Two-Dimensional Polymer-Based Cultures Expand Cord Blood-Derived Hematopoietic Stem Cells and Support Engraftment of NSG Mice

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Currently, *ex vivo* expansion of hematopoietic stem cells (HSC) is still insufficient. Traditional approaches for HSC expansion include the use of stromal cultures, growth factors, and/or bioreactors. Biomaterial-based strategies provide new perspectives. We focus on identifying promising two-dimensional (2D) polymer candidates for HSC expansion. After a 7-day culture period with cytokine supplementation, 2D fibrin, poly(D,L-lactico-glycolic acid; Resomer[®] RG503), and Poly(ϵ -caprolactone; PCL) substrates supported expansion of cord blood (CB)-derived CD34⁺ cells *ex vivo*. Fibrin cultures achieved the highest proliferation rates (>8700-fold increase of total nuclear cells, $p < 0.001$), high total colony-forming units (3.6-fold increase, $p < 0.001$), and highest engraftment in NSG mice (7.69-fold more donor cells compared with tissue culture polystyrene, $p < 0.001$). In addition, the presence of multiple human hematopoietic lineages such as myeloid (CD13⁺), erythroid (GypC⁺), and lymphoid (CD20⁺/CD56⁺) in murine transplant recipients confirmed the multilineage engraftment potential of fibrin-based cultures. Filopodia development in fibrin-expanded cells was a further indicator for superior cell adhesion capacities. We propose application of fibrin, Resomer[®] RG503, and PCL for future strategies of CB-CD34⁺ cell expansion. Suitable polymers for HSC expansion might also be appropriate for future drug discovery applications or for studies aimed to develop hematological therapies.

Introduction

HEMATOPOIETIC STEM CELLS (HSC) represent the top of the hematopoietic hierarchy. They are characterized by their ability for self-renewal and clonal differentiation into all lymphoid and myeloid lineages.^{1,2} Adult HSC reside in the bone marrow (BM) niche, a highly specialized microenvironment consisting of three main compartments (1) localized stroma, (2) extracellular matrix (ECM), and (3) soluble factors.³ *In vivo*, HSC interactions with their surroundings are crucial for maintenance and regulation of the unique HSC properties, though to mimic *ex vivo* the complex niche microenvironment multiple aspects have to be considered.⁴ Singbrant *et al.* reviewed some of the recent advances that indicate osteoblasts, adipocytes, vasculature, nerves, and a variety of hematopoietic progenies to be active in the BM

niche.⁵ As most of the abnormalities in patients with hematopoietic disorders reside in their BM, the present treatment for a series of hematopathologies includes BM transplantation. However, appropriate BM donors are often not available for elderly high-risk patients. More than twenty years ago, cord blood (CB) was recognized as a reliable source of HSC and hematopoietic progenitor cells. The first successful CB transplant was performed in 1988 by Gluckman *et al.* in a pediatric patient suffering from Fanconi anemia.⁶ Currently, the use of CB for HSC transplantation (HSCT) is an established and safe procedure and CB banking spreads worldwide.^{7,8} The use of CB for HSCT benefits from (1) easy collection; (2) less stringent donor-recipient HLA matching; (3) weaker GVHD response; and (4) higher percentage of immature and mature HSC per graft, compared with BM and mobilized peripheral blood (PB).⁹ However, the number

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of CB-HSC per donor-graft is limited and often insufficient for transplantation in adults. The use of two CB grafts per recipient is feasible,¹⁰ but a robust *ex vivo* expansion strategy for HSC remains a long sought-after goal. Among the most successful trials using expanded CB cells De Lima *et al.*, reported a tetraethylenepentamine-based expansion strategy for CB-CD133⁺ cells that resulted in 90% engraftment in patients with advanced hematological malignancies during a Phase I/II trial,¹¹ while Delaney *et al.* reported a Notch-mediated system for CB-CD34⁺ progenitor expansion that substantially shortened neutrophil recovery during a Phase I trial.¹²

Applying biomaterials in tissue engineering is a common practice nowadays.^{13,14} Biomaterial-based strategies for enhancement of HSC proliferation are, however, a poorly described topic with a restricted number of studies addressing the question so far.^{15–20} Identification of suitable biomaterials for HSC expansion has been an iterative process, that means polyethylene terephthalate (PET) structures, for instance, are used directly for study, at a time, then upgraded for the next study.^{15,18} While the rationale for choosing some biomaterials rather than others for HSC expansion is not clear, we propose biomaterial identification to start with the assessment of a series of basic compatibility parameters. Previous work from our group included a systematic evaluation of HSC viability, cytotoxicity, and apoptosis after exposure to different biomaterials. Sixteen two-dimensional (2D) bio- and synthetic polymers from our biomaterial bank were simultaneously tested using standard cytotoxicity protocols and only six polymers were found to be suitable for CB-HSC proliferation.²¹ Basic compatibility tests showed that poly(vinylidene fluoride; PVDF), texin[®] 950, poly(L-lactic-co-D,L-lactic; PLLA-co-PDLLA; Resomer[®] LR704), poly(D,L-lactic-co-glycolic acid; Resomer[®] RG503), Poly(ϵ -caprolactone; PCL), and fibrin supported HSC cultures in being not cytotoxic or apoptotic after long-term exposure *ex vivo*. In the present study, we further analyzed the efficiency of the six previously investigated 2D polymers for HSC expansion in comparison to tissue culture polystyrene (TCPS). CB-derived CD34⁺ cells were freshly isolated from human CB samples and expanded for a 7-day culture period in the presence of specific growth factors. A systematic morphological and functional analysis of the expanded cells was done using *ex vivo* and *in vivo* assays. Our study identified fibrin as the most efficient polymer for CB-HSC expansion, followed by Resomer[®] RG503 and PCL. All three polymers are promising candidates for future strategies aiming at the *ex vivo* modeling of the BM niche.

Materials and Methods

Biomaterials preparation

One biopolymer (fibrin) and five synthetic polymers (degradable ones: Resomer[®] LR704, Resomer[®] RG503, and PCL; nondegradable ones: PVDF and texin[®] 950) were used. First, 2D biomaterial foils were prepared and then small samples ($\varnothing$ 15 mm) were cut out and cleaned. Lastly, the samples were placed in 24-well plates. Procedures for biomaterial preparation and cleaning were conducted as previously described.²²

Fibrin. Fibrin was prepared under sterile conditions by mixing 5 μ L Thrombin (20 U/mL; Sigma-Aldrich) with 90 μ L

of a human fibrinogen suspension consisting of 830 μ L fibrinogen (20 mg/mL; Sigma-Aldrich), 50 μ L CaCl₂ (50 mM; Roche), and 20 μ L GBSH₅ buffer (without glucose and Ca²⁺). Fibrin had to polymerize for 20 min at 37°C (20% O₂, 5% CO₂) before cell seeding.

PLLA-co-PDLLA, Resomer[®] LR704. Granules of PLLA-co-PDLLA were purchased from Boehringer Ingelheim Pharma GmbH & Co. KG and consisted of an L-lactic-D,L-lactic acid ratio of 70:30 (Resomer[®] LR704, 2.0–2.8 dL/g). Foils were prepared by melting-press technique (L.O.T.-Oriol GmbH & Co. KG) using 1.2 g of polymer per foil. Granules were enclosed by Teflon cover foils, then melting temperature was set to 200°C for 5 min and a load of 1 ton was finally applied for 3 min. The foils were cleaned by slightly rinsing in isopropanol, then drying with nitrogen stream followed by vacuum drying for at least 2 h.

Poly(D,L-lactic-co-glycolic acid), Resomer[®] RG503. Poly(D,L-lactic-co-glycolic acid) powder was purchased from Boehringer Ingelheim and consisted of a lactic-glycolic acid ratio of 50:50 (Resomer[®] RG503, 0.32–0.44 dL/g). Foils were prepared and cleaned as done for Resomer[®] LR704, except for the following parameters: melting temperature was 150°C, melting period was 2 min, and loading period was only 1 min.

Poly(ϵ -caprolactone). PCL granules (Mw: 80,000 g/mol) were purchased from Sigma-Aldrich. Foils of 1% PCL were prepared by melting-press technique. Three g of PCL per foil were enclosed on Teflon cover foils and melted at 85°C for 5 min. Afterward, a load of 1 ton was applied for 1 min. PCL samples were cleaned as described for Resomer[®] LR704 and RG503.

Poly(vinylidene fluoride). Unsterile 0.05 mm thick foils of PVDF were acquired from Goodfellow GmbH. PVDF samples were sterilized by extraction in a Soxhlet apparatus using EtOH/Hexane (21:79 v/v) for 2 h. Samples were then dried at 60°C–80°C for 1 h followed by drying in an evaporator for 24 h.

Polyurethane, texin[®] 950. Aromatic Polyether-based thermoplastic polyurethane (PU), texin[®] 950 granules were bought from Bayer MaterialScience AG. Foils with 0.23 mm thickness were prepared by melting-press technique. About 3 g of granules per foil were melted at 200°C for 5 min and a load of 5 tons was then applied for 10 s. Samples were cleaned according to the procedure described for PVDF.

Ex vivo expansion of CB-CD34⁺ cells

CD34⁺ cells were obtained from fresh human CB samples collected after informed consent according to the guidelines approved by the local Ethical Committee of the University Clinics RWTH Aachen (permit number EK187/08). CB samples were collected in sterile collection bags containing approx. 25% citrate phosphate dextrose anticoagulant solution and processed within 3 h after collection. Mononuclear cells (MNC) were first separated by Ficoll-Hypaque gradient centrifugation (density 1.077 g/mL), followed by magnetic separation of the CD34⁺ fraction using the MACS CD34

progenitor cell isolation kit (Miltenyi Biotec), according to the manufacturer's instructions. A pure population ranging from 90%–95% was detected by flow cytometry, as described before.²³

CD34⁺ cells were seeded on top of the 2D polymers in 24-well plates (Nunc) at an approx. density of 0.1×10^5 cells/well. StemSpan serum-free medium (StemCell Technologies, Inc.) supplemented with $1 \times$ penicillin and streptomycin (Gibco Invitrogen Corporation), 10 ng/mL stem cell factor (SCF), 20 ng/mL thrombopoietin (TPO), 10 ng/mL fibroblast growth factor 1 (FGF-1), 10 μ L/mL heparin, 100 ng/mL insulin-like growth factor-binding protein-2 (IGFBP₂), and 500 ng/mL angiopoietin-like 5 (Angptl-5) was used for culture.²⁴ IGFBP₂ was purchased from R&D Systems, Angptl-5 from Tebu-Bio, heparin was from Ratiopharm, and recombinant human growth factors SCF, TPO, and FGF-1 were all from Peprotech.

Cells were cultured for 7 days in a humidified atmosphere at 37°C with 20% O₂ and 5% CO₂. Fresh medium was added every 4 days and cell density was maintained at 50,000–100,000 cells/well. Cells were seeded on TCPS as controls. Regularly, cell morphology was investigated by inverted light microscopy (Leica) and cell growth determined with a CASY1 electronic cell counter (Schaefer Systems). Cumulative growth corresponds to total nucleated cell numbers (electronically counted) normalized in relation to the number of starting cells—which was set to 1×10^6 cells—for every independent experiment.

Cell imaging and live/dead stainings

Expanded cells were observed with an inverted light microscope (Leica) and photographed with a Cool Snap™ HQ2 digital camera (Photometrics).

For assessment of cytotoxicity, live/dead stainings according to the 10993–5 protocols of the International Standardization Organization (ISO) were used as described before.²² Twenty four hours after cell seeding, plates were centrifuged and 1.28 mL Ringer solution (Delta-Pharma) consisting of 20 μ L of fluoresceindiacetate (FDA; 0.1 mg in 20 mL acetone) and 20 μ L propidium iodide (PI; 0.01 mg in 20 mL phosphate buffered saline [PBS]; PI and PBS both Sigma-Aldrich) was added to the cell/biomaterial 24 well-formatted constructs. FDA/PI solution was incubated for 20 s, after which viable cells appeared green fluorescent and dead cells appeared red fluorescent. Photographs were taken with a DM IRB microscope (Leica) and a KY-F75U digital camera (JVC).

Carboxyfluorescein diacetate N-succinimidyl ester cell proliferation assay

Cell proliferation was monitored using carboxyfluorescein diacetate N-succinimidyl ester (CFSE; Sigma-Aldrich), as described before.²⁵ Labeling was done directly after CD34⁺ cell isolation and the procedure consisted briefly of resuspending the cells in 2.5 μ M of CFSE diluted in PBS (Gibco Invitrogen Corporation) with 0.1% fetal calf serum (FCS; PAN Biotech) followed by an incubation period of 10 min at 37°C. Next, at least 5 volumes of ice-cold StemSpan supplemented with 10% FCS were added to the cells which incubated 5 min on ice to stop the staining reaction. Before seeding cells were washed once in PBS. After 7 days of cul-

ture CFSE intensity was measured by flow cytometry using FACSCanto™ II flow cytometer (Becton Dickinson). For control, 24 h after labeling cells with CFSE an aliquot of cells was used to evaluate initial CFSE intensity.

Immunophenotype of expanded cells

The surface phenotype of the expanded cell population was analyzed by flow cytometry. Cells were harvested from culture and washed once in PBS then stained with appropriate monoclonal antibodies against human epitopes on PBS with 1% FCS for 30 min at 4°C (1:100 antibody dilution). Cells were analyzed by five-color flow cytometry using FACSCanto™ II flow cytometer (Becton Dickinson) running on FACSDiva software (Becton Dickinson). At least 20,000 events were acquired. Antibodies used were CD34-FITC (Miltenyi Biotec), CD38-PE, CD13-APC, CD45-PerCpCy5.5, and CD56-PeCy7 (all Becton Dickinson). Isotype controls were included to confirm specificity and for compensation settings. Subsequent data evaluation was done using FlowJo software (Tree Star, Inc.).

Colony-forming unit assays

Aliquots of 10,000 cells expanded for 7 days in different biomaterials were seeded per 35-mm Petri dish containing serum-free MethoCult SF H4236 (Stem Cell Technologies). SCF, TPO, FGF-1, IGFBP₂, and Agptl-5 were then manually added to the methylcellulose medium, as described before.²³ Cells in methylcellulose (two duplicates per condition) were incubated for 14 days in a humidified atmosphere at 37°C and 5% CO₂. Afterward the number of granulopoietic colonies (colony-forming unit granulocyte-macrophages [CFU-GM]) and multilineage colonies (colony-forming unit granulocyte-erythrocyte-macrophage-megakaryocyte [CFU-GEMM]) were scored using an inverted light microscope (Leica). CFU numbers were calculated as follows: (number of scored colonies per dish/number of cells plated per dish) $\times$ total number of cells in culture. Freshly isolated CD34⁺ cells (1000 cells per methylcellulose dish) were evaluated for control.

NSG mice engraftment assay

NOD-scid IL2Rg^{null} mice (NSG) were bred, housed, and handled at the Animal Facility of University Clinics RWTH Aachen, according to institutional regulations. Mice at 6–8 weeks of age were irradiated with 2.5 Gy of whole-body irradiation. Within 24 h mice were injected via tail vein with approx. 50,000 freshly isolated CD34⁺ cells or with their expanded progeny (electronic cell counting from different conditions before injection indicated 30,000–130,000 cells/per mouse). Three mice per group were used in each experiment. Groups of three nonmanipulated, three irradiated noninjected, and three irradiated PBS-injected mice were used as negative controls. Six to eight weeks post-transplantation mice were sacrificed and BM, PB, and spleen (SP) were harvested for further analysis. PB, SP, and BM cells were prepared according to protocols previously described,²³ and analyzed by flow cytometry. In brief, cells were recovered from the respective organs and stained for 30 min at 4°C with antibodies specific for human CD45-APC, CD34-APC, CD13-PE, CD19-FITC, CD3-PerCP-Cy5.5, and

mouse CD45–APC–Cy7 (all Becton Dickinson). At least 500,000 events were acquired per probe and successful engraftment was defined by the presence of at least 0.1% human CD45⁺ cells in mouse BM.

Immunohistochemistry

Mouse BM samples were decalcified, dehydrated, embedded, and sliced, as described before.²³ Immunohistochemical analysis was performed using primary antibodies specific for human CD13, CD20, CD34, CD45, Glycophorin C (all Dako), and CD56 (Novocastra). Epitope retrieval treatment was performed by using Tris/EDTA buffer, pH 9 (Dako) for 30 min in a 95°C–99°C preheated water bath. Stainings were carried out on Dako Autostainer Plus (Dakocytomation) by using the Dako REAL™ Detection System Peroxidase/DAB+ reagents kit. Slide preparations were counterstained with hematoxylin, dehydrated, and finally mounted in Vitro-Clud (Langenbrinck). Stained specimens were observed by microscopy using an Axiophot 2 microscope (Carl Zeiss), photographed with a Cool Snap™ HQ2 digital camera (Photometrics), and photos analyzed using VisiVIEW® Imaging software (Visitron Systems).

Scanning electron microscopy

Expanded cells were washed in PBS and fixed in 3% glutaraldehyde (Sigma-Aldrich) diluted in 0.1 M Soerensen's phosphate buffer for at least 24 h. Samples were postfixed with 1% osmium tetroxide (Sigma-Aldrich) followed by dehydration in a graded ethanol series (30%, 50%, 70%, 90%, and 100%). Ethanol dehydration excluded PCL samples. Hexamethyldisilazane (Sigma-Aldrich) was further used for sample drying. Lastly, samples were fixed on scanning electron microscopy (SEM) stubs, sputter coated with gold, and analyzed using a field emission SEM microscope (ESEM XL 30 FEG, FEI, PHILIPS) in a high vacuum environment.

Statistical analysis

Data presented correspond to mean ± SD. A minimum of three independent donors were used per experiment. In addition three biomaterial repeats per condition were done. Statistical significance of data results from one-way analysis of variance was used followed by Tukey's *post hoc* test (analysis of three or more groups). Significant differences were considered when $p < 0.05$.

Results

Cell growth and viability

To evaluate HSC toxicity after short-term contact to polymers, we performed live-dead stainings. Freshly isolated CB-derived CD34⁺ cells were directly seeded on top of PVDF, texin® 950, Resomer® LR704, Resomer® RG503, PCL, and fibrin. A general scheme of the culture systems tested is shown in Fig. 1. Twenty four hours after cell seeding, FDA/PI staining was used to microscopically distinguish viable from dead cells. Fluorescence images revealed high viability in fibrin cultures ($\approx 90\%$ more living cells than TCPS control; Fig. 2S, T). Comparable cell viability was observed on Resomer® RG503, Resomer® LR704, PVDF, and TCPS control cultures (Fig. 2A and B, G and H, J and K, M and N).

FIG. 1. Schematic illustration of two different strategies for hematopoietic stem cell (HSC) expansion. Instead of traditional 2D tissue culture polystyrene (TCPS) (i), a two-dimensional (2D)/three-dimensional (3D) biomaterial-based strategy (ii) is our suggested alternative for HSC *ex vivo* expansion.

Cells in contact with texin® 950 (Fig. 2P, Q) showed reduced cell viability ($\approx 6\%$ less viable cells than control). PCL autofluorescence did not allow for quantification of live/dead stainings. Presence of cells at the well border (Fig. 2D, E, insert) confirmed highly substrate-derived fluorescence. To overcome method-related limitations, viable proliferating cells on PCL were investigated in parallel by CFSE labeling (section 'cell proliferation and morphology').

After 7 days of polymer-based expansion it became apparent that all substrates (1) support cell growth (Fig. 2C, F, I, L, O, R, U) but, (2) expansion efficiency differed polymer-dependent with fibrin and PCL showing the highest growth rate (Fig. 3A). In fact, fold expansion of total cells after 14 days of culture was superior on fibrin and PCL (expansion: >950000 -fold and >100000 -fold, respectively) than on TCPS (approx. 940-fold). Resomer® RG503 resulted in cell expansion rates similar to TCPS (approx. 950-fold increase), while PVDF, texin® 950, and Resomer® LR704 did not improve total cell expansion.

Similarly, maintenance of the primitive phenotypes CD34⁺ and CD34⁺CD38[−] was significantly higher on fibrin-, PCL-, and Resomer® RG503-expanded cells compared with TCPS-expanded cells (Fig. 3B).

Cell proliferation and morphology

Next, the cell division history of cells cultured under different 2D conditions was monitored by CFSE analysis. CFSE is a cell-tracking reagent, which passively diffuses into cells. Upon cell division it is equally divided into the daughter cells.²⁶ CB-derived CD34⁺ cells were freshly isolated, CFSE-stained, and expanded for 7 days using different polymers. Analysis of CFSE intensities—which are inversely proportional to the number of cell divisions—showed that Resomer® LR704, PVDF, and texin® 950 did not support cell proliferation (Fig. 4A). On the other hand, Resomer® RG503 slightly increased proliferation in relation to TCPS (nine cell divisions vs. seven), while PCL and fibrin greatly stimulated cell proliferation—reaching at least ten cell divisions (Fig. 4B). Maintenance of a more primitive phenotype is directly related to cell proliferation potential.² Next, CD34, CD38, and CD133 surface expressions were analyzed in combination with CFSE to detect immunophenotypical changes during expansion. Figure 4B showed representative dot plots indicating that fibrin maintained a primitive immunophenotype (CD34⁺CD38[−], CD34⁺CD133⁺) for more

FIG. 2. Imaging of cord blood (CB) derived-CD34⁺ cells cultured on different polymers. Panels A and B, D and E, G and H, J and K, M and N, P and Q, and S and T show live/dead stainings of freshly isolated CB-CD34⁺ cells cultured for 24 h on different polymers (scale bar = 500 μ m).

Fluoresceindiacetate (FDA) was used to detect green-fluorescent viable cells while propidium iodide (PI) was used to detect red-fluorescent dead cells. Cells on uncoated TCPS served as controls. (C, F, I, L, O, R, U) show light micrographs of 7 day-expanded cells on different biomaterials (scale bar = 100 μ m). Inserts (D) and (F) show presence of cells at the border of the well containing Poly(ϵ -caprolactone) (PCL). Images shown are from one single representative experiment ($n=3$). PVDF, poly(vinylidene fluoride).

cell divisions than Resomer[®] RG503. Texin[®] 950-expanded cells apparently achieved the highest CD34⁺ expression among conditions. Nevertheless, the number of texin[®] 950-expanded cells gated as CFSE[±]/CD34[±]/CD38[±]/CD133[±] proliferating cells by flow cytometry was never sufficient to obtain sustained populations in our experiments. Therefore, maintenance of a

primitive cell status was alternatively assessed using flow cytometry analysis (section 'cell lineage differentiation').

SEM analysis of 7 day-expanded cells (Fig. 5) revealed that polymer substrates significantly affect cell morphology, and that cell spreading visibly differs due to the polymer substrates. In addition, we observed that cell adhesion is absent

FIG. 3. CB-CD34⁺ cell growth on different polymer-based cultures. **(A)** Cumulative growth given by total nucleated cell (TNC) count estimated by electronic counting over a culture period of 14 days. Data derived from six independent experiments performed in triplicates. **(B)** Expansion of CD34⁺ and CD34⁺CD38⁻ populations after culture of CB-HSC for 7 days. Fold expansion defined as the ratio of amount of cells in culture expressing the CD34⁺/CD34⁺CD38⁻ phenotype at day 7 over the amount of unexpanded cells expressing the corresponding phenotype (as assayed flow cytometry). Data derived from four independent experiments performed in triplicates. *** $p < 0.001$ compared with TCPS.

with most 2D substrates, except for texin® 950 and fibrin, meaning that expanded cells did not adhere to TCPS either (Fig. 5A, B). As a consequence, imaging analysis was restricted to small cell numbers present on the polymer substrates after preparation for SEM, a limitation often reported.^{17,27} Superior cell densities and homogeneous cell organization were yet observed on PCL, Resomer® RG503, and fibrin (Fig. 5C, E, M), corroborating previous CFSE proliferation data. On the other hand, texin® 950 appeared to support cell clustering (Fig. 5K, insert) while focal cell adhesion was occasionally observed (Fig. 5K, L). Fibrin-expanded cells specifically benefited from a three-dimensional (3D)-like structure organization (Fig. 5M, insert), even if one attempts to produce a very thin layer of polymer. A group of defined fibers incorporated the fibrin matrix to which cells can directly attach using prominent filopodia (Fig. 5N). Further, we noticed that cells seeded on top of fibrin gels migrated inside the matrix and tightly interacted with matrix fibers (Fig. 5M, insert).

Cell lineage differentiation

Phenotypic characterization of 7day-expanded cells indicated that progenitor cell numbers were significantly better maintained over time when texin® 950 was used as a substrate (CD34⁺ expression: 49.2% vs. 31.7% on TCPS, CD34⁺CD38⁻ expression: 4.3% vs. 2.5% on TCPS, $p < 0.001$; Table 1). Resomer® LR704-cultured cells significantly downregulated surface expression of progenitor cell markers (CD34⁺ expression 16.1%, CD34⁺CD38⁻ expression 1.4%, $p < 0.01$). Primitive CD34⁺/CD34⁺CD38⁻ markers were expressed on TCPS, PCL, fibrin, and Resomer® RG503-cultured cells without significant differences. This might be consequence of including a very powerful cytokine combination in the culture, as described before.²⁴ In fact, addition of cytokines to HSC culture is directly related to loss of cell stemness. TCPS-expanded cells in fact appear to better maintain primitive phenotypes (CD34⁺, CD34⁺CD38⁻) compared with the other conditions. However, TCPS-based cells under-

performed both in terms of CFU counts and NSG engraftment (sections 'clonogenic potential' and 'NSG mice transplantation and engraftment'), which must be probably due to loss of HSC activity. Cell maturation was delayed on texin® 950. In fact, expression of the CD13⁺ myeloid marker in texin® 950-expanded cells was significantly down-regulated in comparison with TCPS (41.8% vs. 69.1%, $p < 0.001$). For none of the other polymers myeloid differentiation was affected. Finally, the CD45⁺ pan-leucocyte marker in combination with the CD56⁺ NK cell marker was used to monitor lymphoid differentiation. None of the polymer-based cultures did, however, significantly affect CD45⁺CD56⁺/CD45⁺CD56⁻ expression compared with TCPS.

Clonogenic potential

To evaluate hematopoietic progenitor cell frequencies, we performed CFU assays with cells that were expanded for 7 days on different polymers. Again, the results demonstrated similar or improved CFU expansion performance of PCL-, fibrin- and Resomer® RG503-expanded cells in comparison to TCPS-expanded cells (Fig. 6A). Among all tested polymers, PCL was the most efficient in terms of CFU-GM (4.32-fold increase over TCPS cultures, $p < 0.001$), CFU-GEMM (3.60-fold, $p < 0.01$), and CFU-total (4.00-fold, $p < 0.001$) yields. Similar to PCL, fibrin-cultures achieved significantly higher CFU counts than TCPS-cultures yielding a 3.96-, 3.15- and 3.6-fold increase in CFU-GM, CFU-GEMM and CFU-total respectively ($p < 0.01$). Resomer® RG503-based cultures reached smaller clonogenic expansion than fibrin, in the range of 1.12–2.7-fold increase in relation to TCPS. None of the other polymers performed better than TCPS, except for the texin® 950-based cultures with more CFU-GEMMs (1.40-fold increase in relation to TCPS). In contrast to all the other cultures, texin® 950-expanded cells generated higher CFU-GEMM than CFU-GM counts. Such expansion of CFU-GEMM, a more primitive population with the capacity of generating multiple cell types was in line with the upregulation of CD34⁺/CD34⁺CD38⁻ expression on texin® 950

FIG. 4. Proliferation profiles of CB-CD34⁺ cells expanded on different polymers. **(A)** Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) intensities of 7 day-expanded cultures were compared with day 0 cultures. CFSE intensity is inversely proportional to the number of cell divisions. Data derived from three independent experiments performed in triplicates. * $p < 0.05$ and *** $p < 0.001$ compared with TCPS. **(B)** Shown are combined CFSE and CD34, CD38 or CD133 profiles of 7 day-expanded cells. Cells were cultured in 24-well plates containing different 2D polymers. Data derived from one representative experiment. Color images available online at www.liebertpub.com/tec

FIG. 5. Representative scanning electron microscopy of 7-day expanded CD34⁺ cells on different polymers. Low magnified images (A, C, E, G, I, K, M) (scale bar = 500 μm) show that CD34⁺ expanded cells spread differently according to distinct substrates. High magnified images (B, D, F, H, J, L, N) (scale bar = 5 μm) indicate that cell morphology is also affected by the polymer-contact cultures. Typical cell clustering on texin substrates is visible (K insert). Tight interactions between cells and fibers of the fibrin matrix are shown in detail (M insert).

(Table 1). Unexpanded cells were simultaneously tested for their CFU capacity, as control. However, none of the expanded conditions increased the number of CFU-cells obtained by the unexpanded condition.

NSG mice transplantation and engraftment

To assess the *in vivo* engraftment potential, cultured cells were transplanted into NSG recipients. This experimental paradigm represents the most stringent test to analyze the engraftment potential of HSC.²⁸ For transplantation, we selected three of the six polymers. Given their promising expansion impacts *ex vivo*, PCL, Resomer[®] RG503, and fibrin were selected for further *in vivo* testing. The whole progeny of approx. 0.5×10^5 starting CD34⁺ cells per well was recovered from 7 day cultures, washed, and intravenously injected into sublethally irradiated NSG mice. Unexpanded and TCPS-expanded cells were also transplanted as controls. All control mice (except nonmanipulated) died within 15 days after irradiation, most probably due to irradiation-induced pancytopenia. Assessment of total human engraftment was done 6–8 weeks post-transplantations and was performed by flow cytometry quantification of CD45⁺ counts in BM. The presence of at least 0.10% of CD45⁺ human cells in BM was considered as successful engraftment. In our experiments, all recipients transplanted with 0.1 – 1.3×10^5 cells generated at least 1% CD45⁺ human cells (Fig. 6B). Results from human CD45⁺ engraftment in BM revealed that: (1) fibrin-expanded cells engrafted significantly better than cells cultured with other conditions (24.60%, $p < 0.001$ compared with TCPS), (2) unexpanded grafts had the second highest mean engraftment (5.90%), (3) PCL-based grafts yielded 5.80% of mean engraftment, a percentage comparable to unexpanded grafts, and iv) Resomer[®] RG503-based grafts achieved the lowest mean engraftment (2%). Likewise, human CD34⁺ expression in mice BM (Fig. 6C) was superior in animals transplanted with fibrin-expanded grafts (mean of 5.90%), followed by unexpanded grafts (mean of 4.20%). All other conditions resulted in decreased CD34⁺ expressions, as confirmed by immunohistochemistry (Fig. 7).

Next, hematopoietic multilineage reconstitution in mouse BM was analyzed by flow cytometry and immunohistochemistry. Presence of myeloid cells was examined by CD13⁺ (granulocyte, monocyte, mast cell, and GM-progenitor cell marker) and Glycophorin C (erythrocyte marker) staining. CD13⁺ expression among conditions ranged from 7%–14% (Fig. 6C), within that higher percentages correspond to unexpanded, TCPS, and fibrin-expanded cells (Fig. 7). Terminal erythroid differentiation was successfully achieved (Fig. 7) and its expression follows the trend described before for myeloid cell expression. In addition to myeloid, CD19⁺/CD20⁺ (mature B-cell and B-cell precursor marker) and CD56⁺ (natural killer cell marker) expression showed that unexpanded, TCPS-, and fibrin-expanded grafts support development of a lymphoid cell compartment, too (Figs. 6C and 7).

Overall, the confirmation that multiple human hematopoietic lineages, such as myeloid, erythroid, and lymphoid, were present in the NSG recipient BM after 6–8 weeks is a strong indicator of successful transplantations.

To gain more insights into multiorgan engraftment, mice SP (Fig. 8A) and PB (Fig. 8B) were analyzed. In general, myeloid expression was more pronounced in PB while

TABLE 1. SURFACE MARKERS EXPRESSION (%) AFTER 7 DAYS OF *Ex Vivo* EXPANSION OF CB-CD34⁺ CELLS ON DIFFERENT POLYMERS

Condition	CD34 ⁺ CD38 ⁻	CD34 ⁺	CD13 ⁺	CD45 ⁺ CD56 ⁻	CD45 ⁺ CD56 ⁺
Unexpanded	11.5±4.2	92.7±5.1	—	—	—
TCPS	2.6±0.7	31.7±7.9	69.1±15.6	13.6±9.5	4.2±3.2
PCL	2.3±1.1	26.8±7.0	64.0±18.6	22.4±7.7	5.5±5.0
Resomer [®] RG503	2.1±1.9	22.7±10.4	67.4±15.3	17.8±7.7	3.2±2.9
Resomer [®] LR704	1.4±1.8 ^a	16.1±8.7 ^a	64.4±12.1	16.6±7.5	4.0±3.9
PVDF	2.2±1.2	26.3±14.2	67.3±16.2	15.2±7.1	5.2±4.0
Texin	4.3±1.5 ^b	49.2±13.2 ^b	41.8±12.9 ^b	16.2±7.3	3.7±3.1
Fibrin	2.2±1.5	28.6±6.7	67.3±9.6	15.2±9.9	3.8±3.1

^a*p*<0.01 and ^b*p*<0.001 compared with TCPS.

Results are mean±SD of four independent experiments.

CB, cord blood; PCL, poly(ε-caprolactone); PVDF, poly(vinylidene fluoride); TCPS, tissue culture polystyrene.

lymphoid expression was in SP. Similarly to BM, fibrin-expanded grafts were preferred over the other polymer-based conditions, as proved by the superior percentage of CD45⁺ engrafted cells in SP (13.8%) and in PB (4.88%) and superior capacity for myeloid and lymphoid-cell development. Unexpanded cells were the second best condition in terms of multilineage SP and PB engraftment.

Discussion

We have identified three 2D polymer substrates suitable for HSC expansion. Fibrin, Resomer[®] RG503, and PCL successfully supported expansion of CB-derived CD34⁺ cells after a 7 day culture period with cytokine supplementation. Our data indicate that polymer chemistry and topographical features play a role in HSC expansion.

FIG. 6. Colony-forming cell expansion *ex vivo* and bone marrow (BM) engraftment in transplanted NSG mice. **(A)** Colony-forming cell expansion of CB-derived CD34⁺ cells after expansion on different biomaterials. After 7 days of expansion, 10,000 cells/dish were seeded into methylcellulose medium. As controls, 1000 unexpanded cells were plated into methylcellulose medium. Methylcellulose cultures run for 14 days before colony-forming unit (CFU) cells were counted and colonies identified as follows: CFU granulocyte-macrophage (GM), CFU granulocyte-erythrocyte-macrophage-megakaryocyte (GEMM), and CFU total are shown. Results express mean±SD of four independent experiments (*n*=3). **(B)** Percentage of human CD45⁺ engrafted cells harvested from the BM of NSG transplanted mice. Around 50,000 freshly isolated CD34⁺ cells and whole expanded progenies were injected per mouse via tail vein. Each mouse is represented by a symbol (*n*=2–3 mice per group). Lines (–) indicate total means per condition. Data derived from three independent experiments. ***p*<0.01 and ****p*<0.001 compared with TCPS. **(C)** Representative human CD45⁺, CD34⁺, CD13⁺, and CD19⁺ engraftment in the BM of NSG transplanted mice. Color images available online at www.liebertpub.com/tec

FIG. 7. Immunohistochemistry of NSG mice BM transplanted with expanded human CD34⁺ cells. BM slides of NSG recipients transplanted with 7 day expanded human CB-CD34⁺ cells were stained for expression of hematopoietic markers 6–8 weeks post-transplantation. The polymers-types used for expansion and the hematopoietic markers are indicated. Representative stainings are shown. Positive controls are unexpanded cells while negative controls are cells from unmanipulated mice. Brown or brownish-stained cells are positive for the respective markers. Slides of mice transplanted with fibrin-expanded cells show similar marker expression to unexpanded cells. Marker expression is stronger on immunohistochemical stained slides than on corresponding flow cytometry samples (most probably due to different method specificities). Scale bar=100 μ m. Color images available online at www.liebertpub.com/tec

Up to now, only a few systems for HSC culture using biomaterials have been proposed, including the use of collagen,^{17,19,29,30} nylon meshes,³¹ tantalum-coated carbon fibers,³² PET matrices,^{15,16,18} polyethylene-*alt*-maleic anhydride,³³ PLGA, and PU.²⁰ However, biomaterial-based studies in general resulted in highly variable outcomes, mainly due to concerns such as: (1) the choice of using biomaterial types is nearly arbitrary and (2) the effect of using different culture media in combination with biomaterials is itself not well characterized. Our study introduced the rational application of specific polymers in HSC expansion strategies as a result of a step-by-step analysis, starting with assessment of basic compatibility parameters, followed by the evaluation of *ex vivo* efficiency, and final *in vivo* testing. We suggest fibrin as optimal substrate for CB-derived CD34⁺ cell expansion. In fact, we have shown that fibrin-based cultures supported HSC (1) proliferation, (2) differentiation, (3) adhesion, (4) migration, and (5) homing engraftment *in vivo*. First, proliferation and differentiation of HSC was shown by cumulative growth counting, CFSE staining, CFU assessment, and immunophenotype analysis after using flow cytometry. Total nuclear cell expansion of

about 1020-fold was achieved by fibrin- rather than by TCPS-based cultures, after an expansion period of 14 days. HSC analyzed after 7 days of fibrin-based expansion resulted in a mean of ten cell divisions (instead of seven on TCPS). In terms of CFU-counts, fibrin-based cultures performed about fourfold higher than TCPS-based cultures ($p < 0.001$), meaning that a higher amount of primitive cells were able to undergo differentiation into the granulocyte, macrophage, and monocyte lineages. Second, HSC adhesion and migration were investigated by SEM. Microscopic images revealed that HSC superiorly interacted with fibrin fibers, by developing cell filopodia and adhesive characteristics. Previous studies have shown that HSC proliferation and differentiation often co-occurs with morphological changes such as substrate adherence and filopodia production,^{18,27,34–36} and this is in line with our findings. In addition, fibrin typically consisted of a 3D-like matrix of fibers, very likely resembling the ECM responsible for HSC maintenance *in vivo*. Third, HSC homing was studied by NSG mice transplantation experiments. Percentage of human CD45⁺ engraftment in NSG mice BM was significantly higher when fibrin- rather than TCPS-expanded grafts ($p < 0.001$) or unexpanded grafts ($p < 0.05$)

FIG. 8. Multilineage engraftment in different organs of transplanted NSG mice. Seven-day expanded CD34+ cells on different polymers were transplanted into NSG mice. About 6–8 weeks post-transplantation engraftment was assessed. Representative flow cytometry plots showing mouse CD45 versus human CD45/CD13/CD3/CD19 expression in NSG mice. Engraftment was analyzed in (A) spleen (SP) and (B) peripheral blood (PB). Fibrin-expanded cells supported reconstitution of the myeloid (CD13+) and lymphoid (CD3+ and CD19+) compartments in the SP and PB of transplanted mice, similar to TCPS-expanded cells. Overall, typical myeloid circulating populations were present in higher degree in PB while lymphoid populations were more pronounced in SP corresponding to the balanced situation *in vivo*. Color images available online at www.liebertpub.com/tec

were used. About 24.6% mean CD45⁺ engraftment was obtained after transplantation of CD34⁺ fibrin-expanded cells, which is superior to the results achieved by other expansion strategies in similar conditions. In fact, CB-HSC expanded by conventional methods (using stroma-based cultures²⁵ or chromatin-modifying agents,³⁷ for instance) have failed to show significantly improved *in vivo* engraftment compared with unexpanded cells. In contrast, expansion of CB-CD34⁺ cells using a fibrin-based protocol expanded the number of SCID-repopulating cells to such an extent as about four-fold higher than nonmanipulated cells. Our fibrin-based strategy (that achieved an increase of CD34⁺ cells superior to 800-fold) might eventually outperform good manufacturing practice (GMP)-compliant protocols recently reported in clinical trials, where a 150-fold expansion of CD34⁺ cells was achieved by Delta1^{ext-IgG}-expanded cultures.¹²

Structural organization and material origin are known to affect cell behavior.^{38,39} Here, we analogously suggest that the success achieved by fibrin-expanded HSC cultures mostly relies on fibrin's structural organization and on degradation rate. That means (1) fibrin consists of a 3D-like matrix that simultaneously provides structural support and opportunity for cell migration and adhesion, therefore more closely mimicking native BM conditions and (2) fibrin is a fast degradation blood-clotting protein with high hydrophilic character; therefore, preferred for HSC growth (as discussed further on).

Fibrin was found to be not suitable for mouse HSC growth in previous work.²² Here, we have shown that compatibility of HSC from different species to the very same substrates is not predictable and fibrin is indeed advantageous for human CB-HSC expansion. The use of fibrin for a series of tissue engineering applications is common and includes wound healing, vascular grafting,⁴⁰ heart valve applications, and others. Fibrin can potentially be produced as autologous scaffold material (derived from the patient's own blood) and that is advantageous for diverse hematological therapies and for clinical translation into a GMP-compliant product. Media formulations containing cytokines not yet GMP-grade available, such as FGF-1, IGFBP₂, and Angptl-5, might be problematic for translational purposes. Alternatively, fibrin-based cultures could be used for clinical trials if a combination of clinical-grade cytokines such as SCF, TPO, interleukin (IL)-3, IL-6, and fetal liver tyrosine kinase 3 (Flt-3) ligand is used, as in a recent trial.¹²

Additionally, we propose Resomer[®] RG503, and PCL for future HSC expansion applications. Both substrates were able to successfully expand the number of CB-derived CD34⁺ cells, yet with distinctive efficiencies and less powerfully than fibrin. While PCL performed consistently better than TCPS for all *ex vivo* and *in vivo* analyses, Resomer[®] RG503 resulted only in higher CFSE proliferation ($p < 0.05$) and total CFU's ($p < 0.001$) compared with TCPS. Discrepancies of *ex vivo* and *in vivo* results for Resomer[®] RG503 might be due to specific polymer-induced variability or due to the size of the animal group. Again, we hypothesize that a higher number of transplanted animals could possibly discern better hematopoietic engraftment *in vivo*.²⁵ In general, none of the two polymers did, however, show improvements in relation to the unexpanded controls, contrasting to fibrin. That might be due to the fact that both Resomer[®] RG503- and PCL-expanded cells lack structural support. Hence, we

postulate that producing Resomer[®] RG503 and PCL in a 3D format might perform better than TCPS, and eventually than unexpanded cells, under culture conditions similar to ours. The superiority of PCL over Resomer[®] RG503 supporting CB-derived CD34⁺ cell expansion might reside on the distinctive physicochemical attributes of each polymer. We suggest a positive correlation between polymer degradability and HSC expansion. Both PCL and Resomer[®] RG503 are synthetic degradable polymers that can simply experience hydrolysis during long-term *ex vivo* culture.³⁹ Accordingly, different degradation rates will affect cell proliferation differently.⁴¹ This fact might have contributed to the improved HSC expansion efficiency of PCL in comparison with Resomer[®] RG503 with higher degradation time. In theory, suitable biomaterials for HSC expansion *ex vivo* do not implicitly require biodegradability, as they are not intended for transplantation.⁴² However, polymer degradation typically generates smaller degradation products, which can *per se* affect cell behavior, and that has to be considered. One other factor known to affect polymer degradation is hydrophobicity.⁴³ Cells can indeed use membrane receptors to sense the hydrophobic character of a polymeric surface,⁴⁴ or alternatively of adsorbed proteins.⁴⁵ Resomer[®] RG503 and PCL have both intrinsic hydrophobic properties, with PCL being more hydrophobic than Resomer[®] RG503.³⁹ So less hydrophobic surfaces are more favorable for HSC expansion, as previously suggested for CB-derived MNC.²⁰ Direct application of Resomer[®] RG503 and PCL for expansion strategies as part of clinical trials is indeed possible as both are FDA-approved polymers.

In the light of actual knowledge, it is difficult to predict the molecular effect of polymeric substrates on biological performance of cells. Based on our results, it is, however, reasonable to hypothesize that surface chemistry of fibrin, Resomer[®] RG503, and PCL may interact with serum proteins included in the media (such as BSA, insulin, and transferrin present in StemSpan medium) engaging with proper HSC surface receptors, therefore supporting HSC expansion, as suggested in Mei *et al.*'s report on development of biomaterials for clonal growth of human pluripotent stem cells.⁴⁶

Here, we demonstrate a highly efficient strategy for expansion of human CB-derived CD34⁺ cells using fibrin-based cultures in presence of SCF, TPO, FGF-1, IGFBP₂, and Angptl-5. Fibrin-based cultures clearly (1) facilitated cell adhesion and (2) provided a matrix-like structure enabling cell migration, proliferation, and differentiation comparable to spongy BM. Further, we have shown that PCL and Resomer[®] RG503 can also support HSC expansion, but future expansion strategies might include enhancement of polymer surface properties and/or structure.

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Disclosure Statement

None of the authors declare conflict of interests.

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