean absolute number of cells isolated per PB is $3{\cdot}93 \times 10^5$ compared to $16{\cdot}97 \times 10^5$ per UCB. The percentage of isolated cells per total nucleated cells for UCB and PB source samples is presented in Fig. 1a. A significantly higher ($P < 0{\cdot}0001$) yield was found for UCB ($1{\cdot}33 \pm 0{\cdot}74\%$) compared to PB ($0{\cdot}06 \pm 0{\cdot}02\%$). Proliferation in static culture was significantly faster for UCB-derived HSC compared to PB ($P < 0{\cdot}0001$ on D7, Fig. 1b). As expected, proliferation decreased when the TPO, SCF and IL3 concentration were changed on D7 (Fig. 1b). Consequently, CD34 expression decreased (Fig. 1c) while expression of CD42b/CD41 markers increased (Fig. 1d). The fraction of CD42b/CD41 double positive events was significantly lower in cultures from PB than in UCB on D10, 11 & 12 (Fig. 1d) with a difference of $44 \pm 9\%$ vs $76 \pm 11\%$ CD42b/CD41 positive events on D12 for PB and UCB, respectively ($P < 0{\cdot}0001$).

Production of platelet-like particles

On D12 of culture, cells were perfused for two hours resulting in cell anchorage, elongation and release of PLP, starting from both UCB and PB (Fig. 2a and b). The PLP yield defined as the number of PLP over the number of input cells was significantly higher for PB ($7{\cdot}4 \pm 4{\cdot}4$) than for UCB ($4{\cdot}2 \pm 2{\cdot}2$) ($P = 0{\cdot}02$) (Fig. 2c). The PLP yield was independent of the proportion of mature MK when defined as events positive for CD41/CD42b present in the input sample. This held for cells isolated from UCB ($r = -0{\cdot}13$, $P = 0{\cdot}62$) (Fig. 2d) and from PB ($r = 0{\cdot}03$, $P = 0{\cdot}85$) (Fig. 2e) at D12.

Platelet receptor expression

The fraction of events in the morphology gate (Fig. S1) staining positive for either CD41, CD42b or both was comparable between PLP from UCB or PB sources (Fig. 3a and b). Consequently, based on our definition (Fig. S1) approximately 60% of PLP could be defined as platelets (Fig. 3c). Expression of CD61 was also comparable between both HSC sources (Fig. 3d). The mean number of CD42b, CD61 and CD49b receptors per PLP did not significantly differ between both stem cell sources and is comparable to the number of receptors on bPLT (Fig. 4). The number of CD42b receptors per PLP is on average 28 000 per PLP (Fig. 4a) while this is approximately 57 000 and 5500 per PLP for CD61 and CD49b, respectively (Fig. 4b and c).

Morphology and cytoskeleton stain

Cell suspensions after perfusion were analysed by DIC microscopy at 1000× magnification. All suspensions contained a heterogeneous cell population by size. Particles structurally comparable to platelets were found both in UCB and PB derived samples (Fig. 5a and b). These contained what appeared to be granules as intense dark zones inside and near the cell's periphery. Alongside these, the suspension also contained large cells (*) characteristic of remainder MK. The ratio of remaining MK compared to PLP was approximately 7:3 and was indistinguishable between UCB or PB. Particles smaller than typical platelets could either be extracellular vesicles (also called microparticles) or cell debris. Reference bPLT were prepared as a control sample (Fig. 5c). Additionally, the cytoskeleton protein actin was visualized on the DIC images (Fig. S2). All cells from the bPLT suspension stained positive for actin while in the cell suspension from UCB only particles with comparable structure to platelets were found positive for actin.

Table 1 Absolute number of total nucleated cells before isolation and of $CD34^+$ cells after isolation from PB or UCB

	UCB	PB
Absolute number of total nucleated cells per sample before isolation ($\times 10^8$)	$1{\cdot}49 \pm 0{\cdot}65$	$6{\cdot}93 \pm 1{\cdot}73$
Absolute number of $CD34^+$ cells per sample after isolation ($\times 10^5$)	$16{\cdot}97 \pm 6{\cdot}49$	$3{\cdot}93 \pm 1{\cdot}35$

Data are shown as mean ± SD ($n \geq 14$).

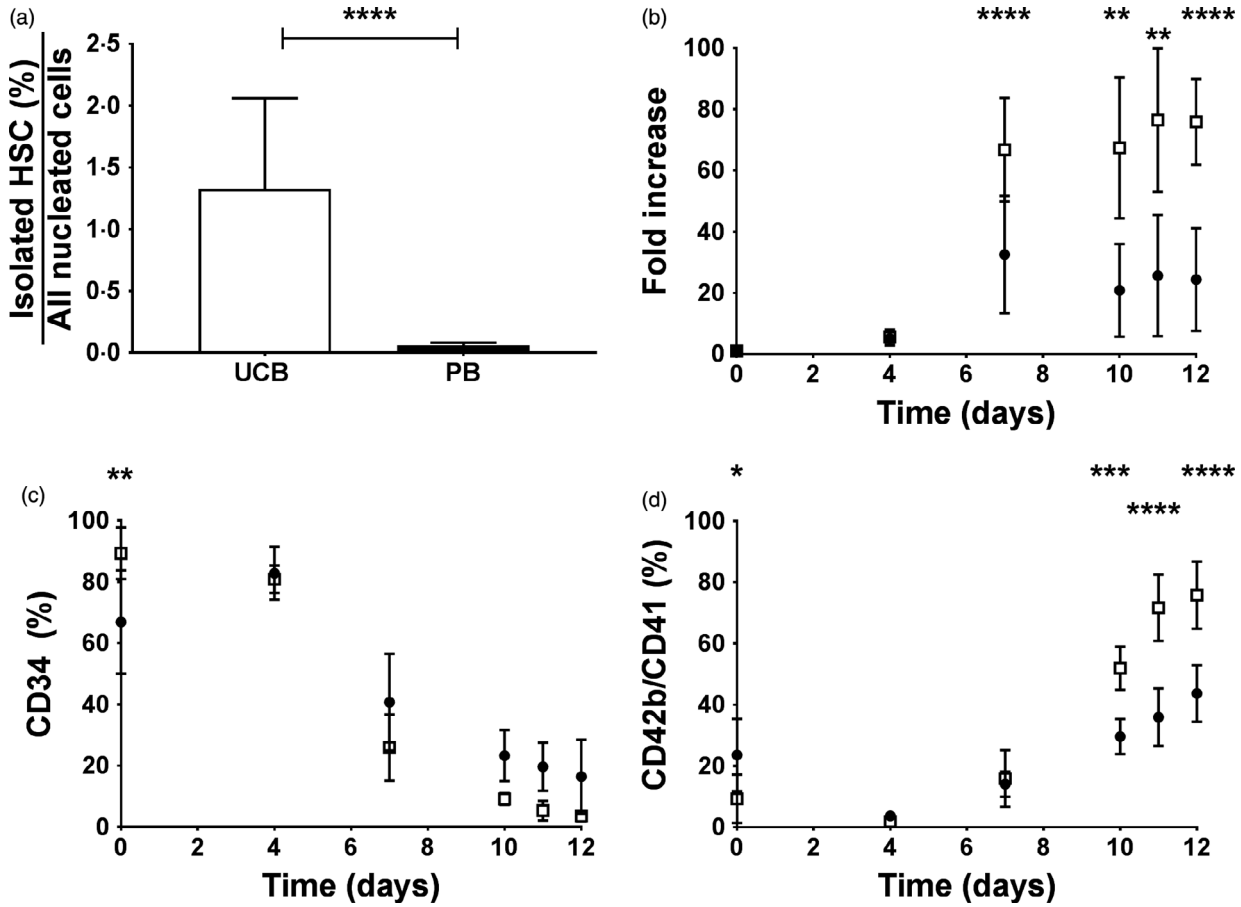

Fig. 1 Static cell culture of HSC derived from PB or UCB. (a) The percentage HSC obtained by magnetic isolation per total nucleated cells in UCB (□) or PB (●) samples. (b) The proliferation (all cells), in function of time, expressed as fold increase relative to the number of cells seeded on D0. (c) Expression of CD34 and (d) CD42b/CD41 markers on cultured cells as measured by flow cytometry as a function of time. Data are shown as mean with SD ($n \geq 5$). Statistical analysis results are shown as $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$ and $^{****}P < 0{\cdot}0001$.

Discussion

To manufacture platelets *in vitro*, three major steps are needed [28]: first, appropriate stem cells are expanded in tissue culture. In a second step, these are differentiated to generate haematopoietic and MK precursor cells. As an alternative, immortalized MK can be used as demonstrated by Nakamura *et al.* [11]. In any case, the final step is to produce platelets from these MK in an efficient and safe manner.

Ultimately, the technology will use self-renewable stem cell sources like hESC or iPSC although some advocate the use of UCB and PB as well [29]. Ethical concerns limit the use of hESC [30] and current platelet yields obtained from iPSC are not (yet) reaching platelet yields obtained *in vivo* [5, 10, 11, 31]. Therefore, to enable research and development to the final step in the process (platelet production), primary HSC can still be used and offer an inexpensive and accessible source. Furthermore, for rare phenotypes, PB may still be needed as a source of autologous stem cells. Bearing this in mind, this study's objective was to compare the yield and quality of platelets produced in hydrodynamic flow conditions using precursor cells from either UCB or PB and to compare these to current standard of care bPLT.

As expected from research by van den Oudenrijn *et al.* [13], *in vitro* expansion of stem cells in static conditions was more efficient from UCB than from PB. Relative to D0, a 68-fold and 32-fold expansion was found on D7 for UCB and PB, respectively. From D7 on, the TPO concentration was raised to promote differentiation to MK. In those conditions, platelet production happens spontaneously, albeit slowly and asynchronously [16, 32, 33]. In our flow chambers, the combination of shear and immobilized VWF results in synchronized and efficient platelet production [14, 27]. BSA gradient centrifugation was performed on culture cell prior to perfusion in the flow chamber to remove PLP produced during static culture

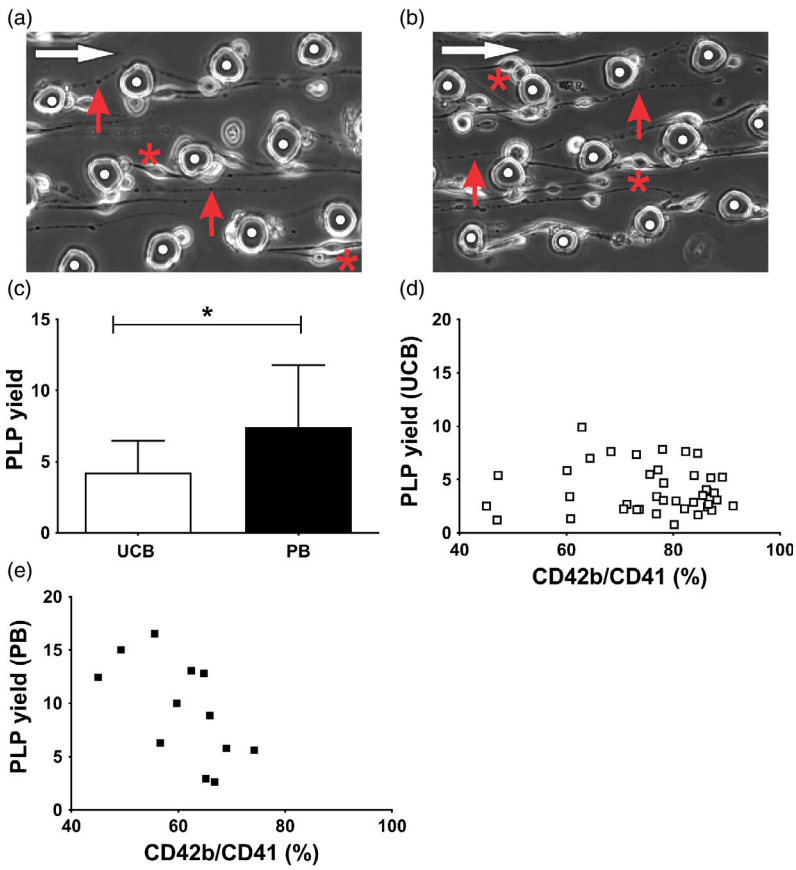

Fig. 2 Platelet production in hydrodynamic flow. (a) Representative image of cell anchorage (*) to VWF-coated pillars (white dots) of a cultured cell (D12) started from UCB and (b) PB. Cell elongation and (pro)platelet formation is indicated by red arrows. The flow direction is shown by a white arrow. (c) The number of PLP produced per input cell during the perfusion experiment. Data are given as mean with SD ($n \geq 12$). Statistical analysis results are shown as $^*P < 0{\cdot}05$. (d and e) The PLP yield was plotted as a function of the number of CD42b/CD41 double positive input cells for (d) UCB ($n = 36$) and (e) PB ($n = 13$).

conditions and focus investigation on PLP produced in the flow chamber. When comparing precursor cells sourced from UCB and PB, a higher platelet yield for PB was found. These data are comparable to findings in static conditions [19]. Hence, phenotypic differences between source cells are translated in the same way in static as in dynamic conditions. MK ploidy has been suggested to correlate with platelet production [19], albeit not in all conditions [34, 35]. We hypothesized that the fraction of CD42b/CD41 positive MK in the input suspension could predict PLP yield but the data show this was not the case. Consequently, although these markers are specific for platelets, these do not predict platelet production efficiency in our system. But expression of CD42b/CD41 as a predictor for PLP production cannot be ruled out completely because the exact expression levels of CD42b/CD41 on the MK may still be correlating. Despite differences in HSC expansion and platelet production efficiency, platelet phenotypic characteristics are quite similar between UCB and PB. Foremost, these are also comparable to standard bPLT, although a trend was found for lower GPIbα (CD42b) and higher integrin β_3 (CD61 or GPIIIa) expression. Decreased GPIbα expression levels have also been reported in murine platelets produced *in vitro* in static conditions from mouse ESC [36]. This was caused by ectodomain shedding and could be prevented by co-incubation with a metalloproteinase inhibitor. Importantly, GPIbα levels were not decreased to levels relevant to impede haemostasis [37]. Contrary to GPIbα, an increase in integrin β_3 expression was found. The β_3 integrin dynamically forms heterodimers with α_{IIb} and α_V, the former being utmost important for platelet aggregation as the fibrinogen receptor. Platelet aggregation studies are required next to demonstrate whether this moderate increase translates into altered function compared with bPLT.

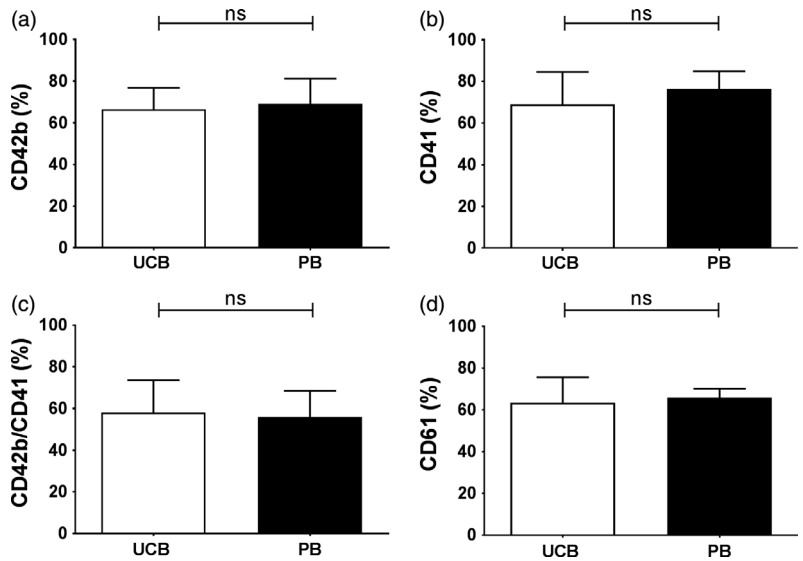

Fig. 3 Expression of platelet markers. The fraction of PLP that was staining positive for (a) CD42b, (b) CD41, (c) CD42b/CD41 and (d) CD61 produced from UCB (open bars) and PB (closed bars). Data are shown as mean with SD ($n \geq 5$).

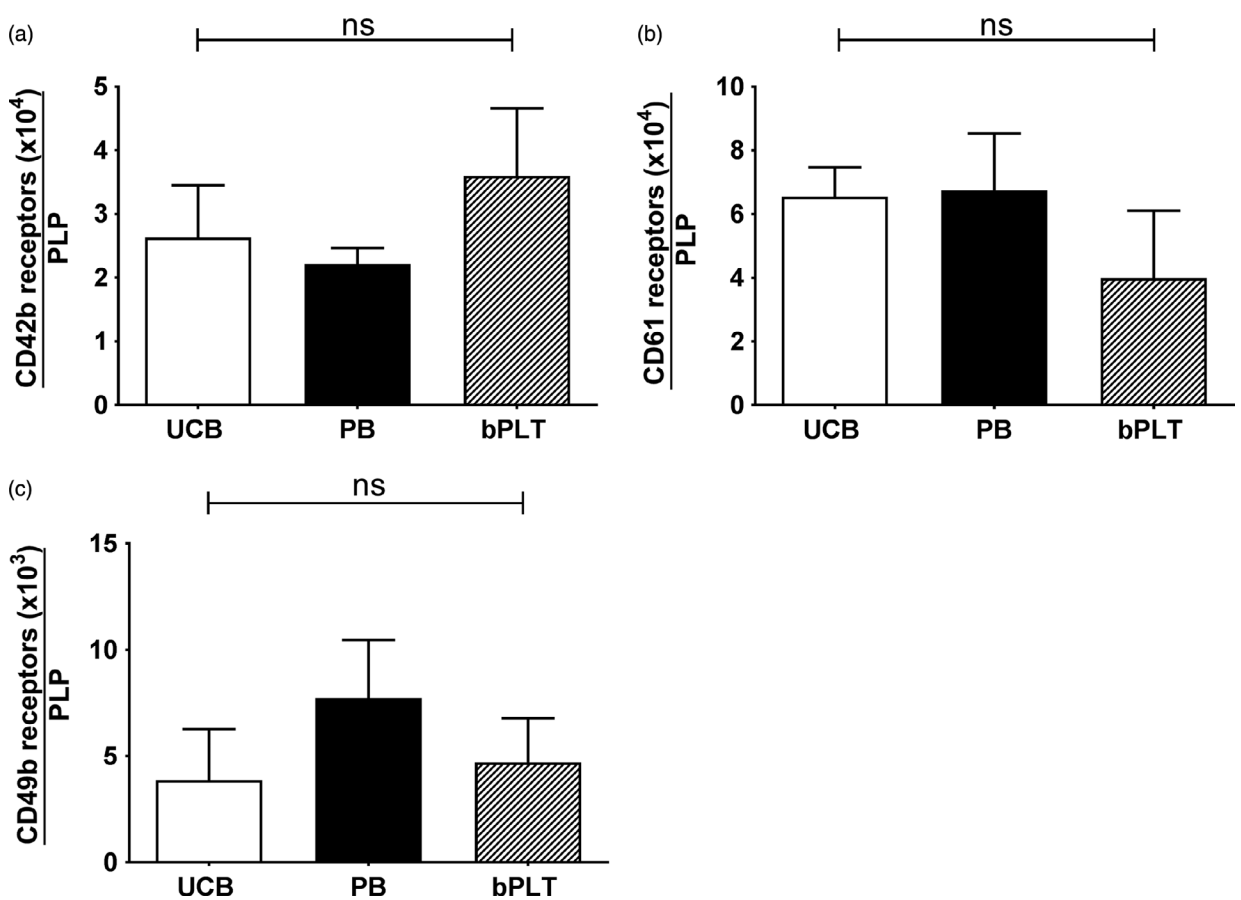

Fig. 4 Number of platelet receptors. The number of (a) CD42b, (b) CD61 and (c) CD49b receptors was determined using GP screen kit on PLP produced from UCB (open bars) and PB (closed bars). Data were compared to the levels in bPLT (hatched bars). Data are shown as mean with SD ($n = 4$).

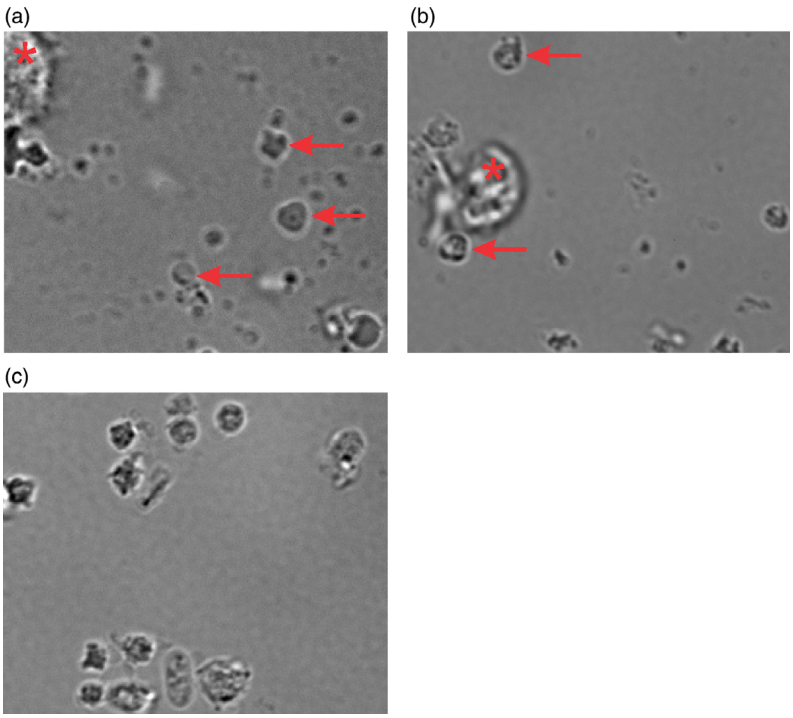

Fig. 5 Differential interference microscopy. Representative images of the cell suspensions from (a) UCB or (b) PB after perfusion and double centrifugation. PLP are indicated by arrows. The larger cells were most likely MK (*). (c) Representative image of bPLT prepared in the same way as the PLP. Pictures were taken from DIC microscopy studies 1000× magnification.

The input cell suspension contains MK in several stages of maturation [33, 38, 39] and senescence. After the 2 h of perfusion to produce platelets, a double centrifugation is performed to remove remainder MK and concentrate PLP. Our microscopic images indicate that large cells as well as small particles are nonetheless still found. Using flow cytometry, a fraction of 70% was consequently defined as platelets while the other 30% are remaining MK (with exclusion cellular debris and microparticles). The same ratio of PLP and MK was found in the microscopic images for both UCB and PB. Therefore, future clinical applications will require optimized filtration and concentration steps of the outflow [40]. Such alternatives are spinning-membrane filtration devices [41] or microfluidic-based separations whether or not in combination with acoustopheresis [42].

However, the microscopic images indicate mainly platelet-like cells. These are similar in size and aspect to bPLT with this imaging technique. These platelet-like particles were present in both suspensions from UCB and PB. Furthermore, actin staining showed that these platelet-like cells are positively stained while no actin was detected in smaller particles. Ultrastructural analyses using electron microscopy must be used to further identify the subcellular organelles as alpha and/or dense granules characteristic of healthy platelets.

We conclude that platelet production efficiency by perfusion of cultured cells in VWF-coated microfluidic flow chambers is higher starting from PB compared to UCB, like in static conditions. Despite this, the absolute overall PLP number is higher starting from UCB because of higher number of isolated cells and higher proliferation rates in the preceding static culture. Preliminary platelet characterization indicates surface receptor expression and morphology are comparable to reference bPLT. Future experiments should focus on platelet ultrastructure and platelet function. In the long run, increasing the platelet yield and lowering production costs are essential prior to proceeding to clinical trials comparing these *in vitro* produced platelets to standard PC.

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Author contributions

KRS & RD performed the experiments; KRS, GS, HBF & DB interpreted results; HBF, VC & DB designed the research; VC & DB provided essential materials and supported the study; KRS & HBF wrote the manuscript. All authors critically reviewed the manuscript and agreed upon submission.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:
Fig. S1 Representative scatter plots to define PLP and platelets.
Fig. S2 Cytoskeleton staining.