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Combined influence of biophysical and biochemical cues on maintenance and proliferation of hematopoietic stem cells

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Homeostasis of hematopoietic stem and progenitor cells (HSPC) is controlled by a combination of biochemical and biophysical environmental cues in the bone marrow (BM) niche, where a tight balance of quiescence and proliferation of HSPC is maintained. Specifically, alongside soluble factors and extracellular matrix (ECM) proteins, spatial confinement and ECM stiffness have been recognized to be critical for regulation of HSPC fate. Here we employ a modular, glycosaminoglycan (GAG)-based biohybrid hydrogel system to balance proliferation of human HSPC and maintenance of quiescent hematopoietic stem cells (HSC) through simultaneous regulation of exogenous biochemical and biophysical cues. Our results demonstrate that HSPC respond to increased spatial confinement with lowered proliferation and cell cycling, which results in higher frequency of quiescent LTC-IC (long-term culture initiating cells), while GAG-rich 3D environments further support maintenance of the cells.

1. Introduction

Hematopoietic stem cells (HSC) are at the root of a complex hierarchy of specialized progenitors which give rise to all types of mature blood cells. With respect to their functionality, HSC are hierarchically organized into long-term repopulating HSC (LT-HSC) – infrequently dividing, quiescent cells functioning as a reserve population, and short-term HSC (ST-HSC) with relatively low self-renewal ability which further give rise to the early progenitors[1–3].

The complex dynamic interplay of soluble and mechanical signals in the BM niche ensures the balance between quiescence, cell cycling, proliferation and differentiation of HSC in a spatially and temporally regulated manner[1,4,5]. Due to their role in life-long hematopoiesis and their marked regenerative potential, HSC transplantation has become a common clinical therapy of several diseases, such as leukemia[6,7]. Unfortunately, failure of host engraftment, limited regeneration of the host hematopoietic system, as well as the lack of successful *ex vivo* expansion strategies limit the success of HSC-based therapies[8–10]. These limitations ask for an increased knowledge on HSC regulation within their niche in order to develop effective methods to maintain or expand HSC *ex vivo*.

Although *in vivo* models for investigating HSC niche function are established, as they reflect the full complexity of the BM microenvironment, they are hard to interpret in terms of dissecting the individual parameters which guide HSC fate decisions. In recent years, *in vitro* models applying bioengineered scaffolds have been successfully demonstrated to be instrumental in selectively addressing specific niche parameters[11–14]. Currently existing scaffold engineering strategies to recapitulate the BM niche environment are prevalently based on microstructured, porous or fibrous scaffold architecture and functionalization with bioactive signaling molecules[7,12,13,15,16]. In order to mimic the 3D niche environment more closely, encapsulation of cells in natural or synthetic hydrogels has become a promising strategy to guide cell fate decisions[11,13,14,17].

For example, 3D encapsulation has been demonstrated to direct stem cell fate decisions in cell types such as mesenchymal stromal cells (MSC)[18–21]. Furthermore, encapsulation of induced pluripotent stem cells (iPSC) in 3D matrices has been shown to accelerate induction of pluripotency. Similarly, 3D microenvironment was demonstrated to promote maintenance of undifferentiated state in the ESC *ex vivo*[22–24]. These studies strongly suggest that spatial confinement of the encapsulated cells in 3D microenvironment promotes their pluripotency in synergy with other parameters, such as material stiffness, soluble factors, as well as cell secreted paracrine and autocrine signals. Our previous studies have shown the important

impact of spatial constraints on HSPC fate decisions in cavity based platforms[15,25,26], motivating us to further explore the role of mechanical confinement by full encapsulation of HSPC in a 3D *ex vivo* culture.

In the present study, we combined GAG-mediated factor presentation with varying degrees of spatial constraint using a cell encapsulation strategy to assess the interplay of biophysical cues, GAG, and growth factor presentation on HSPC fate decisions in a 3D culture environment. For this aim, we used an *in situ* forming starPEG-heparin hydrogel system, assembled by Michael type addition reaction, to create a 3D culture environment mimicking GAG-rich BM and facilitate GAG-mediated growth factor presentation to the cultured cells[27–31]. In addition, GAG-mediated growth factor presentation could be adjusted by partial desulfation of the heparin in the hydrogels[32]. Furthermore, starPEG-heparin biohybrid gels allow for independent modulation of biophysical and biochemical parameters, which allows for dissecting both exogenous signaling cues[30,33].

Using the starPEG-heparin hydrogel platform, we demonstrate that LTC-IC maintenance can be achieved through combined manipulation of biophysical and biochemical *ex vivo* culture parameters in a 3D environment. The established 3D culture system highlights the role of mechanical signaling in HSC maintenance and can serve as a study model for further exploration of HSC mechanobiology.

2. Materials and methods

2.1 Cell culture

Primary human CD34⁺ cells were isolated from leukapheresis products provided by University Hospital Carl Gustav Carus, Dresden. Leukapheresis and subsequent cell experiments were carried out in accordance with the approved guidelines of the ethics committee of the University Hospital Carl Gustav Carus Dresden after obtaining a written informed consent from healthy donors. Granulocyte colony-stimulating factor (G-CSF) mobilized human HSPC were isolated using magnetic cell isolation (CD34 MicroBead Kit, Miltenyi Biotec, Germany) according to the manufacturer's instructions. Cells were counted using MACSQuant Analyzer (Miltenyi Biotec) and the purity range between 92% and 99% CD34⁺ cells of the cell population was verified. The cells were utilized immediately after isolation or following an overnight culture in serum-free CellGro® SCGM medium (CellGenix, Germany) supplemented with 10 ng/ml of the SCF (Miltenyi Biotec), TPO and FLT3L (both R&D Systems, MN, USA).

2.2 Preparation of the hydrogel cultures

Hydrogels were prepared as previously described[30]. StarPEG containing the MMP-cleavable sequence GCGGPQG↓IWGQGGCG-NH₂ (MW ~15500 g/mol) and fully sulfated or 6ON-desulfated heparin modified with an average of 6 maleimide groups per molecule of heparin were synthesized at IPF. For 3D cultures, CD34⁺ cells were embedded in starPEG-heparin hydrogels at a density of 2.5×10^6 cells per 1 ml gel (corresponding to 5×10^4 cells per scaffold of 20 μ l), unless indicated otherwise. The molar ratio of starPEG to heparin-maleimide (γ) 1.0, 1.25 and 1.5 was used to obtain gels with stiffness ranging from 0.5 kPa to 3 kPa. The scaffolds were functionalized with SCF, TPO and FLT3L by adding cytokines to the heparin fraction at a molar ratio of 1:10000. Upon polymerization, scaffolds were suspended in 1 ml CellGro® SCGM medium. For 2D cultures, hydrogel scaffolds were prepared in a similar manner without adding the cell suspension during the scaffold preparation procedure. Cast gel droplets were flattened to cover the surface of a 24-well tissue culture plate (TCP) using Ethylene-Chlorotrifluoroethylene copolymer (E-CTFE) discs with a diameter of 15 mm which were punched out in advance from 0.025 mm thick E-CTFE films (Goodfellow, Cambridge, England). Scaffolds were allowed to polymerize and the discs were removed. CellGro® SCGM medium containing a defined number of cells corresponding to that embedded in the 3D scaffolds (typically 5×10^4 cells per ml) was then applied on top of 2D scaffolds at an amount of 1 ml per well. Transwell (TW) controls were prepared by placing hydrogel scaffolds preloaded with SCF, FLT3L, and TPO into a TW cell culture insert (pore size 8 μ m, Greiner Bio-One) and submerging the inserts into the TCP well-plates containing 1 ml HSPC suspension. A cell suspension containing a corresponding concentration of CD34⁺ cells and additionally supplemented with 10 ng per ml EAHc was used to prepare TCP controls. All samples were cultured at 37 °C and 5% CO₂ content for a culture period of 7 days and monitored by phase contrast microscopy (LEICA DMIL Inverted Microscope, Leica Microsystems GmbH, Wetzlar, Germany) throughout the culture period. At the end of the culture, 3D and 2D scaffolds were degraded using 0.1 - 0.5 Wünsch U per ml collagenase (SERVA Electrophoresis GmbH, Heidelberg, Germany). All cells were harvested and subjected to flow cytometry analysis (MACSQuant Analyzer, Miltenyi) for the surface markers CD34, CD133 in combination with propidium iodide staining for dead-cell exclusion (all Miltenyi, used according to the manufacturer's instructions). Obtained data was further analyzed and processed using the FlowJo software.

2.3 *Hoechst33342 and Pyronin Y Staining for G0/G1 Separation*

Staining for DNA and RNA was performed as described previously[34,35]. Briefly, the harvested cell pellet was re-suspended in 500 μ l SCGM medium containing 10 μ g/ml Hoechst33342 (Life Technologies, CA, USA) and incubated for 45 minutes at 37 °C. Afterwards, 500 μ l SCGM medium containing 2 μ g/ml Pyronin Y (Sigma-Aldrich) was added directly to the samples to obtain a final concentration of 1 μ g/ml Pyronin Y and the samples were incubated for another 15 minutes at 37 °C. Subsequently, samples were centrifuged, re-suspended in AutoMACS® Running Buffer and analyzed by MACSQuant Analyzer. Single stains (only Hoechst33342 or Pyronin Y) and unstained cells were used to adjust voltages on the flow cytometer prior to the sample measurement. Equal numbers of cells were analyzed in each sample. Obtained data was further analyzed and processed using the FlowJo software.

2.4 *Immunostaining and microscopy*

Samples were fixed with 4% paraformaldehyde for 10 minutes and blocked and permeabilized using 5% goat serum and 0.1% Triton X- 100 in PBS for 1 h. Primary antibody against CD133 (Miltenyi Biotech) was then added at a 1:100 dilution and samples were incubated overnight at 4 °C. Samples were subsequently washed and Alexa Fluor 633 goat anti-mouse (1:200 dilution) was applied for 24 h at 4 °C. Afterwards, samples were washed and incubated with Hoechst 33342 (Life Technologies) at a concentration of 1 mg/ml for 30 min before visualization on a Leica Confocal SP5 microscope (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany). For quantification of proliferative clusters, actin cytoskeleton was labeled using Phalloidin Alexa Fluor 633 (1:50 dilution; Life Technologies). Images were analyzed with the open source software ImageJ (<http://rsbweb.nih.gov>; MD, USA).

2.5 *LTC-IC Assay*

Number of LTC-IC in the hydrogel cultures was determined by a method of quantitation of LTC-IC using bulk cultures. Firstly, freshly isolated CD34+ cells were cultured in 3D and on 2D hydrogel matrices or TCP. After 7 days of culture, cells were harvested by collagenase treatment under sterile conditions. Aliquots of defined size (100 μ l) were collected from the harvested samples to determine cell numbers and frequency of CD34+ cells by flow cytometry. Afterwards, cells were re-suspended in 1 ml LTC-IC medium (MyeloCult H5100, Stemcell Technologies, Vancouver, Canada) supplemented with hydrocortisone (Sigma, Sigma-Aldrich) at a density of 1×10^3 CD34+ cells per ml and transferred to MSC feeder cell layer prepared in advance by culturing MSC in 24-well plates in DMEM + 10% FCS for 4 days. Feeder cell layer was washed once with PBS before

applying the CD34⁺ cell suspension. Half of the medium was replaced weekly with fresh medium, and the number of colony-forming units (CFU) was determined after 5 weeks of LTC-IC culture. For the CFU assay, the cells were harvested by trypsinization and 1×10^3 cells were re-suspended in 1 ml of methylcellulose medium for the CFU assay (Stem MACS HSC CFU complete with EPO human, Miltenyi Biotech). Cultures were incubated at 37 °C in 5% CO₂, and the number of colonies was scored at day 14. For reference, freshly isolated CD34⁺ cells were subjected to the CFU assay by plating 500 CD34⁺ cells per ml in methylcellulose and quantifying after 14 days culture as described earlier.

2.6 Data Analysis

All statistics were performed using GraphPad Prism v6. Analysis of the statistical differences between individual samples was performed using student t-test analyses. Multiple samples were analyzed by one-way or two-way analysis of variance (ANOVA) with post-hoc Tukey's multiple comparisons tests. Wilcoxon rank sum test was performed to analyze statistical differences between the distribution of HSPC clusters in soft and stiff hydrogels according to the cluster size. Asterisks denote statistical significance as follows: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) or **** ($p < 0.0001$).

3. Results

3.1 Properties of the starPEG-heparin hydrogels

The stiffness of the hydrogels (characterized by the storage modulus of the material as obtained from oscillating rheometry) of the starPEG-heparin material was systematically tuned by varying the starPEG to heparin ratio (γ), while keeping the heparin concentration in the swollen hydrogels constant[30]. As described previously, the scaffold stiffness increased proportionally to the starPEG to heparin ratio[30]. Gels with a γ of 1.0, 1.25 and 1.5 were used in the study, resulting in the matrix storage moduli ranging from soft (0.5 ± 0.07 kPa), to intermediate (1.5 ± 0.03 kPa) and stiff (3 ± 0.05 kPa) (SI Fig. 1 A and B).

StarPEG-heparin hydrogels used in the study are responsive to cell-mediated remodeling due to the presence of an MMP-cleavable sequence incorporated into one of the starPEG arms, as previously described[30]. In addition, the presence of the MMP-sensitive sequence enabled the degradation of the hydrogel matrices for further phenotypical and functional analysis of the embedded HSPC.

A typical cytokine tri-cocktail of SCF, FLT3L and TPO was used in the study (collectively Early Acting Hematopoietic Cytokines - EAHC) for HSPC culture. The hydrogels were functionalized by preloading of the heparin fraction with EAHC to ensure

homogenous distribution of the growth factors throughout the scaffolds. Given that all cytokines used in our study effectively bind to heparin[26,36], and the concentration of factors in proximity of an embedded cell is dependent on the concentration of heparin around it, the molar ratio of cytokine to heparin was chosen as a constant criterion to enable similar factor presentation. In particular, a ratio of cytokine to heparin of 1:10000 was established for scaffold loading based on an empirical assessment. This corresponds to 50 ng of SCF and FLT3L and 100 ng of TPO in total per scaffold. Prior to the cell culture experiments, the released levels of cytokines from cell-free scaffolds loaded with EAHC over a period of 7 days were measured by ELISA. Cytokines were detected at day 7 at cumulative concentrations of 10 ng/ml for SCF, and 2 to 3 ng/ml for TPO and FLT3L respectively, while the relevant volume ratio of supernatant to hydrogel as later used for the cell culture experiments was applied (Fig. 1 B). Thus, the release of the cytokines did not exceed or was even lower than the concentration typically used in suspension culture of HSPC (10 ng/ml). As previously reported for other factors[30], the network properties (i.e. the stiffness) of the hydrogel material did not influence concentration or kinetics of the cytokine release, pointing to the fact that only the affinity to the constant number of heparin molecules and not sterical effects influence the transport properties and presentation of the EAHC factors within the matrix (SI Fig. 1C).

Additionally, hydrogels containing partially desulfated heparin, where 6O and N-sulfate groups have been selectively removed (denoted further as 6ON-DSH for desulfated heparin), were used in the study for comparison with hydrogels containing fully sulfated heparin (HEP). Mechanical properties of HEP and 6ON-DSH containing hydrogels were similar as measured by rheometry (SI Fig. 1A). Desulfation of heparin resulted in a moderate increase of EAHC release (SI Fig. 1D) for most growth factors, as was expected since the sulfate groups of the heparin are recognized to influence the affinity of heparin to the factors[32,36].

3.2 *Expansion and viability of HSPC embedded in starPEG-heparin hydrogels*

For the initial cell culture experiments, cells were embedded in 3D soft (storage moduli of 0.5 kPa) hydrogels or cultured on top of 2D hydrogels, both preloaded with EAHC. No additional cytokines were supplemented in the medium in either case. As a reference, HSPC were cultured on TCP and supplemented with 10 ng/ml EAHC. HSPC cultured for seven days in 3D or on 2D hydrogel scaffolds displayed significantly higher viability and approximately 1.5 fold higher expansion compared to the TCP control (Fig. 1 C and D). Expansion of the HSPC in soft 3D hydrogels was found to be similar compared to 2D hydrogel surfaces of the same stiffness.

Fig. 1 Characterization of hydrogel material and HSPC hydrogel culture. **A.** Hydrogel scaffold is formed by covalent crosslinking of starPEG and heparin. The biohybrid hydrogel can be modularly tuned by reversible functionalization with cytokines, covalent conjugation of adhesion ligands, or implementation of enzymatically cleavable crosslinkers to allow for cell-responsive remodeling. **B.** Cumulative release of EAHC from the hydrogel scaffolds over the course of 7 days at different time points. Mean $\pm$ SD of 2 experiments (n=3). **C.** Expansion and **D.** viability of CD34+ cells after 7d of culture on TCP in presence of 10 ng/ml EAHC, or 2D and 3D hydrogels functionalized with EAHC (molar ratio of cytokine to heparin 1:10000). Mean $\pm$ SD 3 donors, n=3, asterisk denotes $p < 0.0001$. **E.** CLSM images of HSPC embedded in the hydrogels and on 2D (**F**) after 48 h of culture. Cells were stained for actin and CD133. Scale bar 20 μ m **G.** Phase contrast microscopy images of HSPC cultured on 2D surfaces for 7 days. Scale bar 50 μ m.

Evaluation of the embedded HSPC by confocal laser scanning microscopy (CLSM) after 48 h of culture revealed CD34+ cells with a confined, round shape and even distribution of CD133 marker, which is indicative of non-polar cell morphology (Fig. 1E). HSPC were represented as single units, with no contact between the cells even when embedded at high densities (Fig. 1E, SI Fig. 2 A and B). In contrast, HSPC on 2D surfaces tend to form populations of clustering cells and display amoeboid shape, with uropod-like protrusions and uneven distribution of CD133 marker, suggesting polar cell morphology (Fig. 1F and G). Proliferation of HSPC in the hydrogels was observed as the formation of clusters containing cells which are tightly confined together. Clusters were found to expand by compression of the surrounding gel matrix and not via enzymatical reorganization of the scaffold (Fig. 2C and

SI Fig. 2E and F). Therefore cell migration in 3D hydrogel was not observed and the cell-cell contact was limited to the immediate progeny derived from a single cell.

The increase of seeding cell density (8×10^4 cells per well) resulted in enhanced proliferation on TCP and 2D cultures compared to the seeding density of 2×10^4 cells per well. Notably, no significant difference could be observed in 3D hydrogel cultures with high or low cell densities (SI Fig. 2C and D).

3.3 *Influence of hydrogel stiffness and spatial confinement on HSPC cell fate*

Motivated by the previous work[15,25,26,37], we herein focused on the role of spatial constraints on HSPC fate decisions in a 3D hydrogel culture. Increasing the starPEG to heparin ratio resulted in more stiff hydrogels and thus a spatially constrained environment for the embedded cells. Thereby we observed that an increase of hydrogel stiffness caused a reduced expansion of HSPC embedded in the 3D matrices. The decrease of proliferation was inversely proportional to the stiffness of the hydrogel, being lowest in stiff (storage moduli of 3kPa) 3D gels (Fig. 2A). Notably, cell viability was not altered by mechanical properties of the material and was significantly higher in all hydrogel cultures compared to TCP independent of the hydrogel stiffness (SI Fig. 3A). Furthermore, an increase of hydrogel stiffness in the 3D culture resulted in significantly higher expression of CD34 marker (Fig. 2B). In contrast, no differences in the cell proliferation were observed when HSPC were cultured on stiff or soft 2D hydrogel matrices, highlighting the impact of the 3D confinement on the cell fate (SI Fig. 3B).

Microscopy analysis of the hydrogels after the period of culture revealed that the total number of formed cell clusters in stiff hydrogels was lower compared to the soft gels (Fig. 2E). In addition, more clusters containing three or more cells were found in the soft scaffolds compared to the stiff counterparts (Fig. 2D). The biggest clusters observed in the stiff gels contained max. 5 cells, whereas a max. of 10 cells per cluster were found in the soft hydrogels. Interestingly, both cultures contained equal numbers of single cells, which supposedly did not enter cell cycle during the culture period (Fig. 2D, SI Fig. 3D).

To identify the frequency of quiescent cells in 3D cultures, we used Hoechst 33342 and Pyronin Y staining and analyzed equal numbers of CD34⁺ cells by flow cytometry after 1 week of culture in 3D hydrogel scaffolds with increasing crosslinking degree. Alternatively, cells were cultured on 2D matrices and TCP for reference. The majority of freshly isolated cells were in G0 (resting) phase of cell cycle (SI Fig. 3C). After 1 week of culture, more than 70% of all cells were found in G1 phase irrespective of the culture type. However, the frequency of the cells in G0 significantly increased in direct correlation to the hydrogel

stiffness in 3D cultures, being lowest on 2D and TCP control (Fig. 2F, SI Fig. 3C). Conversely, the frequency of the cells in G1 steadily decreased from 2D cultures to 3D, corresponding to the increase of hydrogel stiffness and the resulting spatial confinement in the 3D culture (SI Fig. 3C and E). To assess if the high frequency of quiescent cells within stiff gels correlated with an increased primitive HSC phenotype, we performed *in vitro* LTC-IC assay following a seven-day culture of HSPC on 2D cultures or in 3D stiff and soft matrices. Equal numbers of CD34⁺ cells were taken from the cultures for the LTC-IC assay. Maintenance of CD34⁺ cells in long-term cultures (5 weeks) revealed that the cells harvested from stiff hydrogel contained the highest frequency of LTC-IC (Fig. 2G).

Together, these results strongly suggest that an increased stiffness (and subsequent level of spatial confinement) of the 3D environment results in an increased quiescent cell frequency, decreased cell cycling and RNA synthesis, along with increased maintenance of LTC-IC.

3.4 GAG-mediated growth factor delivery and influence of heparin on LTC-IC maintenance

GAG-mediated growth factor presentation[38–40], as well as the interaction between heparin and HSPC-derived factors[26], have been reported to be important in the maintenance of HSPC cultures *in vitro*. Therefore we analyzed the impact of interactions of the HSPC with heparin in the hydrogel matrix, as well as the role of heparin-mediated cytokine presentation.

To deplete the GAG-released fraction of growth factors, 3D HSPC cultures functionalized with EAHC were subjected to daily medium exchanges without further EAHC supplementation. As shown in Fig. 3A, a frequent medium exchange resulted in a strong reduction of cell numbers, as well as a decrease of cell viability (SI Fig. 4A). Consistent with the previous observations, cells proliferated efficiently in 3D scaffolds where medium had not been exchanged over the course of the culture period. Importantly, medium exchange *per se* did not influence HSPC viability or proliferation when cytokines were supplied in a soluble form to the cells embedded in the non-functionalized 3D hydrogel cultures (SI Fig. 4B and C).

Fig. 2 Influence of hydrogel stiffness and increased spatial constraint. **A.** Expansion of CD34+ cells after 7d of culture in 3D hydrogels, or on top of 2D hydrogels, and TCP. Data are presented as mean $\pm$ SD of n=3 and 3 donors. **B.** Distribution of CD34 and CD133 marker expression after 7d of culture in the 3D hydrogel or on top of 2D hydrogels, and TCP. Data are presented as mean $\pm$ SD from 7 donors and n=3 per experiment. Asterisk denotes $p < 0.0001$. **C.** CLSM images of HSPC clusters after 5d of 3D cell culture. Nuclei (blue) and actin cytoskeleton (red) were stained. Scale bar 50 μ m. **D.** Distribution of clusters according to the cluster size. Mean $\pm$ SD of 2 donors, n=2, asterisk denotes $p < 0.001$. **E.** Total number of clusters (regardless of cluster size) in stiff and soft gels. Mean $\pm$ SD 2 donors, n=2, asterisk denotes $p < 0.01$. **F.** The frequency of cells in G0 as a function of hydrogel stiffness after 7d of culture. Mean $\pm$ SD 3 donors, n=3, asterisk denotes $p < 0.001$. **G.** Number of LTC-IC derived CFU as a function of hydrogel stiffness after 7d of HSPC culture. Freshly isolated CD34+ cells were cultured for LTC-IC assay for reference. Mean $\pm$ SD 3 donors, n=3, asterisk denotes $p < 0.0001$.

To test whether cell contact with the loaded hydrogel scaffold was influencing the proliferative activity, HSPC were cultured either on a 2D loaded hydrogel scaffold or in the

presence of a loaded hydrogel placed on a trans-well (TW) cell culture insert and suspended in the culture medium to avoid direct cell-hydrogel contact. TCP culture with medium supplemented with EAHC was used for reference. Consistent with the previous observations, contact with the loaded 2D gel resulted in a higher proliferation compared to TCP samples (Fig. 3B). Lowest proliferation was observed in TW samples with the loaded gels.

To further discriminate if the interaction between the hydrogel and the HSPC contributed to the high proliferative activity of HSPC in hydrogel cultures, similar setting with 2D and TW was prepared with plain gels without functionalization with the growth factors. Soluble cytokines were supplied in the medium at 10 ng/ml as in the TCP culture. No significant differences in proliferation were seen in the cultures with plain gels either on 2D, TW inserts or TCP. (SI Fig. 4D).

To evaluate the impact of the GAG properties on the growth factor presentation by the hydrogel matrices, 2D and TW cultures with 6ON-DSH and HEP-based hydrogels were compared. Similar to the HEP-based hydrogels, contact with the 2D 6ON-DSH hydrogels led to an increased proliferation of the HSPC compared to TCP and TW cultures (SI Fig. 4E). The cell proliferation was slightly higher on 2D 6ON-DSH scaffolds compared to the HEP-based counterparts. Importantly, the difference in proliferation was less distinct than the difference found for the growth factor release between the two scaffold types. We next analyzed the impact of heparin desulfation and subsequent modulation of the GAG capacity for cell-secreted factor binding on maintenance of LTC-IC in the stiff hydrogels, as well as its contribution to stiffness-mediated cell cycle changes. Therefore we evaluated the frequency of quiescent cells in hydrogel cultures containing HEP or 6ON-DSH. Notably, HSPC proliferation was equal in the stiff scaffolds containing HEP and 6ON-DSH (SI Fig. 4F). Cell cycle analysis of the cells extracted from HEP and 6ON-DSH hydrogels showed a similar trend of increased G0 frequency corresponding to an increase of scaffold crosslinking, as previously observed with the HEP-containing scaffolds (Fig. 3C). Furthermore, the frequencies of quiescent cells in HEP tended to be higher than in 6ON-DSH.

To address the contribution of heparin in the maintenance of LTC-IC in the stiff hydrogels, an LTC-IC assay was performed with gels containing 6ON-DSH heparin. A similar trend of increasing LTC-IC frequency proportionally to the scaffold stiffness and subsequent spatial confinement was observed as previously in the HEP-containing scaffolds. However, the maintenance of the LTC-IC in the 6ON-DSH-containing scaffolds was much less expressed with significantly higher maintenance of LTC-IC observed in the stiff HEP scaffolds compared to the stiff 6ON-DSH hydrogels (Fig. 3D).

Fig. 3 GAG-mediated growth factor delivery and influence of heparin on LTC-IC Maintenance. **A.** HSPC expansion after 3D culture in cytokine-functionalized 3D hydrogels and medium exchange every 24 h. Results are presented as mean $\pm$ SD (n=3) of 3 donors. **B.** Expansion of HSPC on 2D hydrogel matrices compared to TW cultures with similar loading but no cell-hydrogel contact. Results are presented as mean $\pm$ SD (n=2) of 3 donors. **C.** Comparison of quiescent cell frequency in HEP and 6ON-DSH-containing gels in stiff and soft hydrogel scaffolds. (Mean $\pm$ SD of 2 donors, n=3 per donor) **D.** LTC-IC assay of HSPC harvested from hydrogels with HEP and 6ON-DSH. Results are presented as mean $\pm$ SD (n=2) of 2 donors. Asterisk denotes $p < 0.01$

Overall, the above results suggest a complex interplay between spatial constraints and the GAG-factor interactions on HSPC maintenance.

4. Discussion

Using rationally designed starPEG-heparin hydrogels for 3D HSPC culture, we evaluated the impact of substrate stiffness and the resulting spatial confinement on fate decisions of the embedded HSPC. The ability to independently modulate biochemical and biophysical parameters in a 3D culture is the hallmark feature of the applied biohybrid hydrogel. Here we took advantage of this unique property to create a GAG-rich 3D environment for HSC maintenance, and simultaneously introduce variable degrees of spatial confinement to regulate HSPC cycling and proliferation without interfering with the GAG-mediated cytokine signaling characteristics. The obtained results reinforce the concept of using spatially defined niches, reflecting the environment inside the BM to control stem cell quiescence and long-

term maintenance. Furthermore, the established starPEG-heparin 3D culture was demonstrated to be a promising tool to elucidate putative mechanisms of biophysical and biochemical signaling pathways involved in regulation of HSC maintenance.

Here we demonstrate that GAG-containing biohybrid hydrogels support HSPC expansion and maintenance through sustained delivery of cytokines reflected by the superior cell viability and cell proliferation in soft 3D and on 2D scaffolds compared to TCP. Importantly, even in the stiff hydrogel scaffolds, the viability of the embedded cells was superior to standard TCP cultures, which is in contrast with previous reports where HSPC responded to stiff substrates with decreased viability[41]. Furthermore, our results clearly demonstrate that the hydrogels can support HSPC culture under overall low cytokine concentrations, which is most likely achieved by obtaining a high local concentration of active cytokines released from the GAGs in the vicinity of the cultured cells. High local cytokine concentration is envisioned to maximize LTC-IC expansion through maintenance of growth factor-receptor signaling at a critical threshold level[42]. Through the sustained delivery of growth factors in accordance to dynamic needs of the embedded cells and maintenance of high local concentration, the hydrogel cultures resemble the autoregulative BM stromal environment and might thus provide more physiological conditions for HSC maintenance in comparison to conventional cultures with soluble cytokines.

To contribute to the deciphering of putative mechanisms of biophysical and biochemical signals on HSPC fate decisions, we systematically increased the hydrogel stiffness of the 3D HSPC culture. Our results show that HSPC expansion and maintenance can be controlled in the 3D hydrogel culture system by varying the stiffness of the matrices. We observed reduced cycling and increased quiescence as a result of the increased stiffness of the hydrogel, and thus the increased spatial confinement of the embedded cells, while keeping the local concentrations of supplied growth factors constant. Remarkably these observations were observed for both GAG types used as hydrogel building blocks (HEP and 6ON-DSH), albeit were more pronounced in HEP-containing hydrogels.

Several studies have suggested that HSC can sense and respond to changes in mechanical properties of their surroundings[41,43,44]. A study by Holst *et al.* suggested that the response of HSC to the substrate elasticity is adhesion-independent, however, transduction of mechanical signals involves the actin-myosin cytoskeleton machinery[43] known to influence stem cell fate decisions[45–48]. Furthermore, the correlation of cytoskeletal dynamics in cell shape and polarity of HSC has been demonstrated[49]. The hydrogel matrices used in this study were not functionalized with specific adhesion ligands (e.g. RGD[50]), and were

therefore almost non-adhesive, however transduction of mechanical signals through confinement of actin cytoskeleton remains a possibility to explain the observed correlation of differences in cell shape between 2D and 3D cultures and the corresponding reduction of cell cycling (i.e. from 2D to 3D, and furthermore in 3D with increasing stiffness). The absence of the impact of substrate stiffness on 2D matrices and TCP further suggests that spatial confinement rather than the material stiffness was the primary cause of the reduced cell cycling in our study. Although, it should be emphasized that HSPC in 2D culture are mostly in suspension and have a relatively limited direct contact with the substrates on non-adhesive surfaces.

Besides cell shape, differential mode of cell-secreted factor signaling emerges as another important aspect which influences pluripotency, self-renewal and proliferation of stem cells upon spatial confinement[26,51]. Interestingly, encapsulation-mediated promotion of pluripotency of iPSC and ESC has been shown to be dependent on cell density, suggesting the role of the synergistic interplay between paracrine signaling and spatial confinement[22,23]. Notably, the increase of maintenance with increased spatial constraints in stiff hydrogels corresponds well with our previous studies with application of bioengineered microcavity arrays, demonstrating single cell cavities to support HSPC maintenance and functionality best[15,25,26]. Based on these reports, we hypothesize that the mechanical constraints of the stiff hydrogels might prevent the growth of proliferative clusters and thus maintain HSPC in a more quiescent state through synergistic action of spatial confinement of the cells and reduced paracrine and juxtacrine signaling among progenitors in the proliferative clusters.

Finally, LTC-IC experiments demonstrated that spatial constraints not only induce quiescence, but more importantly, can maintain LTC-IC, as the highest numbers of LTC-IC were obtained after the culture in the stiff hydrogels compared to the soft matrices, or 2D and TCP cultures. In contrast to our findings on expansion and quiescence, which revealed only small differences between HEP and 6ON-DSH, we found that HEP containing hydrogels performed better than 6ON-DSH counterparts. Notably, we saw superior maintenance of LTC-IC hydrogels containing fully sulfated heparin compared to the desulfated counterpart, which was most pronounced in the stiff hydrogels. Remarkably, after the period of culture in stiff HEP but not in the 6ON-DSH hydrogels, LTC-IC functionality was maintained at almost the same levels compared to non-cultured HSPC. Thus, our results indicate that besides the stiffness, the degree of the GAG sulfation can contribute to LTC-IC maintenance. HEP has been recently demonstrated to scavenge HSPC-secreted factors involved in HSPC fate decisions, such as IL8, ANG-2, GM-CSF, HGF, and PECAM among others, and thereby

contribute to HSC maintenance by modulating autocrine and paracrine signaling[26]. Therefore, it can be suggested that the highly sulfated heparin further supports HSPC maintenance in the stiff 3D culture by sequestering a multitude of cell-secreted signaling molecules.

Fig. 4 Dependence of HSC maintenance on dimensionality, stiffness, and heparin. While HSPC do not respond to changes in stiffness on 2D matrices, in 3D the cells form smaller and less proliferative clusters in stiff cultures, compared to the soft counterparts. Sulfation of heparin has further influence on maintenance of HSPC in the stiff hydrogel matrices, with sulfated heparin showing superior maintenance capacity.

In summary, the established biohybrid hydrogel culture represents a novel tool for long-term maintenance of HSC by enabling the control of cell cycling through independent adjustment of biophysical cues and providing an optimal cytokine environment through the negatively charged heparin component. Our results suggest that the maintenance of HSC in the 3D hydrogel culture is achieved by a synergistic interplay of spatial cell confinement, paracrine signaling, GAG-mediated scavenging of cell secreted factors and high local cytokine concentration. To our knowledge, the model described herein is the first example of the independent modulation of biochemical and biophysical cues through a materials platform to gain a high degree of control on the outcome of long-term human *ex vivo* HSPC culture. Furthermore, the established culture model may be used to further dissect mechanisms of HSPC mechanobiology as well as to further optimize the *ex vivo* culture of HSPC for extending the options of clinical transplantation.

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