Space constraints govern fate of hematopoietic stem and progenitor cells *in vitro*

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Abstract

Deciphering exogenous cues that determine stem cell fate decisions is a persisting challenge of cell biology and bioengineering. In an effort to unravel the role of spatial constraints in the cell-instructive characteristics of bone marrow microenvironments, murine hematopoietic stem and progenitor cells (HSPC) were exposed to fibronectin-coated microcavities *in vitro*. Microcavity sizes were chosen to allow for the inclusion of either individual or multiple cells. Repopulation experiments using lethally irradiated mice showed that the maintenance of functional HSPC in culture critically depends on cavity dimensions. Short-term repopulating hematopoietic stem cells (ST-HSC) were found to be best supported within single-cell sized compartments while long-term repopulating HSC (LT-HSC) were maintained within both cavity sizes. In sum, the reported data reveal spatial restriction to be a simple but powerful means for directing HSPC fate *ex vivo*.

Key Words: ECM, In vivo test, Microstructure, Stem cell

1. Introduction

Hematopoietic stem and progenitor cells (HSPC) of adult mammalian organisms are known to reseed in a specialized microenvironment within the bone marrow referred to as *stem cell niche* [1]. This microenvironment is made up of various cell types, biopolymers of the extracellular matrix (ECM) and signaling molecules, tightly regulating life-long hematopoiesis through a balance of HSPC self-renewal, differentiation, dormancy and apoptosis [2]. Although the hematopoietic system is studied already for more than four decades, our knowledge about the control of HSPC function within their niche(s) is still very limited and effective methods to maintain and expand HSPC for applications in regenerative medicine are lacking [3,4].

Microenvironmental cues critical for HSPC maintenance in the bone marrow (BM) consist of specific receptor interactions with neighboring cells (e.g. cadherins) and matrix structures (e.g. osteopontin, heparan sulfate, fibronectin (FN)) as well as soluble cytokines (e.g. stromal cell derived factor 1, stem cell factor) and metabolic parameters (e.g. oxygen, glucose) [5–21]. However, not only the presence of any of these signals but further details including concentration levels and gradients, ligand clustering and synergistic action of signaling events need to be carefully considered for the control of HSPC fate [20,22–24]. Accordingly, spatial proximity of HSPC is likely to be an important parameter in the regulation of paracrine and autocrine signals [25–27].

Although *in vivo* models for investigating HSPC niche function are advantageous by comprising the full complexity of the BM microenvironment [20], the related approaches hardly allow for modulating and dissecting individual parameters. Therefore, *in vitro* models can be instrumental to selectively address specific niche parameters [28–30]. Beyond that, recapitulating signals of the BM niche *in vitro* can be used for modulating HSPC for therapeutic purposes. In related studies, adhesive interactions, presentation of immobilized cytokines, availability of cell-cell receptors and receptor ligand clustering were shown to trigger HSPC fate in culture. Specifically, we recently demonstrated surface immobilized FN to result in a more dormant state of human HSPC (hHSPC). Spatial restriction of individual hHSPC within FN coated microcavities was observed to better maintain a CD34+ and CD133+ phenotype. However, as surface markers are only of limited indicative value [31], functional *in vivo* repopulation experiments are required to prove the potential of HSPC populations obtained after exposure to those conditions.

Thus, competitive repopulation studies in mice, representing the 'gold standard' in the analysis of HSPC functionality were applied within this study to evaluate the stemness of cells after *in vitro* culture in adhesive single-cell and multi-cell microcavities, respectively. The obtained data for the first time allowed to conclude on the role of spatial constraints and adhesive interactions for HSPC maintenance *in vitro*.

2. Materials and methods

2.1 Fabrication of FN functionalized PDMS culture carriers

Fabrication of PDMS microcavity arrays and following functionalization with FN or heparin was performed as described previously and exemplary illustrated for FN in Fig.1 [32,33]. In brief, silicon masters were designed with symmetric arrays of pillars using photolithographic etching (GeSiM, Rossendorf, Germany) and were fluorosilanized via gas phase with (heptadecafluoro-1,1,2,2-tetrahydrodecyl)-dimethylchlorosilane (ABCR) prior to PDMS

casting. Microcavity cell culture carriers were created by replication of thin films of PDMS (Sylgard 184 silicone elastomer kit, Dow Corning, Wiesbaden, Germany) from the microstructured silicon wafers with a diameter of 15 or 40 μm , a height of 10 μm , and an intercolumniation of 3 μm . After curing for 2 h at 120 °C, PDMS replicas were carefully peeled off and the unreacted contents of polymer or initiator were removed by swelling in heptane (Merck, Germany) for 24 hours. Subsequently, heptane was entirely removed by storage of the PDMS replicas for 24 hours in a vacuum oven at 60 °C. PDMS microwell arrays were punched with a 11 mm punch press and glued on top of cleaned glass slides ($\varnothing$ 11 mm). To functionalize the surface of the PDMS microcavities with thin copolymer films, PDMS surfaces were treated with medium pressure oxygen plasma (PDC-002, Harrick Plasma, NY, USA) for 60 s and incubated in a solution of 20 mM 3-aminopropyl-triethoxysilane (ABCR) dissolved in ethanol (Sigma-Aldrich) for 2 h. Subsequently PDMS molds were washed once in ethanol and finally annealed for 1 h at 120 °C. Films of poly(ethylene-*alt*-maleic anhydride) (PEMA) (MW 125000, Sigma-Aldrich) were produced by adding 40 μL of a 0.3_{wt}% solution of PEMA dissolved in ethanol on the amino-functionalized surface. The polymer was covalently linked to the surface by annealing for 2 h at 120 °C and the excess of PEMA removed by autoclaving in MilliQ for 20 min at 120 °C (autoclave system, DX23, Systec, Wetzlar, Germany). Finally, FN (Roche, Germany) was covalently immobilized to PEMA coated PDMS carriers at a concentration of 40 $\mu\text{g mL}^{-1}$. Therefore the anhydride moieties on the surface of PEMA coated PDMS carriers were regenerated for 2 h at 120 °C and incubated for 1 h in a solution of FN dissolved in phosphate buffered saline (PBS) (Sigma-Aldrich).

Covalent immobilization of heparin was enabled by modification of recycled anhydride moieties with amine moieties by incubation for 4 min in a 0.1 M 1,4-diaminobutane (Fluka, Munich, Germany) solution in isopropanol. Subsequently the surfaces were rinsed in isopropanol, deionized water, 0.01 M hydrochloric acid (Sigma), and finally once more in deionized water. Afterwards amide bonds were converted into imide groups by heating at 120 °C for 1 h. Subsequent aminofunctionalized surfaces were incubated for 4 h in 1 mg mL^{-1} heparin solution (MW14000, Merck, Darmstadt, Germany) in 0.1 M borate-buffer, pH 8, containing 500 mM N-(3-dimethylaminopropyl)-n-ethylcarbodiimide hydrochloride (Sigma). Non-covalently bound heparin was removed by rinsing in deionized water.

Afterwards, both sets of microcavity carriers were rinsed three times with sterile PBS and incubated in StemSpan™ (StemCell Technologies, Grenoble, France) medium prior culture.

2.2. Mice

C57BL/6 (B6), and B6.SJL-Ptprc^a Pep3^b/BoyJ (B6.SJL) mice were purchased (The Jackson Laboratory) and bred and maintained under specific pathogen-free conditions in the animal facility at the Medical Theoretical Center of the Technische Universität Dresden. Experiments were performed in accordance with German animal welfare legislation, and were approved by the relevant authorities, the Landesdirektion Dresden.

2.3. Cell culture of lineage negative bone marrow cells

Murine BM cells were isolated from femura and tibia of 6 week old mice and lineage negative (Lin-) cells prepared using magnetic microbeads (Miltenyi Biotec) and a MACS® LD column (Miltenyi Biotec) in combination with lineage antibodies (clones in brackets) CD3-bio(145-2C11), CD19-bio(eBio1D3), NK1.1-bio(PK136), Ter119-bio(TER-119), CD11b-bio(M1/70), Gr1-bio(RB6-8C5), B220-bio(RA3-6B2) (all eBioscience, San Diego, CA, USA). To avoid unspecific binding of the antibodies to the cell surface additionally Fc block CD16/32 pure

(eBioscience) and rat IgG (Dianova, Germany) were added. The derived Lin⁻ mouse BM cells were cultured TCP (control, 48 suspension well plate) and FN coated PDMS microcavity arrays with cavity diameters of 15 or 40 μm . The culture medium consisted at 50% of StemSpan™ and at 50% of hAngptl2 enriched StemSpan™ medium. Furthermore, the medium was supplemented with 10 $\mu\text{g ml}^{-1}$ murine heparin (5000 U ml^{-1}) (Biochrom AG, Berlin, Germany), 10 ng ml^{-1} of each mTPO, mSCF, mFGF-1, and 20 ng ml^{-1} mIGF-2 (all R&D). The medium was partly exchanged after 3 d (-150 μL and +400 μL), 5 d, and 6 d (both: -400 μL and +400 μL). After 7 d of culture, cells were detached using accutase® (Thermo Electron Cooperation, Waltham, Australia) (10 min at 37 °C), washed and after resuspension counted and analyzed for CD117 and Sca-1 expression.

2.4. Transplantation

For **competitive repopulation** cells from 10 culture carriers per condition were pooled and competitively transplanted into 8 weeks old recipient mice (Harlan® Laboratories, Venray, Netherlands). Therefore, three 3 recipient mice per condition were lethally irradiated (8.5 Gy, X-ray source: MaxiShot, Yxlon International GMBH, Hamburg, Germany) and a mixture of 1×10^6 culture derived cells and 5×10^5 total BM competitor cells were transplanted. The contribution of donor cells to blood neutrophils was analyzed in approx. 4 week intervals over 21 weeks.

CFU-S₈: Three recipient mice (Harlan®) per condition were irradiated (8.5 Gy) and 5×10^4 cells, derived from two pooled surfaces from culture, were retro-orbital injected in each mouse. After 8 d the mice were sacrificed and the spleen was removed and fixed in Bouin's solution (Sigma-Aldrich). Finally, the numbers of macroscopically visible spleen colonies (CFU-S₈) were counted. The mice were kept under specific pathogen-free conditions in the animal facility at the Medical Theoretical Center of the TU Dresden and neomycin was added to the drinking water (3 weeks, 1.17 g L^{-1} , Sigma-Aldrich) to prevent infections.

2.5. Flow Cytometry

Cell samples from the lineage depletion and cell culture were analyzed with a LSR II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The contribution of donor cells to blood neutrophils was measured with a FACS Calibur flow cytometer (Becton Dickinson). Overall, after blocking (CD16/32 and rat IgG) the cells were stained in PBS/5% (vol/vol) FCS using the following antibodies (clones in brackets): CD117-APC(2B8), Sca-1-PE(D7), Gr-1-PerCP-Cy5(RB6-8C5), CD11b-PE(M1/70), CD45.1-APC(A20) and CD45.2-FITC(104). Lin⁻ cells were identified by lack of CD3, CD11b, CD19, CD45R, Gr-1, Nk1.1, and Ter119 surface marker expression. Dead cells were excluded using DAPI (Life Technologies, USA) (1 $\mu\text{g mL}^{-1}$). Obtained data were analyzed and processed using the software FlowJo (FlowJo software, Tree Star Inc., Ashland, OR, USA).

2.6. Statistical Analysis

All data were analyzed using either GraphPad Prism 6 (GraphPad Software Inc., USA) or the open source software R (<http://www.r-project.org/>). Analysis of the statistical differences between two samples was performed either by one-way ANOVA or two-way ANOVA (PMN contribution) and Tukey-Kramer's post hoc test. Asterisk denote statistical significance as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. All data are presented as mean values $\pm$ standard deviation.

3. Results

3.1. Proliferation and differentiation of HSPC in FN coated microcavities

As human CD133⁺ HSPC were previously observed to exhibit altered cycling in adhesive microcavities hosting individual cells (15 μ m diameter), we herein first asked whether and how murine bone marrow (BM) cells similarly respond to such culture conditions [28].

Sets of poly(dimethyl siloxane) (PDMS) culture carriers containing 10 μ m deep cavities with dense hexagonal packing and diameters of 15 and 40 μ m were reliably replicated by soft-lithography, functionalized with thin layers of maleic anhydride copolymers, and homogenously coated with FN (or heparin) as reported by Franke *et al.*[32] (Fig.1A). Murine immature hematopoietic cells lacking the expression of antigens indicative for a mature cell lineage, designated as lineage negative (Lin⁻) HSPC, were cultivated for a period of 7 days on top of the functionalized culture carriers (Fig.2A). As the population of CD117⁺ (Kit), Sca-1⁺, and Lin⁻ (KSL) cells is known to contain the main HSPC fraction, total cell numbers and the KSL fraction were evaluated by flow cytometry.

Fig.1: Preparation of adhesive microcavity arrays and setup for HSPC *in vitro* culture & repopulation experiments. (A) 1) Schematic view of a PDMS microcavity array (grey) functionalized with thin films of reactive polymer (blue) and FN (green). 2) Surface chemistry used for FN immobilization. FN was covalently linked to maleic acid copolymer layers, which in turn were immobilized via imide bonds to aminosilane functionalized PDMS surfaces. (B) Confocal laser scanning microscopy (CLSM) image of a PDMS micropattern, demonstrating the homogeneous immobilization of FN (green). (C) Scanning electron microscopy (SEM) image of a PDMS microarray containing wells in a dense hexagonal arrangement. (D) Lin⁻ cells were isolated and cultured in serum free medium on the PDMS-FN microcavities during 7 days, 1×10^6 cultured cells (CD45.1) were injected into lethally irradiated recipients (CD45.2) with 5×10^5 total bone marrow cells (CD45.1xCD45.2), peripheral blood chimerism was analyzed by flow cytometry.

Significant expansion rates (≥ 3 -fold) and cell vitalities $> 97\%$ were obtained for HSPC grown on the microcavity arrays as well as on control carriers (tissue culture plastic (TCP) and heparin coated microcavity arrays) (Fig.2). The highest expansion rate was obtained for cells cultured on top of FN coated microcavity arrays containing cavities of $40\ \mu\text{m}$ in diameter. In contrast expansion rates of cells grown on heparin coated microcavity arrays were independent of spatial constraints and similar to TCP (Fig.SI1A). Analyzing the KSL subset at day 7 we observed a drastic, about 3-fold, increase of the KSL fraction of cells grown on FN functionalized microcavity arrays and an up to 6-fold increase on TCP. No influence of cavity size on the frequency of KSL cells was detected. These findings may imply that although adhesion to FN supports proliferation of Lin⁻ cells, HSPC on FN are more quiescent than on TCP.

Fig.2: Expansion and CD marker expression of murine BM derived Lin⁻ cells on day 7. (A) HSPC cultivated for 2 d on top of FN coated microcavity arrays with cavities of 15 (left) and $40\ \mu\text{m}$ (right) diameter (B) Overall cell numbers of murine BM derived Lin⁻ cells cultivated for 7 d on top of TCP or FN coated PDMS microcavity arrays with various diameters. (C) Plots show the gating strategy for the cytometry analysis of the HSPC population before (left) and after (right) 7 days of culture. (D) Percentage of cells from the KSL subset in the total cell population. All data are presented as mean $\pm$ SD from $n = 7$ per condition derived from four donors.

3.2. Repopulation studies using HSPC after culture on FN coated microcavity arrays

Next we analyzed the repopulation efficacy of the *in vitro* cultured HSPC. Adherent cells were detached from the microcavity arrays on day 7 of the cultures. Cell numbers and KSL fraction were determined and 1×10^6 of the whole harvested population combined with 5×10^5 freshly isolated Lin⁻ competitor cells were transplanted into lethally irradiated wild type recipient mice. Cells from up to 10 individual microcavity arrays were pooled and transplanted into three individual recipients. The success of the repopulation was monitored over a time period of up to 162 days by analyzing the relative contribution of donor cells to short-lived polymorphonuclear neutrophils (PMN) using flow cytometry. As PMN have a high turn-over rate donor cell contribution to this compartment is indicative for the donor cell contribution to the hematopoietic stem cell compartment.

Cells grown in contact with FN coated microcavity arrays were found to repopulate significantly better than TCP controls with respect to their contribution to hematopoiesis in recipient mice in the long term (Fig.3B). During the first 28 days after transplantation cells that had been exposed to small cavities in culture showed a significantly higher relative contribution to PMN compared to cells grown in large cavities, reflecting the effective reconstitution of the HSPC compartment by donor cells [34]. Controls of cells grown on TCP or heparin coated microcavity arrays showed similar, cavity independent, and the smallest contribution to the repopulation (Fig.3B-C, Fig.S11B). These findings indicate an influence of both surface coating and cavity size on the short-term engraftment efficiencies. Monitoring the relative PMN contribution over the following weeks we observed an improved donor contribution if the donor cells were grown on FN functionalized microcavities in comparison to TCP and heparin functionalized microcavities. However, cavity size had no strong impact on long-term HSC (LT-HSC), as no differences between the contribution of cells cultured on FN (or heparin) functionalized arrays of 15 μm and 40 μm diameter microcavities was detected after a period of 56 days (Fig.3C, Fig.S11B). Taking into account that 1×10^6 cells were transplanted as harvested from culture, irrespective of their differentiation status, the total number of transplanted KSL cells was drastically lower for FN coated PDMS microcavities of both diameters (approx. 1.4×10^5) in comparison to TCP (approx. 2.7×10^5). Considering these facts, FN functionalization and size of microcavities show significant effects on repopulation (Fig.3C). Since the effect of the small cavities was most pronounced at early time points, we determined in an additional experiment the ability of progenitor cells to give rise to macroscopic spleen colonies after transplantation into irradiated recipient mice (CFU-S₈) [35], testing the number of spleen colonies 8 days after transplantation [36]. For that purpose, cells were collected after 7 days of culture on FN coated microcavities or TCP and transplanted into irradiated recipients. 8 days after transplantation the number of visible surface colonies in the spleen was evaluated (Fig.3D). Cells cultured for 7 days *ex vivo* were able to form macroscopically visible colonies in the spleen. The number of CFU-S₈ was significantly lower if cells were cultivated on 15 μm cavities compared with 40 μm cavities and TCP. The spleen colony assay supports the data from transplantation experiments, suggesting that small cavities better maintain primitive precursors but not multipotent (or no description) progenitors without CFU-S₈ activity.

4. Discussion

In an effort to further dissect the cell-instructive characteristics of BM niches, we set out to explore the role of spatial constraints created by adhesive microenvironments in controlling HSPC fate. For that purpose, a set of defined FN and heparin coated microcavity arrays was prepared and applied for HSPC cultures, which were subsequently evaluated by *in vivo* repopulation studies in mice.

Our data show that murine HSPC expansion can be controlled by exposure to FN, but not heparin, coated microcavities above or below a particular size as previously observed for CD133+ human HSPC in culture [28]. The expansion rates of murine Lin- cells on FN coated surfaces were found to be higher than earlier reported for human CD133+ HSPC [28]. However, while human CD133+ cells and murine KSL cells originate from two different species and differ in composition, both subsets contain LT-HSC and ST-HSC, which are indispensable for successful repopulation of a depleted hematopoietic system. Thus, similar mechanisms can be reasonably assumed to be involved in the decreased proliferative activity of HSPC constrained in smaller microcavities.

Fig.3: Competitive repopulation and colony forming ability in spleen determined to assess HSPC functionality after 7 days of culture. (A) Plots show the gating strategy for the cytometry analysis of the contribution of donor cells in PMN. (B) Murine Lin⁻ cells (CD45.1) grown on top of FN functionalized 15 μ m or 40 μ m sized cavities and TCP for 7 days were collected and 10^6 test cells together with 5×10^5 freshly isolated competitor BM cells (CD45.1xCD45.2) transplanted into each lethally irradiated recipient mouse (CD45.2). The relative contribution of donor cells to PMN of the peripheral blood was analyzed via cytometry. Results are presented as mean $\pm$ SD from 8 or 9 mice (B):1,2,3). (C) Relative PMN contribution of donor cells 28 days and 56 days after transplantation into lethally irradiated recipients (left). Results of relative PMN contribution of donor cells set into relation to transplanted KSL cells (right). Results are presented as mean $\pm$ SD of data obtained from three mice and three independent experiments (see (b):1,2,3). (D) 5×10^4 cells collected after 7 days of culture on top of ECM functionalized culture carriers or TCP were transplanted into lethally irradiated recipients. The numbers of macroscopically visible colonies in the spleen were counted 8 days after transplantation (colony forming unit-spleen day 8, CFU-S₈). Results are presented as mean $\pm$ SD of $n \leq 3$ mice per condition.

Repopulation experiments demonstrated the functionality of HSPC cultured on FN coated microcavity arrays in terms of their ability to contribute to the formation of PMN in the recipient [19]. Although the total KSL number of HSPC was lower after culture on either type of FN coated carrier compared to TCP we found a drastically improved repopulation potential of these cells, confirming the supportive effect of adhesive interactions in the maintenance of HSPC *in vitro*. Furthermore, our data suggest that enhanced adhesion of the cells in small microcavities favors ST-HSC over LT-HSC. For cells grown under these conditions, we obtained the highest contribution to PMN at 28 days after transplantation, in agreement with reduced lodgment to the spleen.

Improved homing and engraftment of HSPC cultured on adhesive microcavity arrays is concluded to be triggered by the expression of high levels of the integrins VLA-4 and VLA-5. Several previous studies have documented that the integrin superfamily of cell adhesion

molecules are regulators of adhesion, engraftment and migration [37–40]. It was furthermore reported that maintenance of the regenerative capacity of HSPC during *ex vivo* culture can be improved if HSPC are cultured in presence of immobilized FN [41,42]. Results of *in vitro* experiments with a murine hematopoietic progenitor cell line (FDCP-mix) and KSL cells suggested that pre-exposure to FN was necessary for cytokine induced migration [43]. Recently, the role of adhesion molecules VLA-4 and CD44 in homing and release to BM and spleen was analyzed, demonstrating that CD44 is involved in CFU-S lodging in both BM and spleen, but VLA-4 in BM lodgment only [38]. In addition, integrins such as α_2 , α_6 , β_3 were successfully used to identify and isolate LT-HSC, indicating a distinct change in the integrin expression pattern and cell-matrix interactions in dependence of the HSC subset.[44–46]

Hence, culture of HSPC on small, FN coated cavities does obviously promote VLA-4 expression, especially in ST-HSC, due to spatially maximized exposure of the cells to immobilized FN, likely resulting in a preference of the VLA-4/VCAM-1 adhesion pathway *in vivo*. In line with this, these cells show a clear preference to relocate after transplantation in the BM rather than the spleen. Additionally, ST-HSC are promoted over LT-HSC due to the differences in integrin expression pattern in this situation, too.

Taken together, our data suggest that *ex vivo* cultivation of HSPC on small, adhesive microcavities efficiently prevents differentiation into more committed progenitors selectively maintaining ST-HSC and LT-HSC. As demonstrated by this report, the developed set of cell-adhesive microcavity arrays provides a useful tool for studies on the role of spatial constraints and adhesive interactions in stem and progenitor cell fate control *in vitro*. Beyond that, it can be reasonably assumed that spatial constraints govern the balance of autocrine and paracrine signals of cells in culture. Within small cavities secreted factors are expected to accumulate more efficiently in contrast to large cavities, attenuating autocrine signaling pathways over paracrine ones. In addition adsorption of factors to the culture carrier followed by intense interactions between the attached factors and cells is assumed to be more likely under limited spatial constraints. The introduced format of culture carriers may therefore allow for modulating another important aspect of the microenvironmental control of HSPC in a defined *in vitro* setting. Ongoing experiments aim at exploring this option.

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