Hematopoietic Stem Cell Differentiation inside Extracellular Matrix Functionalized Microcavities

(Differenzierung von Hämatopoietischen Stammzellen in Extrazellulärmatrix-Mikrokavitäten)

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Kurzbeschreibung


Um die poröse Struktur des Knochenmarks in vivo-ähnlich darzustellen, wurde eine Zellkultur Plattform mit Mikrokavitäten verschiedener Dimensionen von Multi- bis Einzelzellgröße entwickelt und mit Molekülen der extrazellulären Matrix beschichtet. Die Vorteile dieser Plattform liegen in der offenen 3D-Geometrie dieses mikrokavitäten Kultursystems, die den Zellen ermögliche verschiedene Wachstumsbedingungen bezüglich Homing, Migration, Adhäsion oder Suspension frei zu erkunden. Das leicht zugängliche Setup eignete sich zudem hervorragend für die zytometrische Analyse der Zellen oder die quantitative Mikroskopie.


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1 CD34 und CD133
2 Niedrige Zytokinkonzentration: 10 ng jeweils, hohe Zytokinkonzentration: 30 ng je TPO, SCF, FL3
bestätigen ferner obenstehende These, dass Zytokin-induzierte Zellexpansion durch erhöhte Zelladhäsions-vermittelte Signale überschrieben wird.


Abstract

The bone marrow (BM) niche provides hematopoietic stem (HSC) and progenitor cells with many exogenous cues that tightly regulate homeostasis. These cues orchestrate cellular decisions, which are difficult to dissect and analyze in vivo. This thesis introduces a novel in vitro platform that permits systematic studies of BM-relevant factors that regulate homeostasis. Specifically, the role of 3D patterned adhesion ligands and soluble cytokines were studied in a combinatorial fashion. Analysis of human HSC differentiation and proliferation at both population and single cell level showed synergistic and antagonistic effects of adhesion- and cytokine-related signals. Those effects were dependent on the cytokine concentration and the distribution and number of adhesion ligands.

The aim of this thesis was to model the in vivo bone marrow with its porous 3D structure and different sized niche compartments using a microcavity culture carrier. The developed culture system presented extracellular matrix (ECM) adhesion ligands to the HSCs in various defined dimensions ranging from single- to multi-cell capacity. The 3D open well geometry of the microcavity carriers also allowed HSCs to freely explore different scenarios including homing, migration, adhesion, or suspension. Furthermore, the developed setup offered straightforward accessibility to analytical methods like cytometry and quantitative microscopy.

Single cell analysis of adherent HSCs showed decreased DNA synthesis and higher levels of stem cell marker expression within single cell microcavities under low cytokine conditions. This effect was reflected in a decline of proliferation and differentiation with decreasing microcavity size. When the cytokine concentration was increased beyond physiological levels the inhibitory effect on proliferation and differentiation due to single-cell-microcavity adherence was diminished. This result highlighted the fine balance between adhesion related and soluble cues regulating HSC fate. Within small microcavities more adhesion related receptors were engaged due to the 3D character of the culture carrier compared to multi-cell wells or conventional 2D cell culture plates. This study demonstrated that adhesion-related signal activation leads to reduced proliferation and differentiation. This geometry-based effect could be reversed by increased cytokine supplementation in the culture media. For plane substrates, HSCs attachment to fibronectin or heparin initiated early cell cycle entry compared to non-adherent cells during the initial 24 h. Cytokine supplemented media favored integrin activation that induced fast adhesion, ultimately leading to early cell cycle activation. However, after prolonged cell culture the system balanced itself with a lower cycling rate of adherent versus non-adherent HSCs. Furthermore, HSCs within the 3-dimensionality of the microcavities cycled less than 2D adherent cells. These findings additionally supported the above stated idea of limited HSC proliferation as a consequence of more adhesion-related signals overwriting cytokine driven expansion.

To complement the various in vitro studies, an in vivo repopulation study was performed. Cultured HSCs derived from single cell microcavities outperformed freshly isolated HSCs in a competitive repopulation assay, indicating that carefully engineered substrates are capable of preserving stem cell potential.

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3 Namely CD34 and CD133
4 Low cytokine conditions: 10 ng each; High cytokine conditions: 30 ng each TPO, SCF, and FL3
Overall the reported findings provide a promising *in vitro* culture strategy that allows the stem cell field to gain a better understanding of the impact of distinct exogenous signals on human HSCs, which discloses new concepts for the wide scientific community working towards tissue engineering and regenerative medicine.
1 Introduction

The unique properties of stem cells – self-renewal and differentiation into tissue specific cells - offer great potentials for regenerative therapies in medicine. Stem cells are distinguished by their regenerative potential between the all-rounder embryonic stem cells that are able to form all cell types and tissue specific adult stem cells. Regeneration after injury and tissue renewal e.g. daily blood and intestinal cell formation requires functional adult stem cells. Studies of these cells enable the identification of the essential properties yielding therapeutic potential unlike specialized cell types. One of the best studied adult stem cell populations are blood forming hematopoietic stem and progenitor cells (HSC). Elucidation of the regulation of these stem cells is expected to aid the understanding of developmental and repair mechanisms in tissues, which are crucial for essential medical procedures such as bone marrow transplantation.

1.1 Motivation

The main implications for bone marrow transplantation are various hematopoietic cancers, such as leukemia and lymphomas, or the high dose chemotherapy for cancers in other organs, typically responsible for the eradication of the native blood-forming environment. Further indications are genetic or acquired bone marrow failure, e.g. aplastic anemia, thalassemia, sickle cell anemia, and autoimmune diseases. Therapeutic delivery of sufficient amounts of viable HSCs is of critical importance; therefore their successful in vitro expansion remains one of the major goals in contemporary hematopoietic research. The development of a culture platform for HSCs to control their fate and provide favorable conditions for expansion is expected to considerably improve the outcome of ongoing transplantation procedures.

1.2 Objective

Inside the bone marrow, a specialized microenvironment known as the 'stem cell niche' supports maintenance and maturation of HSC [1]. Interaction between HSCs and their niche involves the interplay of many factors, including extracellular matrix (ECM) molecules, cell–cell contacts, and cytokines. This interplay critically balances cell cycling and quiescence, leading to proliferation or apoptosis and self-renewal or differentiation [2, 3, 4], respectively. Due to this complexity of the in vivo HSC microenvironment the in vitro induction of HSC proliferation on a variety of supports mimicking the structural and functional characteristics of the bone marrow remains an intriguing conundrum. HSC expand in vivo, but they usually differentiate in vitro [5]. Even though HSC are the best-studied adult stem cells the reconstitution of the in vivo conditions into the cell culture dish has proved to be quite challenging by far [3].

Understanding the implication of distinct exogenous signals and their orchestration in HSC fate decisions is a prerequisite for successful tissue engineering strategies and improving clinically-applied cell replacement therapies. But stem cell properties and their fate are difficult to prove and track in vivo.
Therefore the aim of this work was to dissect the effect of geometrical constraints and adhesive interactions on HSCs during cytokine-driven expansion in an *in vitro* set-up. For this a set of culture carriers with defined micrometer-sized cavities that were 10 µm deep and 15 to 80 µm in diameter was prepared and functionalized with bone marrow relevant extracellular matrix (ECM) molecules. This setup is aimed to model the *in vivo* bone marrow HSC niche with its highly porous structure [6, 7, 8] and different sized niche compartments, which range from single to multi-cell capacity as this parameter is expected to have a high impact on HSC fate inside a possible stem cell niche.

![Figure 1 Niche Relations.](image)

The objective of this work is to gain insight into the relation of proliferation and differentiation with dependence on matrix adhesion and spatial constraints by providing experimental tools to analyze and control HSC fate decisions *in vitro*.

Human CD133+ HSCs from peripheral blood, which are early progenitors [9] with multilineage [10] and repopulative capacity [11], were cultured on these ECM-coated microcavity structured substrates. Analysis of HSC proliferation and differentiation at both population and single cell levels was applied to elucidate the interplay of spatial constraints, adhesive interactions, and cytokine concentrations on HSC fate decisions *in vitro* (Fig. 1).
2 Basics

Stem cells play a central role in an organism. They have the potential to develop into many different cell types during early life and growth. Additional stem cells serve as internal repair system for damaged tissue and replacement of worn-out tissue. Further on cancer may also arise from stem cells going into abnormal proliferation; therefore it is essential to elucidate their control mechanisms.

2.1 Stem Cells and their Role in Life

Stem cells are distinguished from other cells by two important features. They are unspecialized cells capable of self-renewal and they can be induced to differentiate. In some organs, e.g. bone marrow and gut, stem cells regularly divide to replace and repair tissue. In other organs, such as pancreas and heart, they only divide under special conditions. To accomplish self-renewal and/or differentiation stem cells use either symmetric or asymmetric division strategies [12]. Symmetric division is defined by the acquisition of the same fate by both daughter cells e.g. in the case of self-renewal. Asymmetric division features both stem cell characteristics by generating one daughter with stem cell fate and another one that differentiates [13]. It is believed that asymmetric cell division of HSCs coincides with primitive stem cell function [14]. Two main mechanisms govern asymmetric divisions by regulating the orientation of the mitotic spindle: The first ‘intrinsic’ mechanism is based on the asymmetric distribution of inner cell components, while the second ‘extrinsic’ mechanism involves the asymmetric exposure of daughter cells to external cues [12] (Fig. 2). However, the rare number of adult stem cells proves it difficult to technically verify actual asymmetric division via direct imaging.

Figure 2 Stem Cell Divisional Strategies. (A) Each stem cell (orange) can divide either symmetrically or asymmetrically. Intrinsic control of asymmetric division includes (B) the assembly of cell polarity factors or (C) the segregation of cell fate determinants to one daughter cell. These factors can be associated with the cell membrane, the cytoplasm, centrosome, or any other cell component distributed between the two daughter cells. (D) One daughter cell is retained in the stem-cell niche (red) and is maintained as stem cell by extrinsic cues whereas the other daughter differentiates (green). This may also function the other way around by exposing one daughter to differentiation inducing signals (modified after [12]).

Different kinds of stem cells are described and worked with in science: pluripotent embryonic stem cells (ESCs), non-embryonic multipotent ‘somatic’ or ‘adult’ stem cells, and induced pluripotent stem cells (iPSCs). The stem cell potency can be ordered hierarchically (Fig. 3):
Totipotent cells are able to build a fully viable organism by forming ESCs and the extraembryonic tissue of the blastocyst. Only cells of the first few divisions of a fertilized egg are totipotent.

ESCs are of pluripotent type and derived of the inner cell mass of the blastocyst at a stage before it would implant in the uterine wall, self-replicate and give rise to cells from all three germ layers (ectoderm, mesoderm, and endoderm) [15].

IPSCs are adult cells ‘reprogrammed’ genetically to enter an ESC-like state demonstrating all important characteristics of pluripotent stem cells, including the expression of stem cell markers, the formation of all three germ layers, and the ability to contribute to different tissues when injected into mouse embryos at an early stage in development [16, 17, 18].

**Figure 3 The Stem Cell Hierarchy.** The totipotent zygote formed by the fusion of egg and sperm divides to form inner cell mass (ICM) and extra embryonic tissue (EE) of the blastocyst. When isolated the cells of the ICM can be maintained in vitro as pluripotent embryonic stem cells. During development the ICM cells become increasingly restricted in their lineage potential and generate tissue specific multipotent stem cells [15].

Somatic or adult stem cells are undifferentiated cells found in differentiated tissue that are able to self-renew and generate the cell types of the respective tissue they reside [19]. However there are controversy debates that stem cells from one tissue may give rise to cell types of a complete different tissue [20], which is referred to as ‘plasticity’ [21, 22]. Sources of adult stem cells have been found in the bone marrow, blood stream, cornea and retina of the eye, the
dental pulp of the tooth, liver, skin, gastrointestinal tract, and pancreas. The primary functions of adult stem cells are to maintain steady state functioning of their respective tissue and replace cells that die because of injury. Adult stem cells are rare (e.g., 1 in 10,000 to 15,000 cells in the bone marrow is a hematopoietic stem cell HSC [23]) and in contrast to ESCs the exact origin of adult stem cells in any mature tissue remains unknown. Before they reach their fully differentiated state, stem cells generate intermediate cells which are called precursors or progenitor cells and already regarded as committed [24]. However, these cells are capable of undergoing cell division resulting in two different specialized cells. Once adult stem cells are removed from the body, their division capacity is limited and further complicating their expansion. Methods used for identification of stem cells are; (a) the labeling of cells and then to score the cell types they generated and (b) the determination of the repopulation capacity of their tissue of origin in another animal.

**Stem Cells and their Niches**

Small regions inside a given tissue are commonly assumed to maintain and control stem cell activity [13, 25]. These specialized microenvironments known as stem cell ‘niches’ can be defined as three-dimensional spatial structures in which one or several stem cells [26] can reside for an indefinite period of time and produce progeny cells while self-renewing [27]. Very recent models suggest separate but adjacent niche sites supporting the coexistence of either actively cycling or quiescent stem cell subpopulations [25].

Despite the growing knowledge, a lot of key questions in adult stem cell research still remain: What are the characteristics of the ‘niche’ that control their behavior in vivo [28]? And, how are stem cells influenced by these characteristics? Are stem cells instructed by special environmental cues to undergo certain fate decisions or are they already determined and only permit particular signals to influence them?

2.1.1 **Hematopoietic Stem Cells**

Hematopoietic stem cells (HSCs) are blood forming cells which were recognized more than 40 years ago [29]. They are capable of self-renewal and give rise to all kinds of blood cells in the body. One single HSC may regenerate the entire hematopoietic system of an organism [30]. HSC develop into two major progenitor lineages; common myeloid (CMP) and common lymphoid progenitors (CLP). CMPs again give either rise to megakaryocyte/erythroid progenitors developing into platelet producing megakaryocytes and erythrocytes or granulocyte/macrophage progenitors generating mast cells, neutrophils, eosinophils, and macrophages. CLPs will mature into B-lymphocytes, T-lymphocytes, and natural killer cells [31] (Fig. 4).

Within adults the majority of HSCs reside in the bone marrow (BM) with few cells constantly cycling through the peripheral blood. During development hematopoietic sites are also found in the aorta-gonad-mesonephros region, fetal liver, and neonatal BM [32]. For clinical purposes usually granulocyte-colony stimulating factor (G-CSF) mobilized HSCs out of the peripheral blood are used, since this is less invasive and less painful for the donor than harvesting bone marrow. Mobilized cells are passed through a device that separates out cells expressing CD34
on their surface and returns the remaining blood to the donor’s circulation. This procedure is known as apheresis.

Many combinations of surface markers have been used to identify, purify, and isolate HSCs derived from peripheral blood or bone marrow. Such markers can be tagged with monoclonal magnetic or fluorescently labeled antibodies to be selected out of the bone marrow or peripheral blood and detected via magnetic cell sorting (MACS) and/or fluorescence-activated cell sorting (FACS). Most important markers are presented below (Tab. 1). Lineage marker (lin) include 8 to 14 different monoclonal antibodies that recognize surface proteins present on differentiated hematopoietic lineages [33, 34].

Table 1 Surface marker expression of mouse and human HSCs summarized after [35, 36, 37].

<table>
<thead>
<tr>
<th>Mouse Surface Marker</th>
<th>Use</th>
<th>Human Surface Marker</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>lin-</td>
<td>present on differentiated hematopoietic cells</td>
<td>lin-</td>
<td>present on differentiated hematopoietic cells</td>
</tr>
<tr>
<td>Sca-1+</td>
<td>expressed by stem cells and progenitors, excludes myeloid-cell-biased progenitors</td>
<td>CD34+</td>
<td>expressed by hematopoietic stem/progenitor cells, endothelial cells</td>
</tr>
<tr>
<td>CD27-</td>
<td>member of TNF receptor family expressed by many progenitors</td>
<td>CD38-</td>
<td>expressed by myeloid, B- and T-cell progenitors, monocytes, plasma cells</td>
</tr>
<tr>
<td>CD34-</td>
<td>expressed by progenitors</td>
<td>CD45+</td>
<td>all hematopoietic cells</td>
</tr>
<tr>
<td>CD90.1 (Thy-1.1)+</td>
<td>lost at the multipotent progenitor state</td>
<td>CD90 (Thy1)</td>
<td>hematopoietic stem cells, T cells, fibroblasts, stromal cells</td>
</tr>
<tr>
<td>CD117 (KIT)+</td>
<td>expression levels determine immaturity of cells</td>
<td>CD109-</td>
<td>T cells, endothelial cells, and activated platelets</td>
</tr>
<tr>
<td>CD93 (AA4.1)</td>
<td>highly expressed by fetal HSCs and lymphoid progenitors</td>
<td>CD117+ (c-kit)</td>
<td>expressed by hematopoietic stem cells and progenitors</td>
</tr>
<tr>
<td>CD135 (FLT3, FLK2)</td>
<td>up regulated by short term HSCs, lymphoid biased cells</td>
<td>CD133+ (Prom-1)</td>
<td>hematopoietic stem cells, endothelial progenitor cells, neuronal- and glial stem cells</td>
</tr>
<tr>
<td>CD150 (SLAM)+</td>
<td>specifies long term HSCs if used with CD48- and CD41-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Typically, the combination of three to five surface markers recognizes functional stem cell activity and yields a higher purification of these. Murine hematopoietic stem cells are typically isolated for high expressions of stem-cell-antigen 1 (Sca-1) and CD117 (KIT) lacking lineage markers (lin) and are therefore called LSK (lin-, Sca-1+, KIT+) subset [2], which may be further purified using SLAM marker CD150 [38, 39] and the exclusion of CD34 and CD135 (FLT3) [40, 41]. For the purification of human HSCs marker combinations such as CD34+ [42], CD133+ [11], CD90+ [43] and the exclusion of CD38 [44] and lineage markers may be used [28]. As already mentioned, clinical use usually employs only CD34, which is a population enriched for HSC and progenitors but still contains other blood cells.

However, no definite HSC marker or marker combination has been found so far. Recent progress in genomic sequencing, genome wide analysis of RNA expression and protein levels, and their comparisons among various types of stem cells may be used to identify sets of genes defining ‘true’ stemness and specify different stem cell populations [28, 15].

Methods often used for stem cell identification in vivo are [28]: (1) Molecular markers within living tissue get labeled and the specialized cell types generated are determined. (2) Cells
removed from one animal are labeled and transplanted into another animal to ascertain whether they repopulate their tissue of origin. (3) The gold standard to prove stem cell identity are competitive repopulation experiments which assay long term reconstitution and thus self-renewal potential of putative stem cells [45]. Irradiated recipient mice are injected with a constant number of 'helper' bone marrow cells (usually recipient cells, just enough to ensure survival) mixed with gradually increasing number of donor HSCs of a distinct lymphocyte surface antigen (Ly) e.g. CD45 expressed by different alleles can be recognized by specific monoclonal antibodies which distinguish between the variants. Myeloid and lymphoid cells are identified after 8 to 10 weeks. The repopulation capacity of donor cells is assessed by the ratio of donor and recipient HSCs (distinguished via their Ly) and the number of implanted donor HSCs. PCR-markers, chromosomal markers, and enzyme markers may also be used to distinguish host and donor cells. Serial transplantation in which reconstituted HSC from the primary host are transplanted into a second irradiated mouse measures lifespan and expansion limits of the stem cells. (4) Immunologically-incompetent mice allow the testing of human cells in mice either with or without human fetal bone or thymus implants. Severe Combined Immunodeficiency (SCID) or Non-Obese Diabetic (NOD)-SCID mice serve as model animals. Frequently used in vitro assays include the Long-Term Culture-Initiating Cell assay (LTC-IC) and Cobble-stone Area Forming Cell (CAFC) assays. The LTC-IC measures whether HSCs still exist after five to seven weeks culture period which is proved afterwards by their colony forming capability (Fig. 12). The CAFC recognizes the ability of HSC to maintain underneath stromal cell layer in tissue culture over five to seven weeks easily detectable with light microscopy as a dark cobblestone-like patch. Tissue culture approaches are particularly convenient when working with human HSCs.

2.1.2 **Hematopoietic Stem Cell Niche**

Of all cell types in the body, blood cells survive for the shortest period of time [28]. Thus the life of an organism depends on their replenishment, which occurs in the bone marrow, where HSCs reside, self-renew, and differentiate into all the blood cell types (Fig. 4).

HSCs are constantly regenerated in the BM where they differentiate into all types of mature blood cells [1] (Fig. 4). Within the BM, the so called ‘hematopoietic stem cell niche’ is suggested to balance the homeostasis of HSCs by acting on homing, quiescence, and mobilization as well as regulating apoptosis, proliferation, and differentiation [4]. Many factors of the niche microenvironment interplay with HSC behavior such as extracellular matrix (ECM) molecules and soluble factors secreted by all cells residing within the bone marrow and cell-cell contacts of HSCs with stromal cells and among each other [46] (Fig. 5). BM stromal cells, which are discussed to be derived from mesenchymal stem cells (MSC) [47], are a mixed non-hematopoietic cell population forming bone, cartilage, fat, fibrous connective tissue, and the network that support HSC blood cell formation.

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5 Progenitors grow in culture for shorter times.
Inside the bone marrow two sets of stem cells reside: hematopoietic stem cells from which all red and white blood cells arise and mesenchymal stem cells which produce chondrocytes (cartilage), osteoblast (bone), adipocytes and skeletal muscle.

Two different sites are suggested and heavily discussed to contribute to distinct niches for HSCs within the BM: near the endosteum (endosteal niche) and around sinusoids (vascular niche) [2]. Sinusoids are reticular fenestrated venules that allow cells to pass in and out of circulation. There is evidence that individual dormant HSCs are located more in niches at the endosteum, whereas activated HSCs are in close contact to sinusoids of the BM microvasculature [26]. Further speculation is that, HSC simply pass through these sites during their constant circulation (see 2.1.3) [48, 49]. However, the influence of either niche site, stem cell number, or the possible action of other microenvironments on HSC maintenance and their precise bio-molecular composition is not fully discovered yet [50]. Different stromal cell populations are thought to contribute to either niche site with mainly osteoblasts and osteoclasts collaborating to the endosteal niche and endothelial cells, megacaryocytes, reticular cells, and mesenchymal progenitors governing the vascular niche [48]. Though, as already mentioned, the uncertainty about the ‘true’ stem cell phenotype and technical limitation of single cell imaging in vivo complicates the identification of supporting cells with which adult stem cells interact. There is evidence that osteoblasts are involved in niche formation: An increase in N-cadherin expressing spindleshaped osteoblasts correlates with an increase in HSC number. It was concluded that osteoblasts might support HSC through specific interactions between N-cadherin and β-catenin [51]. Also, increased Jagged-1 expression (osteoblast) and increased Notch activation (HSC) result in higher HSC number [52]. And, metabolic death of osteoblasts is followed by subsequent decrease of HSC [53]. But, otherwise in vivo conditional depletion of Notch receptor and/or its ligand Jagged-1 [54], or depletion of β-catenin [55] is not affecting HSC maintenance [48]. The chemoattractant stromal derived factor-1α (SDF-1α) proposed to regulate HSC maintenance and Angiopoietin-1 (Ang-1)
Basics

conducting HSC quiescence are both expressed by endosteal and perivascular cells [56, 57, 48]. Thus, both niche populations devise different but also similar factors that regulate HSC maintenance. High concentrations of calcium ions (Ca\(^{2+}\)) at the endosteal surface may also contribute to the enrichment of HSC at the endosteal niche, as it has been shown that mice deficient in the Ca\(^{2+}\) receptor (CaR) do not engraft in the BM [58]. Additionally, HSC lacking CaR showed impaired adhesion to the ECM protein collagen I (Col I). Besides cell-cell contacts, ECM components associated with either niche site regulate HSC functionality.

Very recent research provides evidence for an instructed lineage choice of HSCs meaning that cell functionality and differentiation are influenced by their surrounding environment [59]. However, it is still unclear how gene expression dynamics are generated and controlled by cell-cell or cell-matrix interactions [60]. An easy to access in vitro stem cell culture platform with discrete stem cell ‘niche’ factors as described above in chapter 1.1 would allow for long term observation of cells.

Figure 5 Stem Cell Niche Factors [61]. Inside the bone marrow a combination of distinct soluble and membrane bound factors and ECM molecules balance HSC homeostasis and regulate their divisional behavior. The biomolecular and physical impact of the ECM upon the interplay of proliferation and differentiation of HSCs is of particular interest within this work.

2.1.3 The ECM Relevancy

The ECM is crucial to mediate cell attachment, migration, and survival [62, 63], but also binds to growth factors and controls their diffusion [61]. The three-dimensional network of the bone marrow relevant ECM consists of stabilizing collagen fibers of collagen types I and IV, proteoglycans containing chondroitinsulfate (CS), heparan sulfate (HS), and hyaluronic acid (HA) [64, 65, 66], and non-collagen glycoproteins like fibronectin (FN) or laminin (LN) providing recognition sequences for cell adhesion receptors [67, 68]. Most of the data concerning the BM ECM composition were obtained from in vitro co-cultures of HSCs and stromal cells and the elution of the BM. Both techniques harbor the disadvantage of unknown localization inside the BM. A previous study shows the distribution of relevant ECM throughout the BM using immunofluorescence with Col I, Col IV, and FN localized to the endosteum and Col IV and LN associated with vessels [67]. Additionally, FN was also found within the central marrow region but not associated to the vasculature. The different allocation of ECM proteins supports the idea of diverse niches inside the BM. Osteopontin (OPN) synthesized in osteoblasts is suggested to have constraining effects on the HSC number since its absence leads to an increase of stem cells [69, 70]. For proteoglycans, BM and co-culture elution studies show, as mentioned above, the presence of CS, HS and HA [64, 65, 66].
Rabbit BM elution shows chondroitin-6-sulfate as the predominant glycosaminoglycan (GAG) (79%) and the occurrence of 16% HA [66]. The characterization of synthesized proteoglycans by long-term co-cultures of murine BM cells identified HS as the predominant GAG followed by CS [65]. In vitro culture of HSC on immobilized GAGs indicates highly sulfated HS especially 6-O-sulfation of the glucosamine residue of heparin (Hep) to be supportive for HSC long-term maintenance [71, 72]. The supportive and adhesive functions of GAGs for HSCs require the cooperative interaction with growth factors and ECM proteins [73, 74, 71]. The adhesion of HSCs to immobilized Hep after rolling under flow conditions supports the idea of HS proteoglycans being localized around the BM endothelium and thereby being involved in the process of HSC migration to the BM [75]. The glycoproteins thrombospondin and tenascin are both expressed during development and in regenerating organs and found in the BM in the surroundings of maturing HSCs [76, 77, 78].

For ECM recognition the cells express respective adhesion receptors. It was found that normal murine bone marrow lineage depleted, Sca-1+ cells express a number of adhesion receptors, including αL-, α1-, α3-, α4-, α5-, α6-, β1- integrins, L-selectin, CD44, and PECAM [79]. These adhesion receptors are not only important for anchoring HSCs, but also promote specific downstream signaling and cooperate with growth factor signaling pathways [80, 81]. α4 and α5-integrins control HSC proliferation and differentiation either directly or by modulating cytokine-induced signals [46] and are essential for bone marrow repopulation after transplantation, the so called ‘homing’ and ‘engraftment’ process [82, 83].

**Homing and Engraftment to the BM**

Homing is a highly regulated multistep process similar to the migration of leucocytes to inflammatory sites (Fig. 6). This process includes an initial phase of tethering and rolling of the cells to the endothelium of vessels via selectins, firm adhesion via integrins, transendothelial migration, and chemotaxis through the ECM to the endosteal surface [84]. The next phase involves the interaction of surface receptors, such as α4β1 integrins with FN in the ECM and vascular adhesion molecule-1 (VCAM-1), and interactions with soluble, membrane-, or matrix-bound growth factors [84]. The stromal cell-derived factor 1α (SDF-1α) expressed by stroma and endothelial cells and its CXC-Motiv-Chemokinrezeptor 4 (CXCR-4) expressed on HSC play a key role in human HSC trafficking to the BM microenvironment [85]. SDF-1α induces a rapid temporary activation of α4 and α5-integrins enabling the binding of cells to the endothelium and their exit from the circulation [86]. Many cell types, including progenitors and mature blood cells may home to the BM, but only stem cells engraft in their niche and initiate repopulation [87]. A critical cytokine for HSC engraftment and survival in the BM is the stem cell factor (SCF, also called KIT-ligand or steel factor), which is expressed by stromal cells. SCF is a ligand for KIT (in mice) and accordingly c-kit (in human), a receptor tyrosine kinase on the HSC surface. The activation of c-kit is differentially affected by the soluble versus the membrane bound isoforms of SCF [88]. Membrane bound SCF seems to enhance maintenance of long term hematopoiesis [89, 88] and induces the up-regulation of CXCR-4 expression [87].
Basics

The homing process involves the rolling of cells along the vessel walls with the help of selectins, then transendothelial passing and migration to the endosteal surface along a chemotactic gradient of SDF-1 and engraftment to the niche via integrins and c-kit, receptor for SCF.

Sensing the Environment

Studies of SCF stimulated cell proliferation demonstrate that Rac activation is critical for c-kit signaling [90]. Rac belongs to the Rho family of small guanosin triphosphate binding proteins (GTPases), which interact with specific target molecules controlling actin cytoskeleton reorganization, cell shape and polarity to mediate cellular adhesion responses, like migration, cell cycle progression, and survival [91]. Rho GTPases have been shown to integrate stimuli from tyrosine kinase receptors, chemokine receptors, and integrins [84]. The best studied members are Rac, Rho A and Cdc42. In HSCs Rac is activated by stimulation of CXCR-4 via SDF-1α, adhesion via β1 integrins, and stimulation of c-kit via SCF – all pathways important for stem cell engraftment to the BM niche and retention [90]. Rac and Cdc42 lead to actin polymerization at the cell front and cell polarization for directed migration respectively [92], and regulate cell cycle progression through mitogen-activated protein kinase (MAPK) dependent and independent mechanisms leading to cyclin D1 expression which induces G1/S cell cycle transition [93, 94]. In general, for adhesion dependent cells RhoA activity drives actin stress fiber formation and focal adhesion formation, increases cell contractility and thereby increases cyclin D1 expression and G1 phase progression [95]. Since HSC are not necessarily adhesion dependent cells the role of RhoA for HSC engraftment and cell cycling seems diffuse. It has been shown that that decreased RhoA activity resulted in defective α4β1 and α5β1 integrin-mediated adhesion and impaired SDF-1α directed migration [90]. But also, RhoA down-regulation increased HSC engraftment and cell cycle progression [96]. The balance between the degree of activation of the two RhoA downstream targets ROCK and mDia might be responsible for the either G1 progression promoting or inhibiting effect of RhoA [97]. ROCK is inhibitory to G1 progression, whereas mDia is stimulatory. Studies in endothelial cells suggest that RhoA preferentially activates mDia relative to ROCK, and thereby shifts the balance toward cycling. For ROCK activation rather than mDia higher levels of RhoA activity are required [98]. Hence, low RhoA leads to increased cell cycling (via mDia). Thus, cell attachment and following cell polarization, actin fiber polymerization and focal adhesion formation are closely interconnected with the regulation of cell division [97]. For example, the shape of HSCs was found to influence the frequency of cycling; among the slow dividing fraction more HSC had membrane protrusions compared to rather round and fast dividing cells [14]. Slow division in turn is related to a higher repopulation potential [99]. Changes in
cell shape alter cytoskeletal organization and with this the nuclear form [100]. Mechanical
induced changes in nuclear shape can affect nuclear import of transcription factors and initiate
cell cycling [101]. The other way around, the cell cycle status of HSCs modulates their adhesion
receptor expression and thereby adhesion and migration behavior [79, 102].

Neither Rac nor RhoA influence the affinity of adhesion receptors to the ECM but control
assembly and disassembly and thereby the density of integrins within cell adhesion complexes.
With this mechanism the cell is able to regulate the strength of the cell-matrix contact and to
sense elasticity and geometry of the respective environment [103]. Thus, with the help of cell-
matrix adhesions cells may sense the bio-molecular composition of the ECM and also the
physical and geometric properties of their microenvironment [103]. It has been shown that
both the specificity of adhesion ligands and the strength and the geometric distribution of cell
adhesion forces can affect stem cell proliferation and differentiation [104] by regulating cell
cycle entry (see above).

Cell adhesion with its complex intracellular signal transduction network [105] is often
synergizing with signals provided by growth factors [80, 79] via physical linkage of both
adhesion and growth factor receptors [95]. Once activated, integrins recruit actin adaptor
proteins (e.g. vinculin, paxillin, actinin, etc. [106]) and phosphoproteins such as focal adhesion
kinase (FAK) which link to growth factor/ cytokine receptors [95]. HSCs realize this so called
‘outside in’ signaling via Proline-rich tyrosine kinase 2 (Pyk2) which is a FAK homologue [107].
In contrast to the adhesion site located FAK; Pyk2 is distributed throughout the cell plasma
[108] which might at least partly explain the diffuse adhesion behavior of HSC compared to
adhesion related cell types [107]. On the other hand, growth factor/ cytokine receptors in turn
can activate integrins, termed ‘inside out’ signaling, and enhance ECM adhesion, which then
again may influence growth factor signaling [81]. This interplay of adhesion and growth factors
and their effect on the signaling machinery of the cell influence cell cycle regulation.

In summary, cell-ECM interactions, receptor and signaling protein activation, and the resulting
changes in cell shape and tension play an important role in divisional behavior of cells and vice
versa, which then may affect stem cell self-renewal and/ or differentiation and the action of
cytokines upon it.

2.1.4 HSC Relevant Cytokines

Growth factors, such as cytokines and hormones, are capable of stimulating cellular growth,
proliferation, and differentiation. Cytokines are cell signaling molecules secreted by cells and
used for cellular communication, while chemokines are small-sized cytokines which induce
chemotactic behavior in cells (chemotactic cytokines).

Within the BM niche soluble factors secreted locally or at a distance regulate HSCs and their
progeny. The exact signal composition that governs especially self-renewal still remains
unclear [109]. Some insight has been gained from in vitro studies testing cytokines and their
contribution on HSC expansion, self-renewal, and maintenance. As already mentioned above
the chemokine SDF-1 directs HSC homing into the BM niche important for engraftment [85].
Numbers of studies have shown interleukine (IL)-11 and IL-6 to maintain HSC activity. In
contrast, IL-3 induces expansion and differentiation but reduces HSC long-term culture
capability [110]. A variety of cytokine ‘cocktails’ have been used to induce HSC cycling for expansion. The combination of FMS-like tyrosine kinase receptor-3 ligand (FL3), SCF, and thrombopoietin (TPO) has proved to most efficaciously activate HSC cycling with retention of self-renewal capacity [86]. SCF is a critical cytokine for engraftment and maintenance of long-term repopulating cells [111]. Structural similar to SCF is FL3 which acts in a synergistic manner with SCF on HSC proliferation and maintenance [109]. Another important factor for HSC maintenance is TPO which is also responsible for the regulation of megakaryocyte proliferation and differentiation. The only cytokine clinically used is the granulocyte-colony stimulating factor (G-CSF). G-CSF stimulates HSC release into the peripheral blood prior to apheresis as alternative to the painful bone marrow harvest [112].

Many cytokines appear as soluble and membrane bound isoforms inside the bone marrow displaying different actions upon cells. For example, the soluble isoform of SCF exhibits a rather short term effect on HSC survival since the receptor-ligand complex is subsequently internalized and degraded after binding [88]. With sustained high soluble SCF concentration this down-regulates surface c-kit expression leading to desensitization and following mobilization of HSCs to the peripheral blood [113]. Whereas the membrane bound SCF functions as long term survival factor of HSCs as the SCF/c-kit complex doesn’t get internalized keeping them inside the BM niche and additionally supporting integrin mediated engraftment to the ECM [113]. SCF only functions as a dimer which binds two molecules of c-kit leading to receptor dimerization and subsequent intracellular signal transduction [114].

Cytokines influence the chemotactic movement important for HSC homing (chapter 2.1.3.) along the SDF-1α/CXCR-4 axis. For example, HSCs exposed to IL-3 for expansion show reduced response to SDF-1α [115] and impaired engraftment potential [82]. In contrast, as already mentioned above in 2.1.3, the exposure of HSC to the membrane bound isoform of SCF increases CXCR-4 expression [87].

As per definition as growth factors, cytokines induce cell cycling. The critical parameter in HSC cycling and expansion is the retention of engraft ability and the self-renewal capacity. HSCs expanded ex vivo ‘home’ less efficiently to the BM than freshly isolated cells. Transition of HSCs from Go to G1 but not into cell cycle already markedly diminishes repopulation capacity in irradiated recipients [116]. Cycling HSCs show reduced engraftment compared to quiescent cells [117]. Induction of S-phase entry reduces the functional binding of HSC to the ECM due to altered adhesion receptor expression [118]. Expression of α4 and β1 is implicated in defective homing as their appearance declines in culture [119]. Synchronized dividing HSCs show fluctuating adhesion to FN and VCAM-1 during cell cycle transition with reversibly increased adhesion to FN and reciprocal decrease in adhesion to VCAM-1 [120]. Additionally mitogenic activated cells shifted from α4 to predominantly α5 dependent adhesion to FN.

The amounts of cytokines play an important role in HSC maintenance as well. Most of the studies concerning the effect of cytokines single or in combination have been done with concentrations way above physiologically relevant ones. This may lead to contrary conclusions regarding the functionality of cytokines on HSCs. Physiological concentrations of cytokines are suggested to be within picogram range [74]. For the experiments presented below the cytokine concentrations were chosen just at the lowest level for the cells to be comfortable in culture.
2.2 Cell Culture Scaffolds

In recent years the *in vitro* investigation of cell-ECM interactions and their effect on cell behavior in terms of adhesion, migration, and development has ‘migrated’ from a plane two-dimensional (2D) unnatural platforms to rather *in vivo* like three-dimensional (3D) [121] distribution of adhesion and growth relevant factors [122]. New imaging devices which allow for observation of 3D objects [123] and the development of micro- and/or nanometer sized biotechnological setups disclose a number of physiological insights for basic research.

2.2.1 General 2D, 3D

Inside the body cells are surrounded by a variety of extracellular cues influencing their behavior. Cells sense the geometrical distribution of bio-molecular ligands [122] and physical properties like rigidity [124] in a spatial 3D manner. This environmental information is transferred to intracellular signaling cascades directing cell survival, proliferation, differentiation, and movement [63]. Cells behave differently in response to 3D vs. 2D microenvironments not only in terms of adhesion and morphology [121] but also in their reaction to biophysical factors [125]. Particularly the rigidity of e.g. cover slips in 2D is a component of *in vitro* systems, which is not present in living tissues. The texture of substrates may influence differentiation decisions of stem cells. For instance matrices mimicking brain softness induce neuronal lineage specification of MSCs. In contrast stiff collagenous bone-like substrates push osteogenic differentiation [124]. In order to study basic cell behavior in response to their environment it is essential to provide relevant models. Successful artificial substrates are biomimetic viz. they mimic *in vivo* structures [126].

To address the issue of a more *in vivo* like 3D surrounding, encapsulation of cells within biological functionalized hydrogels [127] as well as collagen gels have been applied [125]. In contrast to these 3D embedding strategies the open well precise geometry of the microcavity culture scaffolds presented in this work allows an easy access and escape of HSCs, enabling them to freely explore different growth environments in terms of homing, migration, or suspension. An additional advantage of the proposed setup is its straightforward accessibility to high-resolution analytical methods including cytometry, quantitative microscopy, and further functionality assays.

Apart from basic cell research bioengineered surfaces have found many applications in tissue engineering for transplantation and cell-based sensor devices in e.g. drug discovery screening. These approaches promise to overcome shortage of transplantable donor material as well as more predictive drug discovery processes [128].
2.2.2 Substrate Engineering

Spatially and chemically defined surfaces are fundamental for the precise control and interpretation of cellular behavior as cells detect composition, stiffness, and geometry of their environment.

Immobilization of Biomolecules

The coupling of ECM molecules and growth factors onto cell culture carriers yields a promising setup for the investigation and control of HSC behavior. For example cytokines like SCF appear not only in soluble fashion but also tethered to cell membranes [129] (see 2.1.4). Thus functional immobilization of SCF might lead to long term survival and proliferation of primitive HSCs [130].

Several immobilization strategies are used for surface functionalization: (i) Biomolecules may be adsorbed to the desired surface, meaning they are non-covalently bound via electrostatic interactions, Van der Waals forces, hydrophobic or hydrophilic interactions, and hydrogen bonds. This is advantageous for the functional activity of the biomolecule or when they need to be released on cellular demand. But the nature of the biomolecular tethering might be to loose and they get ripped off by cells or diffuse to the media changing buffer concentrations. Adsorption is applicable for systems which require release of signaling molecules or matrix reassembly by cells. (ii) Covalent binding of biomolecules assures stable attachment but may lead to possible sterical hindrances of the active core of the molecule. Functional groups like –COOH, -OH, -NH₂, and -SiOH are usually applied to tether proteins via their aminofunctionality e.g. side chain of the lysine residue to surfaces. Although the orientation of the active core might still be spatially unfavorable the introduction of chemical spacers like poly ethylene glycol (PEG) prevents sterical hindrances and assures easier access for cells [130].

Figure 7 Scheme of Biomolecule Immobilization. SCF was immobilized covalently to the poly(octadecene-alt-maleic anhydride) POMA (A) or to a polyethylene glycol (PEG7) spacer attached to POMA (B). (C) Functional Fc-chimeric protein attachment onto PEG hydrogel via ProteinA linkage. Modified after [130]and [99], respectively.
setup is exemplified in figure 7 where SCF was covalently bound either directly to the hydrophobic poly(octadecene-alt-maleic anhydride) (POMA) or attached to flexible poly(ethylene glycol) (PEG 7) spacer arm tethered to POMA to provide easier accessibility for the HSC to compare the action of tethered versus soluble SCF upon receptor c-kit kinetics and HSC proliferation. Lutolf et. al achieved functional binding of biomolecules using Fc-chimeric proteins which strongly bind to ProteinA modified hydrogel microwells [99](Fig. 7).

**Surface Patterning to Restrict Cellular Adhesion**

Since *in vivo* ECM molecules and cytokines are not homogenously distributed, but rather patterned, micro-structured cell culture carriers are increasingly used in biotechnology [131]. Practical applications of micro-patterned cell culture substrates range from tissue engineering to cell array based biosensors [132]. The restriction of cellular spreading to a specific surface area has been shown to dramatically affect cellular behavior [122, 133, 134]. Protein-resistant substrates like PEG-terminated surfaces block protein adsorption from solution as well as active adhesion of cells as cell surface molecules are repelled, too. The application of soft lithographic techniques allows the geometric patterning of cell adhesive and non-adhesive PEG areas [131]. Several PEGs and application options (see chapter 3.4) were used in this work to selectively functionalize the microcavities with ECM molecules and passivate the surface in between to restrict HSC adhesion to the spatial confinement of the microcavities.

**Defined ECM-coated 3D Microcavities**

For the precise geometrical control of HSCs, micro structured poly(dimethylsiloxane) (PDMS) moulds replicated from silicone masters were used in this work. PDMS allows for defined structuring down to 100 nm [135] without disadvantageous swelling in aqueous solutions as observed for hydrogels [136] or reconstituted collagen [125] during cell culture. PDMS is applicable for versatile surface patterning strategies; e.g. microcontact printing that employs structured PDMS stamps inked with the desired molecule to create chemically micropatterned surfaces [122, 132]. Furthermore, with a chemically modified surface to bind bioactive molecules the structured PDMS itself can be applied for cell culture. Such PDMS culture carriers may be used e.g. for shape control of single cells [137] or for multicell-arrays [138].

In this work PDMS moulds with microcavities of different sizes ranging from 15 to 80 µm fitting single or several cells were coated with maleic anhydride copolymers to allow the covalent coupling of BM relevant ECM molecules [139, 140]. Proteins were directly bound via lysine side chains to the anhydride moieties of poly(ethene-alt-maleic anhydride) (PEMA) copolymer coating. Hep was immobilised via diaminobutane linkers between the anhydride of the copolymer and carbodiimide activated carboxylic acid groups of the GAG (Fig. 9). These homogenously coated (Fig. 13) microcavities were applied to elucidate the interplay of spatial constraints, adhesive interactions, and cytokine concentrations on HSC fate decisions *in vitro*.

**2.2.3 Co-Culture versus the Artificial 3D Niche**

Co-culture with stromal cells has been shown to effectively support HSCs *in vitro* [141]. Despite that, the complexity of these culture systems impedes the identification and modulation of microenvironment components such as cytokine concentration or ECM composition. The defined three-dimensional HSC culture system presented in this work allows the defined
modification and combination of distinct microenvironmental cues to study their effect on stem cell maintenance and differentiation \textit{in vitro}.

The combined action of spatially constrained adhesive substrates and soluble cytokines was investigated with the 3D ECM functionalized microcavity setup upon HSC proliferation and differentiation.
## 3 Materials and Methods

### 3.1 Chemicals, Reagents and Equipment

<table>
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<tr>
<th>Reagent</th>
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<td>ACK Lysis Buffer</td>
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<td>StemSpan® Serum-Free Expansion Medium (SFEM) Mouse</td>
<td>STEMCELL Technologies SARL, Cologne, Germany</td>
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<td>TPO mouse</td>
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<td>Trypsin-EDTA</td>
<td>Sigma Aldrich, Deisenhofen, Germany</td>
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### Table 3: Antibodies

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<td>CD117 APC</td>
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<td>Mouse IgG1</td>
<td>Miltenyi Biotech</td>
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<tr>
<td>CD133 PE</td>
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<td>anti mouse</td>
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<td>SCF</td>
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### Table 4: Equipment

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<td>Cell Counter Casy 1</td>
<td>Schärfe System GmbH, Reutlingen, Germany</td>
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<tr>
<td>Centrifuge, Heraeus, Labofuge 400R</td>
<td>Heraeus Holding GmbH, Hanau, Germany</td>
</tr>
<tr>
<td>Critical Point Dryer CPD 030</td>
<td>Bal – Tec AG, Balzers, Liechtenstein</td>
</tr>
<tr>
<td>FACS Aria II (10-fluorescence detectors, 3 lasers: 633 nm (red), 488 nm (blue), 405 nm (violet)</td>
<td>Becton Dickinson, Heidelberg, Germany</td>
</tr>
<tr>
<td>FACS Calibur (4-fluorescence detectors, 2 lasers: 633 nm (red), 488 nm (blue))</td>
<td>Becton Dickinson, Heidelberg, Germany</td>
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<tr>
<td>Gold Sputter SCD 050</td>
<td>Bal – Tec AG, Balzers, Liechtenstein</td>
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### Materials and Methods

<table>
<thead>
<tr>
<th>Equipment Description</th>
<th>Company Information</th>
</tr>
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<tr>
<td>Incubator Hera Cell 150</td>
<td>Heraeus Holding GmbH, Hanau, Germany</td>
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<td>LSR II (12-fluorescence detectors, 4 lasers: 633 nm (red), 488 nm (blue), 405 nm (violet), 355 nm (UV))</td>
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<td>Lyophilisator Lyovac GT2</td>
<td>GEA Lyophil GmbH, Hürth, Germany</td>
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<td>Membrane Pump (55l/ min, N 035.1.2.AN18)</td>
<td>Fisher Scientific, KNF Neuberger, Schwerte, Germany</td>
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<td>MilliQ Purification System for deionized water</td>
<td>Millipore, Schwalbach, Germany</td>
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<td>Plasma Cleaner PDC-002</td>
<td>Harrick, Ithaca, NY, USA</td>
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<td>Rotary Evaporator</td>
<td>Büchi Labortechnik AG, Germany</td>
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<td>Spectral Photometer incl. Magellan Software</td>
<td>Tecan GmbH, Crailsheim, Germany</td>
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<td>Spincoater RC5</td>
<td>Süß Microtec AG, Garching, Germany</td>
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### Microscopes

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<td>FEI Company, Hillsboro, OR, USA</td>
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<td>Light Microscope Olympus IX 50</td>
<td>Olympus Europa Holding GmbH, Hamburg, Deutschland</td>
</tr>
<tr>
<td>Laser Scanning Mikroskopes, Leica TCS SP1 &amp; SP5</td>
<td>Leica Microsystems GmbH, Wetzlar, Deutschland</td>
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<td>Fluorescence Microscope Leica DMIRE2</td>
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<td>Axiovert 200 &amp; 200M Zeiss</td>
<td>Zeiss, Jena, Germany</td>
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### Objectives

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<td>Zeiss, Jena, Germany</td>
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<tr>
<td>HC PL FLUOTAR 10x/0.30</td>
<td>Leica Microsystems GmbH, Wetzlar, Deutschland</td>
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<td>HC PL FLUOTAR 20x/0.50</td>
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<td>HCX PL APO 40x/1.25-0.75</td>
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<tr>
<td>HCX PL APO Lbd.bl. 20x/0.70</td>
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</tbody>
</table>
3.2 Wafer Design and Surface Functionalization

A set of silicon masters was designed with square symmetric columns ranging from 15 to 80 µm in diameter with respective distances from 30 to 160 µm and 10 µm in height using photolithographic etching (GeSiM, Rossendorf, Germany). Later on the silicon masters were redesigned with columns of the same dimensions as mentioned above but with a minimum distance in between (Fig. 8).

Figure 8 Waferdesign. (A) Represents the design originally used to replicate PDMS microcavities, which was later modified (B) to prevent cell adhesion in between the cavities.

After fluorosilanization ([Heptadecafluoro-1,1,2,2-tetrahydrodecyl] dimethylchlorosilane) of the masters (gas phase over night), thin silicone films of poly(dimethylsiloxane) PDMS (Sylgard® 184 silicone elastomer kit) were replicated (10:1 curing agent to prepolymer, 5h at 65 °C) from the microstructured masters. The microstructured silicone moulds were glued onto cleaned glass slides (Menzel, Braunschweig, Germany) using a 30 s pretreatment with low pressure oxygen plasma of the cover slip and the plane backside of the silicone structures (Fig. 9).

Figure 9 Replication of the PDMS Culture Carrier. The freshly mixed two component PDMS is poured onto the silanized silicone master (A) and polymerized (B). The cured PDMS structure is then peeled off, glued onto a glass coverslip, and coated with the desired ECM molecules (see also Fig. 10) followed by HSC culture (C).
Thin films of poly(ethene-alt-maleic anhydride) (PEMA) were covalently attached to the aminosilanized silicone microstructures as recently reported [139]. Briefly, silicone microstructured cover slips were treated for 60 s with medium pressure oxygen plasma and placed 2 h into 10 mM 3-aminopropyl-triethoxysilan/ethanol solution, dried for 1 h/120 °C, coated with PEMA/ethanol, dried again 2 h/120 °C, and autoclaved (20 min/120 °C) to wash away unbound PEMA. Prior to FN immobilization, the anhydride moieties were regenerated 2 h at 120 °C to assure covalent binding of FN [140]. FN purified from adult human plasma by a protocol adapted from [142] was diluted to a final concentration of 20 µg/ml in phosphate buffered saline (PBS). The PEMA-coated microstructures were then incubated with FN solution for 1 h and subsequently rinsed several times with PBS (Fig. 10). Col I was immobilized at a final concentration of 0,5 mg/ ml (on ice, 8 parts PureCol, 1 part 10x NaOH, 1 part 10x PBS and diluted to 1 mg/ ml with the respective amount of PBS, pH 7,4) for 3 h and rinsed several times in MilliQ water. Hep immobilization was achieved using 1,4-diaminobutane as linker providing amino groups. Therefore PEMA-coated microcavity cover slips with freshly regenerated anhydride moieties were incubated 4 min in 0,1 M 1,4-diaminobutane/isopropanol solution, rinsed 2 x 1 min in MilliQ water, 1 min in 0,01 M hydrochloric acid, and again rinsed 2 x 1 min in MilliQ water. After drying with nitrogen the formed amide bonds were converted into imide groups by reheating cover slips to 120 °C for 2 hours. Immediately after cooling, the surfaces were incubated over night with freshly prepared (no older than 5 min!) 1 mg/ ml Hep solved in ice cold 0,1 M borate buffer (pH 8,0) containing 200 mM 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 100 mM N-Hydroxysulfosuccinimide (Sulfo-NHS) for carboxylic group activation. Non-bound Hep was removed by rinsing several times and autoclaving 20 min 120 °C in MilliQ water.

To get rid of air bubbles inside the microcavities which are formed during protein coating procedure, the microstructured cover slips were placed into an desiccator, covered with PBS and degassed (55l/ min), prior to the addition of double concentrated protein solution, which is then directly diluted 1:1 on the surface with the degassed PBS. Hep solution was directly degassed.

Prior to cell seeding the surfaces were equilibrated in cell medium w/o additional growth factors over night at 37 °C.

Quality and stability of the coatings were tested using FN fluorescently labeled with Carboxytetramethylrhodamine (TAMRA) FluoReporter. The fluorescence intensity was checked for seven days by confocal laser scanning microscopy (TCS SPi) while incubating the surfaces in 10% fetal bovine serum in PBS to show stability of the immobilized FN against displacement by other proteins. For visualization of Col I and Hep coatings, the immobilization solution was mixed 1:10 with either Col I FITC or Hep FITC, respectively. Additionally Col I fibril formation was controlled with scanning electron microscopy (SEM) after drying the surfaces with nitrogen and sputtering a thin layer of gold (60 mA, 25 s).
Materials and Methods

Figure 10 Surface Modification. Proteins are bound via their lysine side chain to the anhydride moiety of the PEMA (A). Sugars like Heparin are immobilised via diaminobutane linkers between the anhydride of the copolymer and carbodiimide activated carboxylic acid groups of the glycosaminoglycans (B).

Fluorescence intensity was measured using ImageJ software (http://rsbweb.nih.gov/ij/) calculating the Mean Gray Value which is the average gray value within the selection (Fig. 15, dotted lines in the respective pictures). This is the sum of the gray values of all the pixels in the selection divided by the number of pixels.

3.3 Cell Culture and Analysis

After obtaining informed consent, G-CSF (10 µg/ kg body weight) mobilized human HSCs from peripheral blood of healthy donors were immune magnetically isolated for CD133 positive cells after the manufacturer’s instructions (CD133 indirect Isolation Kit, Miltenyi). The manufacturer’s protocol was slightly modified: Instead of the recommended 100 µl/ 10⁸ cells of FcR blocking reagent/ micro bead solution, and 50 µl/ 10⁸ cells antibody, only 125 µl/ ml and 62.5 µl/ ml leucapheresis blood respectively were used. After dilution series this proved to be as effective as the recommended amount considering the isolated cell numbers, with lower impact on healthy appearance of cultured cells. The immune magnetic isolation was carried out as follows: CD133 positive cells are labeled with a biotinylated monoclonal antibody which in a second step was detected by biodegradable streptavidin-coated 50 nm sized magnetic micro beads. Cells were further separated on a MACS® column in a MACS® separator which generates a magnetic field. The flow through was collected as negative fraction depleted of the labeled cells. After the MACS® column was removed from the magnetic field, the retained CD133 positive labeled cells were flushed out. Cells were subsequently used for culture and flow cytometry.

Cells were grown in serum-free CellGro medium supplemented with either 10 ng/ ml (low cytokine concentration) or 30 ng/ ml (high cytokine concentration) of stem cell factor (SCF), thrombopoietin (TPO), and FMS-like tyrosine kinase 3 ligand (FL3). For 14 d cultures half medium was exchanged after 7 d. The ‘standard’ setup for the cell experiments was the low cytokine concentration (10 ng/ ml). As explicitly stated in the results part of the text, only some experiments on cell cycling kinetics were performed at high cytokine concentrations (30 ng/ ml). The viability of cells was tested using 0.01 mg/ ml fluorescein diacetate (FDA) and 2 mg/ ml PI.
Materials and Methods

Freshly isolated cells were directly plated (5 x 10^4 cells/ml) onto ECM-coated microcavities preincubated with media at 37 °C and cultivated at 37 °C, 5 % CO2). All labeling (FACS, LSM) procedures were performed using 10 % FCS/ PBS buffer.

3.3.1 HSC Culture in ECM-functionalized Microcavities

On microcavities for proliferation and differentiation studies, HSCs were cultured for seven and/or 14 days on ECM moulds. Non-adherent and adherent cells were collected separately after 4 min of trypsinization, counted, washed and labeled for FACS using anti-CD133 phycoerythrin (PE), anti-CD34 APC and anti-CD38 fluorescein isothiocyanate (FITC) 10 min 4 °C (see supplements for FACS principle). For immunofluorescence laser scanning microscopy (LSM) (SP5, Leica, pinhole completely opened) of adherent cells after seven days, HSC were additionally secondary labeled with isotype specific Alexa Fluor 546 and Alexa Fluor 647 goat anti-mouse (Invitrogen, 1 h at room temperature) since PE and APC are fast bleaching fluorochromes. Alexa Fluor isotype specificity was verified via FACS.

Figure 11: BrdU Incorporation. The thymidine analog BrdU (A) is incorporated into newly synthesized DNA strands during S-phase of the cell cycle. Though BrdU positive actively cycling cells can be detected FACS (B). Counterstaining with propidium iodide allows separation of the cell cycle stages G1/G0, S, G2 and M (B, C).

The cycling kinetics of cells were detected by 20 μM bromodeoxyuridine (BrdU) incorporation into replicating DNA during the 24 h preceding fixation in 70% ice-cold ethanol at time points of 24 h, three days, and seven days of cell culture. BrdU is a thymidine analogue (Fig. 11 A) which gets integrated into the DNA during synthesis (S-phase) (Fig. 11 C). BrdU incorporation was measured by FACS for both cells adhering on planar surfaces and non-adherent cells (Fig. 11 B). BrdU also was fluorescently visualized for HSCs adhering on FN-coated PDMS moulds (Improvision, Leica) after labeling with anti-BrdU and secondary Alexa Fluor 488 goat antimouse and counterstained with propidium iodide (PI). Cycling versus non-cycling cells were counted using the freely available ImageJ software. For step by step protocol refer to the supplements section.
For analysis of HSC functionality, colony forming assay (CFU) was performed. Therefore, after seven or 14 days of culture on ECM-coated microcavities adherent or supernatant cells were separately harvested and plated (500 cells/surface) into standardized semi-solid media supplemented with growth factors supporting colony-forming units-granulocyte-erythroid-macrophage-megakaryocyte [CFU-GEMM], burst-forming units-erythroid [BFU-E], colony-forming units-erythroid [CFU-E], colony-forming units-granulocyte-macrophage [CFU-GM], and colony-forming units-granulocyte [CFU-G] cells, depending on the potency, which HSCs maintained after culture (Fig. 12). Colonies were scored after another 14 days of culture in CFU media.

![CFU-GEMM](image1)
![CFU-GM](image2)
![CFU-G](image3)
![CFU-E](image4)
![BFU-E](image5)

**Figure 12 Colony Forming Units.** Schematic Diagram of CFU-GM formed by hematopoietic progenitors after two weeks incubation.

### 3.4 Surface Passivation

For the passivation of the surface in between the microcavities in order to block protein anchorage and force cell to stay within microcavities apart from each other’s potentially paracrine stimulation, several blocking approaches were undertaken (Fig. 13).

Different aminofunctionalized poly(ethylene glycol)’s (PEG) and one polyoxazoline (PMOXA) were used, as they are known for their protein antiadhesive properties [143, 144, 131] (Fig. 13).

In all cases, prior to application of PEG the structured PDMS moulds were coated with PEMA as described above (chapter 3.3) and the anhydride moieties were recovered 2 h at 120 °C. The PEGs were applied by either stamping (PDMS or poly(acryl amide) (PAAM) stamp), dropping or dipping (Fig. 13). For exact solvent concentrations and approaches employed for the different PEGs, refer to results chapter 4.1.2.
Materials and Methods

Figure 13 Used PEGs for Surface Passivation. Four different PEGs were used in different approaches to passivate surface in between cavities.

The PDMS stamps were either used unmodified (blank PDMS) or modified with PEMA, coated as described above for the moulds, whereas the anhydride group was not recovered, thus
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preserving the maleic acid groups and allowing only physisorption of PEG. The PAAM stamp was used to try a more wet stamping technique [137] to effectively apply Poly-L-Lysine grafted with Polyethylene glycol (PLL-PEG) onto the surface. Therefore 50 µl of 20 µg/ml PLL-PEG in PBS was mixed to 1 ml PAAM pre-gel (30 % acrylamide/ bis solution 37:5:1). Crosslinking was initiated with 20 µl ammonium persulfate (APS) and accelerated with 4 µl N,N,N,N-tetramethyl ethylenediamine (TEMED). The gel was polymerized by dropping 50 µl onto an even fluorsilanized silicone master ((Heptadecafluoro-1,1,2,2-tetrahydrodecyl)dimethylchlorosilane, gasphase over night in an airtight-sealed petri dish) and putting an acrylsilanized cover slip sandwich-like on top for 20 min. After the PAAM gel stuck to the acrylsilanized cover slip, the fluorsilanized cover slip could be removed easily with a razor blade. Then the structured side of the PDMS moulds was laid on the gel for 15 min loaded with a 5 g weight. Prior, the PEMA-coated PDMS moulds with freshly regenerated anhydride moieties were either pretreated 30 s with medium pressure oxygen plasma or used directly. After, 10 µg/ml TAMRA labeled FN was incubated 1 h 37 °C to prove passivation via confocal microscopy.

The acrylsilanized cover slips were prepared as follows: Cover slips (Ø 19 mm) were cleaned in MilliQ water, ammonium hydroxide and hydrogen peroxide (ratio 5 : 1 : 1, respectively), dried in a stream of nitrogen, transformed 2 h with 20 mM acrylsilane in EtOH (abs.) containing 5 % MilliQ, washed twice in EtOH (abs.), dried with nitrogen, and annealed for 1 h at 120 °C. The cover slips were stored for up to two weeks at 4 °C, prior to use.

3.5 Mouse Bone Marrow Preparation

The donor mice (C57BL6 CD45.2, 6 weeks, kindly provided by Claudia Waskow, CRTD) were sacrificed and dipped into 70 % EtOH. The femurs from the hind legs were taken, cleaned from remaining muscle, and put into 6-well plate in 5 % FCS in PBS on ice for transfer to the sterile bench. The bones were cut at each end and the BM was gently flushed out with a 23G 2 ml luer lock needle syringe using 5 % FCS/ PBS. The BM was then transferred into a 15 ml tube and centrifuged 5 min at 1500 rpm. The supernatant was discarded and cell resuspended in ACK buffer for erythrocyte lysis for max. 15 – 20 s. Subsequently 10 ml 5 % FCS/ PBS were added and washed another 5 min at 1500 rpm. After discarding supernatant, resuspended cells were transferred into 1.5 ml tube with 1 ml 5 % FCS/ PBS counted, washed again, and resuspended in 5 % FCS/ PBS and kept on ice for short time until further analysis or culture.

For lineage depletion prior to culture and for preparation of competitor cells, single msBM cell suspension was labeled (150 µl per msBM) with biotinylated (bio) lineage antibodies (CD3 bio, CD19 bio, NK1.1 bio, Ter119 bio, CD11b bio, Gr1 bio, B220 bio). For Fc block CD16/32 pure and rat Ig was used. KLS cells for culture were additionally labeled with CD117 APC (KIT) and Sca-1 PE. 5% FCS/ PBS buffer was used for dilution (for accordant antibody dilution please refer to Tab. 3) and for washing 2mM EDTA was added to the buffer. After antibody incubation for 40 min on ice cells were washed 2’ at 2000 rpm. One aliquot was taken as positive control. The supernatant was discarded and washing repeated twice. The pellet was then resuspended in 100 µl washing buffer. 5 µl of anti-biotin MicroBeads were incubated for exactly 15’ on ice and washed. Cells were resuspended and filtered using filter mesh (pore size 100 µm) and then applied onto MACS® column (shortly before usage eluted with buffer/ EDTA) in the magnetic
field of the MACS® separator. The negative cells were allowed to pass through and collected with subsequent rinsing (3 times 500 µl) of the column. Afterwards the cells were washed and incubated with Streptavidin/ Pacific Blue as lineage positive control for another 20’ on ice, washed again, and resuspended for FACS sorting. The obtained KSL cells were cultured on microcavity culture carriers or used for competitor cells in the repopulation assay. For isotypic control and instrument setting #1 aliquot native (unlabeled cells) was taken, #2 was labeled anti CD45R PE, #3 CD45R APC, #4 CD45R Pacific Blue (PB), and above mentioned positive control was dyed with streptavidin PB.

Mouse BM cells were cultured on FN-coated microcavities in StemSpan supplemented with 10 µg/ ml Heparin (5000U/ ml), 10 ng/ ml of each TPO, SCF, fibroblast growth factor (FGF-1), and 20 ng/ ml insulin-like growth factor (IGF-2)[145] with half medium exchange after 7 d of culture.

For analysis, after 7 and 14 days of microcavity culture cells were detached using accutase 10 min at 37 °C, washed with 5 % FCS/ PBS, counted, and transferred into v-bottom 96-microwell plate for staining with anti-CD117-APC (1:200), anti-Sca-1-PE (1:100), and anti-CD34-FITC (1:50) all diluted in 5 % FCS/ PBS. Per microwell 50 µl of antibody solution was used. After 45 min incubation on ice cells were washed three times and put into FACS tubes for flow cytometric analysis.

For competitive repopulation lethally irradiated (200 kV, 20 mA, 6.5 min, ≈ 8.5 Gy) eight weeks old SJL CD45.1 recipient mice were engrafted. For the first trial 1 x 10^5 cells from 10 days microcavity culture were mixed in equal amount with competitor cells and retro-orbital injected in 150 µl 5 % FCS/ PBS into one of three mice each for different microcavity size: # 1 served as control with cells cultured in suspension, # 2 cells cultured on 15 µm sized redesigned microcavities, and # 3 cells from 40 µm redesigned microcavities. To prevent infections Neomycin was added to the drinking water (0.295 g/ 250 ml). In vivo repopulation was analyzed after 21, 36, 57, and 78 days. Therefore blood was taken from the retro orbital sinus of the mice and tested for CD11b (PE), Gr-1 (PB), CD3 (APC), Ter119 (Alexa750), and the ratio of CD45.1 (PE-Cy5) and CD45.2 (Alexa700) was assessed Fig. 30) which relates to the contribution of microcavity cultured cells to the blood production of the recipient mice.
4 Results and Discussion

4.1 Scaffold Design and Preparation

4.1.1 Surface Characterization

For the investigation of spatially constrained HSC proliferation and differentiation ECM molecule coated microcavities were used. The microstructured silicone moulds were replicated from lithographically designed silicone master structures. ECM components (fibronectin (FN), heparin (Hep), and fibrils of collagen I (Col I)) were covalently attached onto the PDMS replicas containing cavities of 15 µm to 80 µm in diameter and 10 µm in depth. The surfaces were designed to provide the same ratio of microcavity area to total surface area similar for all diameters. Redesigned microcavities feature the same cavity dimensions with a distance in between as small as possible (Fig. 14). The covalent ECM attachment was achieved using a poly(ethylene-alt-maleic anhydride) pre-coating on top of the PDMS scaffolds. In this way, proteins can directly be bound via lysine side chains to the anhydride moieties of this copolymer coating. Glycosaminoglycans (GAGs) were immobilised via diaminobutane linkers between the anhydride of the copolymer and carbodiimide activated carboxylic acid groups of the GAGs. The homogeneity, stability, and surface density of the ECM layers was analysed using fluorescence microscopy and scanning electron microscopy.

![Image](image_url)

Figure 14 Proof of Surface Quality. Fluorescent images show homogeneous coupling of the ECM molecules FN and Col I and Hep. Col I was further analyzed by SEM and showed evenly distributed fibrils (insets, scale: 10 µm). The green clusters seen in the fluorescence image turned out to be derivates from the FITC staining. In the lower right image HSC adhere to Hep-coated cavities (red actin, blue DNA). Scale: 40 µm
The FN and Hep coatings were homogeneous inside and outside the microcavities. The Col I fibrils were uniformly distributed across the microstructures (Fig. 14 and 15). A protein displacement assay based on the incubation of coated surfaces with 10% fetal bovine serum proved that FN binding was stable after 24 h incubation (Fig. 15) [146] and thus suitable for long term cell culture.

![Figure 15 Surface Homogeneity and Stability. Immunofluorescence images and surface density plots show homogeneous and similar distribution of FN (red) before and after 24 h. The intensity plot displays similar values for both time points. (Dotted lines indicate section taken for intensity plot, scale 80 µm)](image)

The amount of immobilized FN was analyzed previously [147] using HPLC based amino acid analysis of the hydrolyzed protein 24 h after surface coupling and after exposure to protein displacement solution (see above) [146]. Hence, the deployed immobilization solution of 20 µg/ml FN results in a surface concentration of 226 ng/cm² which approx. equates to the theoretical monolayer concentration of roughly 250 ng/cm² when considering tightly packed FN molecules with their long axis parallel to the surface [148, 146]. Therefore a FN ligand density of 6.13 x 10¹¹ per cm² can be calculated. The surface density of Hep as determined afore using X-ray photoelectron spectroscopy (XPS) can be estimated to 5.6 ± 0.5 x 10¹⁴ disaccharide units/cm² [148].
4.1.2 Surface Passivation

Besides the redesign of microcavities (see below 4.1.3) with higher density another approach was implemented to spatially confine the cells inside the microcavities. The chemical modification of the surface in between microcavities was aimed by passivation with PEGs known to repel protein binding due to their hydrophilic nature [143, 144, 131]. Such PEG passivation strategy would also abate the probability of cell-cell communication as assumed within redesigned microcavities (chapter 4.3.2).

Approaches for Surface Passivation

Several approaches were undertaken to prevent protein binding in between microcavities:

(i) The Jeffamine M1000 is a relatively short PEG with an amino functionalization at one end of the PEG chain for covalent linkage to the anhydride of the PEMA and a methyl group at the other end to prevent protein adsorption.

(ii) The PLL-PEG with its-amino terminated lysine groups was expected to densely cover the PEMA in between microcavities for successful protein blocking.

(iii) The 4-Arm-PEG-NHS is a branched PEG with 4 methyl group terminated end chains and was therefore considered to be more effective to block protein binding in between microcavities.

(iv) The PMOXA is considered as a good alternative for PEG with an end-standing methyl group to prevent unwanted protein binding [149].

Efficiency of Surface Passivation

After PEG application the microcavity moulds were coated with fluorescently labeled FN to prove the passivation of the protein via fluorescence microscopy and intensity measurements.

(i) Jeffamine M1000 was applied to the surface in between microcavities using different application and solvent strategies: Jeffamine M1000 was either solved in H2O or in ethanol. Stamping using a PEMA modified or non-modified flat PDMS stamp was not sufficient to completely block protein binding (Fig. 16 A & C). Streaky patches of FN were still visible, which would allow for cell adhesion. To increase passivation efficiency the PDMS mould was turned around and dipped into a flat drop of Jeffamine M1000 solved in H2O (Fig. 16 B). Even in that case, the blocking was incomplete and PEG partially diffused into the microcavities as the fluorescence intensity inside was diminished. In a last trial, a drop of Jeffamine M1000 was incubated in different concentration onto the microcavity structures for different duration. Low Jeffamine M1000 concentration (5 mM) was not sufficient to prevent protein binding. But with further increase of Jeffamine M1000 concentration (10 mM) the surface tension became too low; the solution entered microcavities, and thus also blocked protein linkage inside the cavities. However, the best FN blocking results were achieved using 5 mM in EtOH and a modified stamp (Fig. 16 C).
Figure 16: Approaches for Surface Passivation with JeffamineM1000. The PEG in different concentrations and solvents (see respective picture) was either stamped with PEMA modified (A), unmodified (C) PDMS, dropped onto the microcavities (D), or the microcavity mould was turned around and dipped into a flat drop of JeffamineM1000 solution (B). The best blocking of FN was achieved by applying 5 mM JeffamineM1000 solved in EtOH via a PEMA modified stamp. Scale 40 µm, FN-TAMRA red.
(ii) Very recently the PLL-PEG was found to be most effective in preventing unwanted protein adsorption [137, 132, 150]. However in our case, the PLL-PEG approach for blocking the surface area between microcavities didn’t satisfactorily block protein binding (Fig. 17).

Figure 17 Application of PLL-PEG for Protein Repulsion. Adsorption of PLL-PEG (A) solved at 1 mg/ml in HEPES buffer with different pH values using a PDMS PEMA modified stamp applied onto PEMA coated surfaces (60 min) was first tested via XPS (B). After stamping procedure the surface was washed with either H2O or HEPES at pH 10.5 and then autoclaved or not autoclaved. The concentration of N atoms was highest for stamping PLL-PEG at basic concentration of pH 10.5 and was not affected by autoclaving the samples afterwards. (C) Despite, the surface coverage of PLL-PEG was not enough to block FN binding even though a wetter stamping technique [137] using PAAM hydrogel was tried. Fluorescence: FN-TAMRA red, z-section.

(iii) The protein blocking capability of 4-Arm-PEG-NHS was first tested on plane surfaces using the dropping or stamping method, and washing with different pH 1/15 M phosphate buffers (for detailed procedures see Fig. 18 A). The PEG was solved on ice as described by the manufacturer in 1/15 M phosphate buffer.

The best FN blocking results with fluorescence intensity similar to the negative control were found for the 5 mg/ml drop incubation and washing with H2O (Fig. 18 B). However, culture of adherent 3T3 fibroblasts demonstrated that the surface is still adhesive for cells (Fig. 18 C). Thus the 4-Arm-PEG-NHS was not further tried for the microcavity scaffolds.
Results and Discussion

Figure 18 Surface Passivation Approaches with 4-Arm PEG-NHS. (A) The different approaches applied are described. FN blocking similar to the negative control was achieved by dropping 5 mg/ml 4-Arm-PEG-NHS in 1/15 phosphate buffer and washing with H2O (B). But passivation was not sufficient to prevent fibroblast adhesion (C). Scale 40 µm, FN-TAMRA red, Actin green, and DAPI blue.

(iv) As PEG coatings have been reported to degrade and show decreased long term stability with functional loss in vivo [151] a potential non-cytotoxic [152] polymer alternative to PEG namely poly(2-methyl-2-oxazoline) PMOXA with equal protein-repellent properties to PEG coatings [149] was tested for surface passivation in between the microcavities. The amino terminated PMOXA had high FN TAMRA blocking capabilities with mean gray value intensity below 10 (Fig. 19). However, the even more hydrophilic properties of PMOXA compared to PEG led to diffusion into microcavities and rejection of protein coupling inside.
Figure 19 Surface Passivation with PMOXA. The PMOXA solved at 4.5 mM in H2O was applied to the surface either by turning around the microcavity scaffold and dipping it into PMOXA aqueous solution or by dropping PMOXA onto the mould. Fluorescence intensity analysis of FN TAMRA coupling shows high efficiency of protein blocking. But PMOXA solution also diffused into the microcavities and prevented protein coupling inside.

Only optimal grafting conditions (like grafting density, solvent quality and molecular weight) of PEGs were found adequate to successfully avoid protein binding. For instance, too low solvent concentration was not enough to completely cover the surface area whereas the high concentrations led to sterical hindrances of the molecules and thereby averted sufficient surface coverage.

In summary, thin films of PEGs or other protein repellant polymers might not be appropriate to completely passivate surfaces for protein adsorption and to further prevent cell adhesion as cells might sense minimal amounts of ECM for attachment [133]. Hence, a thicker gel-like PEG layer might be more qualified as protein repellant and will be investigated in future.

4.1.3 Redesigned Microcavities

For more quantitative analysis of the HSC proliferation and differentiation inhibiting effect of matrix under spatial restrictions, the microcavity setup was redesigned since chemical surface passivation was not sufficient (see 4.1.2). The distance in between the microcavities was reduced to a minimum, which forces the cells to adhere inside (Fig. 20).
Cell experiments were repeated inside these redesigned microcavities comparable to the experiments in standard microcavities described in 4.2. Additionally, the CFU-GM forming capability of microcavity cultured cells was measured.

Figure 20 Redesigned Cavities. To prevent cell growth in between the cavities (A) moulds were redesigned (B) with microcavities side by side thus cells necessarily adhere inside.

4.2 Summarized Discussion of the Surface Passivation

The selective patterning with cell active biomolecules is one the major goals of biomaterial engineering in order to improve cellular bioassays for basic understanding of the action of different molecules on differentiation, proliferation, and signaling pathways. As already discussed above, the commitment of stem cells depends on their shape and also on their three-dimensional adhesiveness. The selective functionalization of single cell constraints would be the premise to exclude cell-cell communication or paracrine signaling. Additionally, as suggested by Trumpp et al dormant adult stem cells might home to single cell niches [26].

The soft lithographic techniques presented above (chapter 4.1.2) are not sufficient to effectively block surface binding of proteins. Hence, thicker protein-resistant layers might be applicable, e.g. PEG hydrogels coated onto the area in between microcavities. Full hydrogel replicas of the microcavities are only suitable for bigger multi-cell cavities (> 100 µm) as hydrogels swell in
aqueous environments [136]. Hydrogel stiffness additionally influences stem cell differentiation behavior. The stiffness can be adjusted and used for pushing stem cells into the desired lineage as described for MSC differentiating into neurons on soft brain like tissue or forming bone on an adequate bone-like hard gel [124]. However, due to its fore-mentioned swelling properties it is not applicable for single cell cavity design but might function as thin layer gel on top of PDMS microcavities. Nanoplotting 6 techniques are further alternative to be used in bigger cavities. Due to wetting effects the spotted area will always be greater than 50 µm. Another approach would be the dipping of the silicone master pillars into protein solution prior to PDMS replication. Thus the desired protein stays within microcavities [153]; however, the activity of the protein might be impaired when it is enclosed in the cured PDMS. Likewise Fc-chimeric Proteins were embossed into hydrogel microwells [136]. Therefore PEG-modified ProteinA was inked to the pillars of a PDMS master. During hydrogel casting the ProteinA was transferred to the gel matrix and covalently attached via the PEG linker. Subsequently after gel curing the desired Fc-chimeric protein was incubated and bound selectively to the microwells. In future hydrogel microcavites and nanoplotting techniques should be explored for the generation of selectively patterned microcavities to influence and elucidate stem cell fate decisions.

4.3 HSC Culture inside Microcavities

The three-dimensional arrangement of ECM assemblies in the bone marrow niche serves as scaffolding for HSCs and plays an important role in triggering their fate decisions [105, 154]. In this context, specific interactions between HSCs and ECM components may initiate proliferation and differentiation [3, 155] and were thereon investigated.

Cell numbers and expression of CD133 and CD34 HSC surface markers were determined by flow cytometry after seven days of culture on plane substrates (serving as control) and substrates with microcavities of different dimensions.

In general, HSC viability on all substrates was very high, with over 99% for FACS gating and approximately 90% for immunofluorescence analysis (Suppl. Fig. 33 and 34).

4.3.1 HSC-ECM Interaction Reduces Proliferation

First analysis of HSC-ECM interactions on plane ECM (FN, Col I, and Hep) -coated substrates showed reduced proliferation with the least expansion on FN. The differentiation pattern appeared to be similar, independent of the presence of matrix molecules (Fig. 21). For the case of FN previous reports explain this finding by showing that adhesion through α4-integrin to the heparin binding domain of FN decreased proliferation by inhibitory signaling [156]. This might be also the case for other matrix molecules as it has been shown that HSC adhesion to ECM secreting stroma osteoblasts reduces proliferation and maintains an immature state [157]. Reduced HSC proliferation due to adhesion-related signaling was expected to possibly alter differentiation, but the differentiation pattern appeared to be similar independent of the presence of the distinct matrix molecules on plane substrates (Fig. 21).

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6 Equipment for handling of liquids in submicroliter range for automatic dispensing of micro drops e.g. for microarray design
Results and Discussion

Figure 21 Proliferation and Differentiation of HSCs on planar ECM Coated Surfaces. (A) Proliferation increases over time with reduced proliferation of HSC exposed to matrix molecules on plane substrates compared to control cells in suspension. (B) The differentiation pattern is similar comparing different culture conditions (Data are presented as mean ± SEM).

4.3.2 Population-wide Proliferation and Differentiation of Spatially Constrained HSCs

After seven days of culture on substrates with microcavities of different dimensions the non-adherent and adherent cell fractions were separately harvested from the surfaces and analyzed for CD133 and CD34 HSC surface marker expression.

HSCs Cultured on Microcavities with Distance in Between

In general, HSC expansion was reduced on ECM-coated microcavities (0.5- to 3-fold expansion for FN and Hep, only 1-fold for Col I) in comparison to the 5-fold expansion seen for the control cells on standard tissue culture plastic (Fig. 22 A). After comparing the different cavity sizes the most pronounced decrease in total cell expansion rate was detected in the smallest microcavities for all three used ECM components. For FN substrates proliferation inside the smallest microcavity (15 µm in diameter) fitting only one cell drastically inhibits proliferation whereas the bigger sized cavities support HSC expansion in similar manner. Therefore, the greater engagement of adhesion receptors of HSC adhering inside the small FN microcavities might influence the above described behavior. On Hep-coated microstructures HSC proliferation seems to be highest in intermediate-sized microcavities (30 and 40 µm in diameter). Previous studies show that adhesion of HSCs to Hep is not as prominent as for FN or Col I [158] and therefore cell-cell contacts which are enhanced inside these intermediate-sized microcavities may play a stronger role possibly boosting proliferation. HSCs cultured on Col I fibril surfaces only show a slight increase in proliferation with increasing microcavity size. Since Col I fibrils already display a certain three-dimensionality HSC sensing of the spatial confinement by microcavities is presumably an aftermath of the overlapped effect of both factors.
Results and Discussion

Figure 22 Differentiation of HSCs on ECM Microcavities. (A) The proliferation of HSCs cultured on ECM coated microstructured surfaces was reduced when cultured on small microcavities after 7 days. CD34 and CD133 marker expression showed a higher fraction of undifferentiated cells in the 15 µm cavities. (B) Segregation of the adherent and supernatant cells reveals a higher ‘stemness’ of the FN and Col I adherent cells. No effect on the CD surface marker was observed for Hep surfaces. ‘Input’ cells are freshly isolated cells and data are presented as mean + SEM; n=3-8.

Overall, reduction in differentiation was found for cells cultured on Hep and Col I surfaces as they show higher rate of stem cell marker expression (93.7 % for Hep, 97.8 % for Col I) rather than on FN with 85.9 % stem cell marker expression. Only on the smallest (15 µm) FN-coated microcavities 96.1 % of cells express CD marker. Thus, matrix molecules presented in a three-dimensional manner distinctively influence differentiation behavior of HSCs.

When segregated, FN- and Hep-adherent versus non-adherent cells in the respective supernatant show the same proliferative trend as described above. But the number of CD marker negative cells adhering to FN is half compared to cells in the supernatant. Hence, adhesion signals provided by FN seem to supress differentiation of progenitors. It has been suggested that adhesive interactions of HSCs to FN regulate proliferation and differentiation of HSCs [46]. FN is implicated in the homing process [82] and its receptors α4- and α5-integrin have been shown to control proliferation and differentiation either directly or through modulation of cytokine signaling [159, 160, 161]. Thus engagement and additionally the number of engaged adhesion receptors might play a regulatory role in maintaining a more quiescent state of HSCs. This assumption will be further addressed in the following chapters of this work.

No impact of Hep-adherence compared to cells in suspension was found regarding cell differentiation. GAGs in vivo are mainly associated to proteins, hence rather displaying their action on cell behavior in combination. Additionally, as already mentioned above, adhesion receptor anchorage is not as strong as for protein ligands [148].
Col I adherent HSCs increase in number with increasing microcavity size whereas cells in the respective supernatants show an opposite trend. Furthermore the number of more primitive cells expressing both CD34 and CD133 marker is 2-fold greater for adherent HSCs compared to supernatant cells. Thus Col I adherence contributes to a more immature state of HSCs, too. The effect of Col I on HSC differentiation or proliferation has not been described yet. But the fact that HSCs specifically adhere to Col I [158] and that it is one of the major components of the bone marrow maintaining immature HSCs [67] implies a differentiation and proliferation decelerating role.

**HSCs within Redesigned Microcavities**

For a more quantitative evidence of the above stated differentiation and proliferation decelerating role of the confining of HSCs, PDMS moulds were redesigned to force cells to stay within microcavities (Fig. 20). After 7 and 14 days of culture on substrates with redesigned microcavities of different dimensions the non-adherent and adherent cell fractions were separately harvested from the surfaces and analyzed for CD133 and CD34 HSC surface marker expression as described above.

As described for the scaffolds with defined dimensions in between the microcavities proliferation is reduced inside small single cell microcavities for FN and Hep after 7 days of culture. For Col I a reverse effect appears with higher proliferation inside smaller cavities (Fig. 23 A). When separately examined the adherent cell fractions don’t show any dependence on the size of the spatial confinement regardless of the presented ECM (Fig. 23 B). The trend seen in the overall cell culture studies with reduced proliferation when cultured on small microcavities (for Col I vice versa) can only be observed within the supernatant cell fraction. However, the ratio of CD34 marker expression is higher when HSCs are adherent to the scaffolds approximately 90 % for all surfaces compared to approximately 80 % for cells in the supernatant. The effect seen for small Col I microcavities might be explained by the process of surface preparation; The Col I solution is probably too viscous to enter each of the smallest 15 µm microcavities and the fibrils are often longer than 15 µm thereby building a planar carpet-like network of Col I fibrils.
Results and Discussion

Figure 23 Differentiation after 7 Days of Culture on Redesigned Microcavities. (A) Overall Proliferation is increasing with larger microcavities for FN and Hep surfaces, whereas for Col I the reverse effect can be observed. (B) Segregation of adherent and supernatant cells shows reduced proliferation but also less CD marker negative cells for ECM bound cells compared to supernatant cells. Data are presented as mean + SEM, n=4.

After 14 days in microcavity culture overall proliferation rate is similar for FN and Col I cultured cells (12-fold) and slightly lower on Hep (10-fold) (Suppl. Fig. 35 A). Expansion of CD marker expressing cells doesn’t show any dependency on microcavity size. Segregation of adherent and supernatant cells again shows less CD marker negative cells for the adherent cells and thus the same absolute number of CD marker expressing cells for FN and Col I surfaces and their respective supernatant (Suppl. Fig. 35 B). On Hep surfaces adherent HSCs show almost no expansion which might partly be explained by the technical procedure of cell removal from the culture for analysis. HSCs growing on Hep have a relatively small adhesion area [158] compared to cells adhering to FN or Col I. This might account for the lower adhesion strength and result in the easier washing off the cells when taking out the supernatant fraction. However, the ratio of CD marker expressing cells within the adherent cell fraction is similar as for FN and Col I with approximately 60 % compared to 50 % in the supernatant (Suppl. Fig. 35 B). This proves the above discussed differentiation decelerating role for HSC-ECM-adhesion related signaling. The proliferation inhibitory effect of small microcavities as described in 4.2.1 for the cavities with distance in between is not as pronounced for the redesigned cavities but cognizable for FN- and Hep-coated surfaces. One reason might be the close proximity of cells sitting in neighboring microcavities. The cells possibly still sense each other and thus promote each other’s cycling via paracrine signaling [162] which was also observed for the intermediate-sized Hep microcavities described in 4.2.1.

Therefore both microcavity designs functionalized with FN were compared directly for overall proliferation after 7 days of culture using the same donors. Surprisingly, no dependency of
Results and Discussion

Proliferation upon microcavity size was seen for the cavities with distance in between. For the redesigned microcavities proliferation was reproducibly reduced with decreasing cavity size (Fig. 24). Although paracrine signaling cannot be ruled out for redesigned cavities, it might not be the major contribution, and HSC show reduced proliferation with decreasing microcavity size.

![Figure 24 Comparison of Different Cavity Designs. Microcavities were functionalized with FN and cultured with HSCs from the same donors for seven days. The proliferation of cells growing on cavities with distance in between doesn’t show any dependency on the microcavity size. On the contrary, within redesigned cavities, the proliferation decreased with decreasing microcavity size. Data are represented as mean + SEM, n=5.](image)

4.3.3 Colony-forming Ability of Microcavity Cultures

To score the potency of in vitro microcavity cultured HSCs colony forming unit capability was assessed. After 7 or 14 days of culture on ECM-coated redesigned microcavities, cells were plated into methylcellulose medium which contains a complete mix of cytokines and growth factors to promote forming of colony-forming units-granulocyte-erythroid-macrophage-megakaryocyte [CFU-GEMM], burst-forming units-erythroid [BFU-E], colony-forming units-erythroid [CFU-E], colony-forming units-granulocyte-macrophage [CFU-GM], and colony-forming units-granulocyte [CFU-G] depending on the potency HSC maintained after culture (Fig. 12).

HSCs were grown for 7 or 14 days on ECM covered microcavity culture carriers, detached and adherent and supernatant cells were separately cultured in CFU media by equal number of 500 per cell fraction. CFUs were scored after 14 days.

For HSCs cultured in suspension the CFU forming capability decreased with increased time in culture and decreasing CD34 and CD133 marker expression (Fig. 25). Especially the CFU-G and CFU-E forming units were reduced after 14 days in culture.
Results and Discussion

Figure 25 Colony Forming of HSCs Cultured in Suspension. With increasing time in culture and decreasing stem cell marker expression the number of CFU decreases to one half after 14 days. Data are represented as mean ± SEM, n=5.

For the microcavity adherent cells the ratio of CD marker expression is higher compared to supernatant cells (Fig. 26 A), it was expected that the CFU forming capability of formerly ECM attached cells is higher than for suspension cells.

After 7 days of microcavity culture cells show similar numbers of CFUs when scored for all surfaces independent of matrix coating and microcavity size (Suppl. Fig. 36).

To exclude the contingency, those 7 days of microcavity culture are too short that the effects observed for the CD marker expression is reflected within the CFU forming profile, HSC were cultured additional 7 days and then scored for CFU capability.

Overall CFU forming after 14 days of microcavity culture was reduced to one half as compared to after 7 days. This correlates to the decrease in surface marker expression profile (Fig. 26 and Suppl. Fig. 36). The CFU-M stayed similar with approximately 18 colonies per surface and CFU-E was drastically reduced from approximately 23 colonies to 4 colonies per surface as also seen for control cells in suspension (Fig. 25). This suggests that the selected set of surface markers tested here correlates well with the CFU potency of the cells with the exception of GFU-M which is not related to the CD34/133 expression and possibly a potency kept throughout longer term differentiation.

Unexpectedly colony forming was reduced within the surface adherent cells although the stem cell CD marker expression according to the FACS profile is higher compared to supernatant cells (Fig. 26 A). For FN and Col I the CFU potency was even decreased inside small single cell microcavities which have been shown from FACS profile to maintain stem cell marker expression. One possible explanation might be the trypsinization of adherent cells for surface detachment since supernatant cells weren’t treated with trypsin (Suppl. Fig. 37). Although cells were recovered in 10% FCS/ PBS and no profound effect was found for the cytometric profile,
the trypsinization procedure probably affects HSCs, which is only seen in long term culture. Flushing the cells off the surfaces and ripping them out of the microcavities, although performed as gently as possible is a quite harsh procedure which probably further influences long term survival and potency of HSCs. This also applies for collagenase-treated cells detached from Col I microcavities. Hence no conclusion on the effect of microcavity culture on the CFU forming potency can be drawn.

Figure 26 CFU Forming after 14 Days of Cell Culture on Microcavities. CFU scores indicate reduced potency for surface adherent cells (B) although the FACS profile shows higher CD marker expression (A). Data are represented as mean ± SEM, n=3.

As tested afterwards, trypsin apparently affects long term culture and thus reduces colony forming capability. Therefore the following experiments with mouse BM culture were carried out using accutase instead of trypsin for detaching the cells from the ECM microcavities. Accutase-detached HSCs yield a significantly higher CFU forming potential when directly compared to trypsinized cells although they display a similar cytometric profile (Suppl. Fig. 37). FACS analysis was directly performed after detachment and recovering of cells. Possibly the surface molecule digestion resulting from the trypsinization was not fully preceded during cytometric analysis but impairs long-term CFU cell culture.

4.4 Single Cell Analysis of Differentiation

While the observed effects of microstructured substrates on the entire HSC population provide valuable insights, the applied integral analysis (FACS) could not distinguish between cells growing inside and outside the microcavities. Therefore HSC marker expression was additionally analyzed by individual adherent cells via immunofluorescence.
Results and Discussion

Figure 27 Single Cell Analysis of Differentiation. (A) Immunofluorescent single cell analysis of adherent cells revealed higher stem cells marker expression by HSC residing inside microcavities compared to those outside. (B) Image of fluorescent CD staining of HSC cultured on microcavity surfaces (DAPI – blue, CD133 – red, CD34 – cyan, scale bar 40 µm). Data are presented as mean ± SEM; n=3.

Distinct differences between cells residing inside and outside the microcavities were found (Fig. 27). For all ECM molecules around 96% for FN and even more approximately 99% for Hep and Col I of HSCs adhering inside the microcavities maintained CD133 and CD34 expression after seven days, whereas only 79% for FN, 91% for Hep and 97% for Col I of cells adhering outside showed HSC marker expression. Within these populations, the fraction of cells expressing the early progenitor marker CD133, either together with CD34 or alone, was again higher inside the microcavities (91% on FN, 98% on Hep and Col I) as compared to cells growing outside (68% FN, 90% Hep, 94% Col I). Interestingly, no pronounced dependence on microcavity size was observed for HSC expression of CD133 and CD34, which is why results obtained from different microcavity sizes were presented in a single graph (Fig. 27).

These single cell results resemble the trends observed within the population level in chapter 4.2.2., although these data are not directly comparable, since FACS and LSM analysis display different sensitivity to fluorescence intensities. Summing up, adhesion inside microcavities and hence higher obligation of adhesion receptors promote a more immature state of progenitors.

4.5 Cell Cycling Dependency on Cytokine Level

For further insight into the regulation of HSC behavior by spatial confinement, cycling was investigated at single cell level by repeatedly scoring DNA synthesis of cells on microstructured surfaces. FACS analysis on plane substrates again served as control. Additionally, since cytokine signaling is a critical parameter for the proliferation of HSC cultures and is known for its synergistic effect on adhesion-related signaling [80], another series of experiments was carried out using higher cytokine concentrations (30 ng in comparison to 10 ng each of TPO, SCF, and FL3).
4.5.1 Plane Surfaces

HSC adhesion to ECM-coated planar surfaces for one day led to enhanced cell cycle entry in the case of FN or Hep (Fig. 28 B). This effect was further enhanced by increased cytokine concentration (Fig. 28 B). Compared to adherent cells, non-adhering HSCs always showed delayed cell cycle entry irrespective of the matrix coating and the cytokine concentration (Fig. 28 A). After 3 days HSCs approached a constant cycling state until the end of culture (7 days). In this state cell cycling was no longer dependent on adhesive contacts of the HSCs for low cytokine concentrations (10 ng). At higher cytokine concentrations (30 ng), only about 50% of cells adhering to the FN or Hep layers replicated their DNA within 24 hours, compared to more than 70% of the non-adherent cells after a similar culture period, whereas around 75% of cells on Col I synthesized new DNA independent of surface adherence (Fig. 28 A, B).

Cells adhering to FN and Hep entered into the cell cycle earlier than cells in suspension or adherent to Col I, followed by a deceleration in the cycling kinetics of the adherent cells at later time points. These adhesion-related effects were further amplified by increasing cytokine concentration, suggesting an ‘inside out - outside in’ signaling mechanism through which bound integrins synergize with cytokines to increase cell proliferation as also reported by Lévesque et al. [81]. In contrast, antibody blocking experiments illustrated previously that HSC adhesion to FN via integrins induces proliferation inhibitory signals under culture conditions containing physiological cytokine concentrations [80]. However, this inhibition of adhesion receptor signaling was repressed by increasing cytokine concentration, which drives HSCs to enter S-phase. These results nicely display the synergistic action of adhesion- and cytokine-related signals. Col I slowed down early cell cycle entry but at later time points accelerated cycling compared to FN or Hep independent of cytokine concentration (Fig. 28 A, B). Thus each ECM molecule has its own effect on the cycling machinery of HSC which is influenced by cytokine concentrations. In addition to the conjunction of the adhesion- and cytokine-related intracellular signal transduction involved in cell cycling, the ability of the ECM molecules to bind cytokines might be of further importance. As described in 2.1.4, tethered cytokines affect cell behavior in a different manner than soluble growth factors. FL3 and SCF both cytokines, which appear in vivo as soluble and bound isoforms, were added to this experimental setup and most probably bound partially to the ECM coating. Thereby they are available in solution and also display their action upon HSCs in a surface bound manner. In the case of SCF the soluble isoform is suggested to have a mobilizing effect on HSCs and to be growth promoting. In contrast the tethered isoform enhances adhesion and maintains immature HSC, reviewed in [163]. At the beginning of culture all cytokines are available in solution elevating integrin activation (‘inside-out’ signaling) leading to faster adhesion. In a second step, integrin-ECM binding cooperates with mitogenic growth factor action (‘outside-in’) [81]. This explains the increased DNA synthesis for FN and Hep adherent HSCs after one day of culture which is even more enhanced upon higher cytokine levels. Over time in culture cytokines diffused to the surface and partly bound to ECM molecules which for SCF (and similarly suggested for FL3 but not proved, yet [163]) may lead to enhanced adhesion, hence proliferation inhibition by integrin action [129] and maintenance viz. slower cycling of HSCs. This explains the lower DNA replication rate for adherent cells at high cytokine concentration after 3 and 7 days in culture compared to supernatant cells Fig. 28 B). For Col I at the beginning of culture adhesion related inhibition of proliferation due to the enlarged ligand density resulting from the Col I fiber.
three-dimensionality might overbalance cytokine induced cycling. Due the 3D gel character of Col I, it might also adsorb and retain cytokines in higher amounts in contrast to FN and Hep. In that way, cytokines act upon HSCs on Col I surfaces in a more sustained manner. Furthermore, cytokines induce a dose-dependent receptor activation [172]. For example, higher concentrations of SCF initially cause a more rapid receptor internalization and extracellular cytokine depletion compared to lower concentrations [164]. Such processes are presumably boosted by the synergistic action of cell matrix adhesions leading to even higher cytokine exhaustion within the media, which then in turn permits the action of adhesion related proliferation inhibitory signals after cytokine reduction. Thus HSC adherent to FN or Hep at 30 ng conditions would consume the most cytokines within the shortest period of time explaining the initially high (after 24h) and later (3 and 7 d) lower rate of DNA synthesis compared to control cells or cells exposed to 10 ng cytokines. Concerning the high cytokine conditions on Col I surfaces; the partially adsorbed cytokines might result in a more sustained action upon HSCs, as described above, which led to higher proliferation rates after 7 days, when compared to FN and Hep and low the cytokine data.
Figure 28 DNA Synthesis on Planar ECM-coated Surfaces. (A) Comparison of the cycling rate between surface adherent and supernatant cells shows after one day an increased cycling for FN and Hep adherent cells. Col I adherence and suspension delay cell cycle entry. This effect is further clarified by increasing the cytokine concentration from 10 ng to 30 ng. After three and seven days FN and Hep adherence decelerates cycling compared to respective supernatant cells or Col I adherent HSCs. (B) To point out the differences the rate of DNA synthesis is compared between adhesion to the distinct ECM molecules. (C) After 24 h of BrdU uptake, cell cycling was determined by cytometric analysis. Data are represented as mean ± SEM (A, B), n=1-3.
4.5.2 Microcavities Reduce Cycling Frequency

For the low cytokine scenario (10 ng), after three days in culture, HSC cycling inside FN microcavities was found to decrease with decreasing microcavity size, ranging from approximately 60% for the largest (80 µm) microcavities to approximately 30% for the smallest (15 µm). Cells adhering outside the microcavities showed 60% BrdU incorporation independent of cavity size. After seven days in culture, cell cycling was higher with approximately 70% of HSCs showing BrdU incorporation irrespective of microstructural constraints (Fig. 29 A). In this state cell cycling was no longer dependent on adhesive contacts of the HSCs for low cytokine concentrations. At higher cytokine levels (30 ng), DNA replication occurred in 50% to 60% of HSCs adhering inside microcavities after three days, independent of the cavity dimensions (Fig. 29 A). After 7 days, an increase of 50% to 70% BrdU incorporation into HSCs was observed as microcavity size increased. The significant reduction in cycling seen for cells grown in the smallest microcavities at low cytokine concentrations (10 ng) was not observed at higher cytokine concentrations (30 ng). For Hep and Col I similar tendencies are observed (lower proliferation with decreasing microcavity size, lower cycling rate inside microcavities compared to respective plane outside) however, not as pronounced (Fig. 29 A).

In line with previous reports [80], and the results presented above for cycling rate on plane ECM substrates, adhesion to FN induces proliferation inhibitory signals under culture conditions with low cytokine concentrations. A cycling inhibitory effect is detected with an increase of the cell-FN-adhesion area inside small microcavities, which is suppressed in the presence of high cytokine levels, far beyond physiological conditions [74], as adhesion-related signals are overbalanced under these conditions.

The changes in cycling rate, observed at the single cell level may lead to the pronounced effect on the overall culture population proliferation and differentiation described in chapter 4.3.2, which results from the exponential amplification of the cavity size-dependent cycling rates due to growth kinetics of the cells.

This finding suggests that keeping HSCs in a three-dimensional arrangement of ECM ligands is critical to maintain the cells in a quiescent and multi-potent state. FN which is proved to play an important role in the homing process of stem cells [82, 83] might also play the crucial part in maintaining HSC in an immature state inside the BM, as for the other BM-relevant ECM molecules tested in this setup (Hep and Col I) the proliferation inhibitory effects are not as pronounced. Although Col I might be considered for short-term cultures up to 24 h since it initially inhibited cell cycling (chapter 4.4.1). Or, cytokines are already introduced during Col I curing onto the PEMA surfaces which might provide stable bound growth factors to the cells. Of course, further ECM molecules which are suggested to be functionally important in the BM [165, 77, 66, 166] need to be examined for their ability to influence spatially confined HSCs.
Results and Discussion

Figure 29 DNA Synthesis Inside vs. Outside Cavities. Cell cycling was determined by immunofluorescence analysis after 24 h of BrdU incorporation (B). A decrease in HSC cycling rate occurs at the low cytokine concentration for FN adherent HSCs but is suppressed at higher cytokine concentration. For Hep or Col I no pronounced effect of microcavities as for FN is observed (A). Data are presented as mean ± SEM. Color index in bottom panels B: BrdU – green, PI – red, scale bar 80 µm.

4.6 Mice Repopulation of Microcavity Cultured HSCs

Although, as previously shown (chapter 4.3.2), HSC cultured with small ECM-coated microcavities maintain their stem cell marker, their functionality remains to be proved in vivo. Competitive repopulation studies in mice are planned to verify if microcavity culturing increases repopulation capability.

In a first trial, whole mouse BM was isolated and cultured onto FN-functionalized moulds with and without distance in between microcavities with the cavity sizes of 15 and 40 µm. Total cell expansion and the respective fraction of KSL+ cells was measured. For redesigned microcavities mouse BM cells expand double compared to cavities with distance in between (Fig. 30 A). Also the percentage of KSL+ cells is higher with 8 % for 15 µm cavities and 9 % for
40 µm cavities compared to 6 % and 5 %, respectively (Fig. 30 B). Hence, side by side microcavities show improved maintenance of KSL+ cells and will be used for future transplantation experiments.

In a first repopulation experiment purified KSL+ cells from C57BL/6N mice were cultured onto functionalized redesigned microcavities and transplanted after 10 days into lethally irradiated SJL mice. Therefor, the microcavity sizes 15 and 40 µm were taken incl. control in suspension. The detached cells were measured for the amount of KSL+ and subsequently transplanted into irradiated recipient SJL mice at 1 x 10⁵ cells plus 1 x 10⁵ freshly isolated helper KSL+ from the transplanted mice. Repopulation was analyzed after 21, 36, 57, and 78 days. The cultured and the helper cells within repopulating cells were distinguished via surface CD45.2 (C57BL/6N) and CD45.1 (SJL) markers to prove the functionality of the microcavity cultured KSL+. Eleven weeks after transplantation cells from 15 µm microcavity culture increase in their contribution to approximately 84 % repopulation of SJL mice whereas repopulation is continuously decreasing for 40 µm and suspension cultured KSL to 55 % and 33 % respectively (Fig. 30). Thus the first trend shows functional HSCs after 10 days of culture within 15 µm microcavity confinement which outcompete freshly isolated cells as proved by in vivo competitive repopulation experiments.

Figure 30 Expansion of KSL on Microcavities. (A) Whole mouse BM was grown for 14 days on FN functionalized microcavities. Highest proliferation of cells was achieved on redesigned microcavities. Besides, the percentage of primitive KSL cells was also highest within redesigned microcavity cultures (not shown). (B) Mouse KSL cells were grown on FN functionalized redesigned microcavity for 10 days and transplanted into mice for competitive repopulation. The contribution of KSL is highest for 15 µm microcavities cultured cells compared to 40 µm or control KSL 11 weeks after transplantation. (n=1)
### 4.7 Summarized Discussion of the HSC–ECM Relation

Since the unique characteristics of the bone marrow stem cell microenvironment depend on a delicate balance of soluble and insoluble cues, a defined ECM-coated microcavity assay was used to investigate the interplay of cytokine signaling and adhesion-related signals in controlling HSC fate in vitro.

The results presented here highlight the dependence of HSC proliferation upon microcavity size and demonstrate the important role of spatial confinement upon HSC cycling, when cells are grown on adhesive substrates. The observations made, support the idea of a spatially induced quiescent state over longer time periods inside the BM whereat the greater engagement of adhesion receptors as inside small microcavities is proposed to be one of the main mechanisms responsible for HSC maintenance. Additional contribution by paracrine signaling activated by cell-cell contacts and close proximity of residing HSCs as proposed for intermediate cavity size and redesigned microcavities and described previously [162, 167] might also play a considerable role in stem cell fate decisions. However, the results demonstrate that microstructured adhesive culture carriers can support HSC quiescence in vitro if the size of the constraining cavity is appropriate to host individual cells. Furthermore, the data indicate that in HSC cultures a minor local variation in the cycling rate, observed at the single cell level, leads to a pronounced integral effect on the overall culture population. Such effect most likely results from the exponential amplification of the cavity size-dependent cycling rates when considering the growth kinetics of cells.

![Figure 31 Balance of Cytokine Level and Adhesion in Cell Cycling.](image)

At moderate cytokine levels increasing adhesion overcomes cytokine stimulation of proliferation and inhibits cell cycling. Whereas adhesion related signals are overbalanced from high cytokine concentrations enhancing cell cycling.

HSCs adhering to either FN or Hep entered into the cell cycle earlier than cells in suspension. In contrast cells adherent to Col I show delayed cell cycle entry. These adhesion-related effects for FN and Hep were further amplified by increasing cytokine concentration. Adhesion has been shown to synergize with the mitogenic action of cytokines but also to induce proliferation inhibiting signals [81, 80]. Considering the lower cycling rate of adhesion inside vs. higher cycling outside microcavities in a low cytokine scenario, this clearly proves the interconnection between adhesion- and cytokine signaling. At higher cytokine concentrations far beyond physiological range adhesion-related inhibition of proliferation is overbalanced.
Results and Discussion

(Fig. 31). This statement suggests that the inhibitory effect of small adhesive microcavities on cell cycling manifests itself only at lower cytokine levels, resembling conditions found in the in vivo bone marrow stem cell microenvironment. At the beginning of culture cytokines promote adhesion and in turn adhesion to FN and Hep accelerates cell cycle entry – a process that is additionally supported by higher cytokine levels. Initial adhesion to Col I suppressed early cell cycle entry compared to FN and Hep which might be explained by the higher adhesion ligand density due to the three dimensional nature of Col I fibrils by this providing more adhesion inhibitory signals overwriting the cell cycle stimulation by cytokines. Later on, for all three ECM molecules the culture system balances itself at a steady state of cycling and non-cycling HSCs. Not only the concentration but also the coupling of growth factors to the provided ECM during culture most probably plays a considerable role in HSC cycling additionally supporting the observations made in chapter 4.5. As for the bound isoforms of SCF and FL3 a HSC maintaining and adhesion supporting role is suggested compared to the mobilizing and proliferation stimulating effect of the soluble isoforms [163]. In summary, higher adhesion receptor engagement inhibits cell cycling up to a certain cytokine concentration. Too high cytokine concentrations in turn overbalance the inhibitory adhesion-related effect and stimulate proliferation (Fig. 31).

The finding of enhanced stem cell marker expression in response to restriction of the microcavity size suggests that keeping HSCs in a three-dimensional arrangement of ECM ligands might facilitate the conservation of their immature state in vitro. This observation is particularly important since HSCs in standard cell culture show a tendency to lose stem cell surface markers and differentiate [5]. Thus, it is quite likely that increasing the adhesive cell-matrix contact area, and consequently the number of engaged integrins, is critical to maintain HSCs in a quiescent and multipotent state.

Enhanced cytokine-driven proliferation is known to impair the expression of adhesion receptors and thereby reduces homing efficiency after transplantation [79]. Thus, the observation that engagement of adhesion molecules in a spatially defined manner at moderate cytokine levels decreases proliferation and differentiation may preserve integrin receptors and offer better repopulation capabilities.

The preservation of stem cell markers during culture could also be relevant for gene transduction approaches. Most gene transduction strategies require cell cycle entry and this usually leads to loss of the stemness of cells. Microstructured culture carriers could potentially preserve the transduced subset of cells and optimize the efficiency of HSC gene transduction protocols. For example, it has been shown that after cytokine-driven retroviral transduction, late dividing cells with preserved CD133+ expression and carrying the transgene had the highest mouse-repopulation potential [168].

One factor, which hasn’t been considered in this work, but indeed plays an important role in stem cell fate decisions, is the surface elasticity or stiffness [124]. The BM with its diverse structures features a wide range of different elasticity’s, from soft stroma cells [169] to hard trabeculae [170]. It is hard to predict the niches surface mechanics since its exact position is not known, yet. But it has been found that during mobilization upon G-CSF treatment bone-lining osteoblasts exhibit a flattened appearance with shorter projections into the matrix [171] and thus are stiffer compared to non-treated cells [169]. The different coated PDMS
microcavities presumably provide different elasticity because of the varying ECM layers. Col I is most probably softer than FN or Hep due to its gelling properties within aqueous solutions. This also might play a role for the different proliferation and differentiation patterns of HSCs seen for the diverse matrix coatings. Mechanical surface characteristics will be investigated and considered for future experiments.

The functionality of this 3D microcavity setup is currently investigated in mice repopulation studies. The first trend shows a higher functionality of single cell microcavity cultured cells compared to suspension or multicell-microcavity culture, which will be further investigated.

### 4.8 Future Prospects

Thus for the homeostatic regulation of HSCs within the BM a tight balance between environmental signals is obligatory for the maintenance and differentiation of stem cells. Further aspects like matrix elasticity and of course more BM relevant matrix molecules, e.g. laminin, osteopontin, thrombospondin, hyaluronic acid and BM isolated ECM mixtures will be tested upon their functionality on HSC fate decisions.

Single cell tracking of HSCs cultured on microcavity platforms will give additional information about the divisional behavior of the cells in dependency on matrix and confinement and allows on one hand to follow several generations of daughter cells including their fate decisions and on the other hand for computational modeling of their behavior to strengthen predictions of in vivo events e.g. during repopulation after chemotherapy.

One first functional repopulation study of microcavity cultured mouse HSC showed a higher repopulative ability of single-cell FN microcavity-cultured cells compared to multi-well or suspension cultured HSCs. This of course has to be repeated and proved in ongoing experiments.

Thin film lithographic attempts to make the microcavity culture platform more qualitative for quantitative cell assays like FACS or CFU weren’t sufficiently successful in blocking protein binding and hence cell attachment in between microcavities. Therefore the microcavity moulds were redesigned with the distance in between as small as possible to force the HSCs to stay inside the confining microcavities. The effects on HSC proliferation and differentiation seen within these redesigned microcavities resembled the trends described for microcavities with distance in between, but appeared not as pronounced. This might be due to paracrine signaling in between HSCs residing in close proximity in microcavities next to each other interacting on the cycling behavior. Therefore further strategies will be applied in future for surface passivation in between microcavities. Thick PEG hydrogel layers in between microcavities for protein blocking are one approach. Another trial yields the usage of tagged ECM or signaling proteins which bind to a priory microcavity embossed linker molecule. Such strategy has the additional advantage of the correct orientation of the immobilized protein.
5 Summary

In vivo geometrical constraints ranging from single- to multi-cell compartments are present in the stem cell niche. The aim of this thesis was to (i) examine the impact of geometry on HSC proliferation and differentiation and (ii) the importance of soluble cytokines on geometry induced effects. Therefore a microcavity culture system was designed to recapitulate geometrical constrains found in vivo that accommodated single cells (10 and 15 μm diameter of cavity) and multi-cell-groups (30, 40, and 80 μm). To facilitate cell attachment, the wells were coated with bone marrow relevant ECM (fibronectin, collagen I, heparin) and the system was supplemented with a range of growth factor concentrations. This setup allowed for studying (i) the dependence of HSC proliferation and differentiation on microcavity size, (ii) the extent of integrin activation on cell cycle progression, (iii) the interplay between adhesion- and cytokine related signaling on cell proliferation and stem cell differentiation.

Cells in single-cell microcavities were exposed to more adhesion ligands (i.e. ECM) due to the 3D nature of the tight fitting wells compared to multi-cell wells or plane surfaces. In small cavities a greater engagement of adhesion receptors reduced the number of cycling and proliferating cells when compared to multi-cell compartments or 2D substrates. Inhibition of proliferation due to adhesion was only observed at low cytokine conditions\(^7\). Cytokine concentrations well above the physiological range masked geometry based effects. Thus adhesion and cytokine signaling have an antagonistic effect on cell behavior at 3 and 7 days in culture. However, during the first 24h in culture, adhesion and cytokines also showed synergistic effects by inducing early cell cycling. HSC initially adhering to fibronectin or heparin entered cell cycle early compared to cells in suspension or adherent to collagen type I. This observation could be further augmented by high cytokine concentrations. At the beginning of culture, cytokine signaling promoted adhesion and the adhesion signaling in turn cooperated with cytokine receptor signaling, which led to enhanced cell cycling. Collagen type I fibrils on contrast provided more adhesion sites due to their 3D nature compared to FN or Hep, thereby possibly facilitating adhesion-related inhibitory signals, which overwrote cytokine signaling and inhibited early cell cycle activation. Furthermore, initially boosted proliferation of fibronectin and heparin adherent HSCs presumably led to early cytokine exhaustion and resulted long-term in a lower cell cycling rate. In contrast, the gel-like collagen I substrates probably adsorbed and retained cytokines, which sustained higher cell cycling at prolonged culture.

Considering differentiation, the highest expression rate of the stem cell markers CD34 and CD133 was found for single-cell microcavities for all three investigated ECM molecules. Hence confinement of HSCs preserves a more immature stem cell state.

The defined 3D HSC culture system described in this work allows the systematic investigation of single ECM and signaling molecules and combinations thereof without the complexity of coculture or in vivo studies. The results suggest that confinement of HSCs leads to reduced proliferation in vitro, keeping cells in a more immature state. The developed setup is believed to become a useful in vitro HSC assay platform thus strengthening the predictive power of human HSC culture studies.

\(^7\) Low cytokine conditions: 10 ng each; High cytokine conditions: 30 ng each TPO, SCF, and FL3
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(1) Extracellular matrix (ECM) molecules can be covalently bound to poly(ethene-alt-maleic anhydride) (PEMA) functionalized poly(dimethylsiloxane) (PDMS) microcavities.

(2) Poly ethylene glycol (PEG) and Polyoxazoline (PMOXA) thin layers are not sufficient to block protein binding and thereby doesn’t prevent cell adhesion.

(3) The 3D microcavity setup provides an easy access and is therefore suitable for analytical methods such as cytometry and quantitative microscopy.

(4) Hematopoietic stem cells (HSC) adhere, proliferate and differentiate within fibronectin (FN), collagen I (Col I), and heparin (Hep) coated microcavities.

(5) HSC adhering within small single cell microcavities functionalized with either FN, Hep, or Col I proliferate and differentiate less compared to cells in suspension, or bigger multi cell microcavities.

(6) ECM adhesion of HSC keeps HSC in a more immature state as they express more stem cell surface markers CD34 and CD133 than cell in suspension. This effect is further amplified by adhesion inside microcavities.

(7) The higher the adhesion of HSC the more HSC proliferation and differentiation is inhibited.

(8) The higher the cytokine concentration the more HSC proliferation and differentiation is stimulated.

(9) The balance between adhesion and cytokine concentration tightly regulates cell cycling.

(10) Soluble cytokines initially promote activation of adhesion receptors which in turn synergizes with growth factor signaling resulting in an early cell cycle start of HSC. In turn, once the culture system equilibrates, adhesion vs. cytokine signaling show antagonistic effects on cellular cycling.

(11) HSC cultured in single fibronectin-coated microcavities are functional in competitive repopulation of lethally irradiated mice.
6 Appendices

6.1 FACS Principle

Cells in suspension single-filed in a fluidic stream (via hydrodynamic focusing) get illuminated by laser light whereby they scatter light and emit fluorescence which is detected by a number of detectors (one in line with the light beam: forward scatter FSC, and several perpendicular: sideward scatter SSC and one or more fluorescence detectors) and converted into digital values displayed at the computer. The FSC correlates with the cell volume and the SSC depends on the inner complexity e.g. shape of nucleus, granularity, or membrane roughness. These two parameters are used to set the final cell population to be analyzed. The fluorescence is displayed in dependency on the relative intensity for each cell (Fig. 32).

![Figure 32 FACS Principle. Cells are gated according to their phenotype (volume and granularity). (B) Within a gated population cells can be distinguished with their fluorescence intensity.](image)

6.1.1 HSC Staining for CD Marker and Cell Cycle Kinetics

For FACS detection of surface CD marker supernatant and adherent cells were separately harvested into FACS tubes as described above, counted, blocked with 10 % FCS/ PBS for 30 min, resuspended in 80 µl antibody solution with the respective antibodies diluted in buffer 0,5 % FCS/ PBS (respective dilution in table 3), incubated for 10 min in the dark at 4 °C, washed with 1 ml buffer, and resuspended in 300 µl for detection. Staining was immediately quantified (changed CD marker profile was observed after overnight storage).

For immunofluorescence microscopy, cells were prefixed with 2 % PFA, directly put into the cell media, for 2 min, blocked 3 x 5 min with 10 % FCS/ PBS, labeled with the desired CD antibody for 10 min at 4 °C, washed 3 x 5 min with 1 % FCS/ PBS, labeled with the respective secondary Alexa Fluor antibody for 45 min at room temperature, washed again 3 x 5 min with 1 % FCS/ PBS, and finally covered with mowiol. Samples can be stored in the fridge for a couple of days.

For the analysis of cell cycle kinetics, cells were incubated with 20 µM BrdU 24 h prior fixation in ice-cold 70 % ethanol for 30 min on ice. After fixation samples were washed 3 x 5 min in ice-cold PBS, and incubated with 0,5 % RNase for 30 min at 37 °C (only for FACS, not immunofluorescence) to avoid possible RNA staining with PI. For cell permeabilization and to unwind the DNA strands for better antibody accessibility cells were treated with 2N HCL and 0,5 % Triton X-100/ PBS for 30 min at room temperature. Cells were then washed 3 x 5 min
with buffer 0.5 % FCS/ PBS to remove all HCL (otherwise the antibody might be destroyed),
stained with the primary anti BrdU antibody 30 min in the dark at room temperature, washed
3 x 5 min with buffer, incubated 45 min at room temperature with secondary Alexa Flour 488,
washed again, incubated with 2 µg/ml PI in PBS for 30 min, washed, and either mounted with
mowiol for immunofluorescence microscopy or resuspended in 300 µl buffer for FACS analysis.

6.1.2 Apoptosis Test

The viability of HSCs was proved via freshly prepared 0.01 mg/ml FDA (viable cells)
counterstained with 2 mg/ml PI/ PBS (dead cells) and immediately detected with FACS and
immunofluorescence microscopy. Within the HSC population gate in FACS 99 % of cells are
viable. Immunofluorescence microscopy showed approximately 90 % viability (Fig. 33) for all
surfaces and microcavity designs.

![Figure 33 Proof of Cell Viability. HSC show more than 99 % viability with FACS gating strategy (A, B). Approximately 90 % of the cells are alive within immunofluorescent images (C, green) independent of cavity size.](image)

<table>
<thead>
<tr>
<th>Cavity size [µm]</th>
<th>15</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>80</th>
<th>control</th>
</tr>
</thead>
<tbody>
<tr>
<td>dead cells %</td>
<td>0.31</td>
<td>0.22</td>
<td>0.31</td>
<td>0.21</td>
<td>0.23</td>
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<td>living cells %</td>
<td>99.50</td>
<td>99.69</td>
<td>99.54</td>
<td>99.47</td>
<td>99.34</td>
<td>99.52</td>
</tr>
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</table>
Figure 34 Cell Viability on redesigned microcavities. Immunofluorescent microscopy shows more than 90 % viable cells (A, B green) growing inside microcavities. FACS gating provides ca. 99 % HSC alive. (n=1, green FDA, red PI, scale 80 µm, data presented as mean ± SD)

### 6.2 Differentiation and Proliferation on Redesigned Microcavities

Figure 35 Differentiation after 14 Days of Culture. (A) Overall Proliferation shows no dependency of CD marker expression on microcavity size. HSCs cultured on Hep surfaces show decreased proliferation compared to FN or Col I cultured cells. (B) Separately viewed adherent and
supernatant cell fractions show higher maintenance of stem cell marker expression for surface adherent cells. Data are represented as mean + SEM, n=3-4.

No dependency of proliferation or CD marker expression on microcavity size was seen after 14 d of culture. However, a higher fraction of surface adherent HSC still expresses stem cell CD marker as compared to cells in the supernatant (Fig. 35).

6.3 Colony-forming Capability of Microcavity Cultured Cells

As already stated above, after seven days of microcavity culture cells show similar numbers of CFUs are scored for all surfaces independent of matrix, microcavity size or adhesion, although there are less CD marker negative cells within the adherent fraction (Fig. 36).

6.4 Effect of Trypsin on HSC Properties in Long Term Culture

Since trypsin hydrolyzes surface proteins during detachment procedure of cells and as suggested in the results and discussion part of this work, it might have a harmful long term effect on HSC concerning their stem cell potential. Therefore accutase an alternative for trypsin was tested in direct comparison for FACS staining and CFU-forming capability. No differences were observed between accutase (10 min) and trypsin treatment (4 min) within the FACS profile of CD marker staining (Fig. 37). However, after 14 days CFU cultured cells treated with accutase show a much higher colony forming ability compared to trypsinized ones. Thus trypsin seems to affect the long term potential of HSCs. It is therefore suggested to use accutase for further in vivo studies on HSC cultures within microcavities.
Figure 37 Trypsin Effect on CFU Forming Capability. FACS Profile (A) and CFU forming (B) were compared after trypsin or accutase treatment to detach cells from same donor from similar surfaces. Cells treated with accutase have higher CFU count compared to trypsinized cells (B), although the FACS profile only shows slightly decreased CD34+/CD133+ (approximately 5%) numbers for the trypsinized HSC compared to accutase (A). (Data are presented as mean of a triplicate trial for one donor ± SEM)

6.5 Surface Functionalization with SCF

Glass cover slips were cleaned (70 °C for 10 min in H2O2, NH3, and MilliQ water, 1:1:5 respectively) and aminosilanized 2 h in 20 mM 3-aminopropyltriethoxysilane/isopropanol/MilliQ 9:1 solution, and dried for 1 h / 120 °C. The cover slips were immediately spincoated with 0.16 % poly(octadecene-alt-maleic anhydride) (POMA)/tetrahydrofuran (THF), dried again 2 h/120 °C for anhydride recovery of the POMA and linkage to the surface. To wash away unbound POMA the cover slips were washed twice in THF.

For spacer coupling to the POMA cover slips the tert-butoxycarbonyl (Boc) PEG7 spacer was deprotected by stirring 2 h in dichloromethane and trifluoroacetic acid (TFA) in the absence of air and following removal of TFA in the rotary evaporator. The PEG7 was then dissolved in borate buffer (0.1 M) at a solution concentration of 10 mM, pH 8.0. Freshly annealed (2 h at 120 °C) POMA-coated cover slips were incubated with the PEG7 solution over night at room temperature, rinsed 3 times in MilliQ, dried under nitrogen and annealed again 2 h at 120 °C to convert the formed PEG7 amide into the stable imide.
The SCF was immobilized for 3 days onto freshly annealed POMA or POMA/PEG7 cover slips. Prior to SCF immobilization POMA/PEG7 was activated on ice for 1 h using freshly prepared sterile filtered 50 mM 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 25 mM N-Hydroxysulfosuccinimide (Sulfo - NHS) solved in 1/15 phosphate buffer, pH 7.4, washed twice in MilliQ and subsequently coated with SCF/PBS. Unbound SCF was removed by washing several times in PBS. Before cell culture SCF-coated coverslips were preincubated with CellGro medium over night.

HSCs were cultured in CellGro media supplemented with 10 ng each thrombopoietin (TPO) and FMS-like tyrosine kinase 3 ligand (FL3). For control SCF was added in solution in the same amount as immobilized on the surface though having the same amount of SCF/well for each setup (Tab. 5).

<table>
<thead>
<tr>
<th>SCF/ Well</th>
<th>Surface Density</th>
<th>Immobilization Concentration (µg/ml)</th>
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</thead>
<tbody>
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<td>SCF [ng/ well]</td>
<td>SCF [ng/cm²]</td>
<td>POMA</td>
</tr>
<tr>
<td>20</td>
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<tr>
<td>30</td>
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</tr>
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</table>

6.5.1 Analysis of the HSCs Grown on Immobilized SCF

On SCF-coated cover slips HSCs were cultured for 10 min, 1 d, 3 d, and 7 d and analyzed for proliferation, c-kit receptor expression, and SCF availability. All cells were collected after the desired time point and counted.

For detection of surface c-kit cells were washed and labeled for flow cytometric analysis (FACS) using anti-CD117 allophycocyanin (APC) 10 min at 4 °C in the dark.

Receptor activation was determined via Elisa of the phosphorylated c-kit. HSCs were lysed 5 min on ice in cell lysis buffer supplemented with 1 mM phenylmethylsulfonylfluorid (PMSF) to prevent proteases from digesting the protein. The cell material was then ultrasonicated for 10 s and centrifuged 10 min 4500 rpm, thus the protein was enriched in the supernatant, which was then deep-frozen at 80 °C until analysis. For analysis the cell lysate was diluted in dilution solution (1:1), pipetted and incubated at 4 °C over night in the anti-c-kit-coated Elisa plate. As zero control dilution solution was used. After 4-fold rinsing with wash buffer the lysate was incubated 1 h at 37 °C with biotinylated anti-phosphotyrosine antibody to detect the activated c-kit. After washing, the samples were incubated with horseradish peroxidase (HRP) streptavidin labeled secondary antibody for 30 min at 37 °C. After washing again, tetramethylbenzidine was added as substrate for the HRP for 30 min at room temperature (RT). Subsequently stop solution was added and the optical density of the wells was measured at λ=450 nm within following 30 min (Spectral Photometer incl. Magellan Software), which correlates to the amount of protein in each well.

SCF availability and surface stability was measured via Elisa of the concentration in the medium after HSC culture. After HSC detachment the cell suspension was centrifuged and the supernatant was deep-frozen at -80 °C until analysis. As control and internal standard SCF was diluted at different concentrations (3, 2, 1, 0.5, 0.3, 0 ng/ml) in the manufacturers dilution solution. The sample medium was diluted 1:10 in dilution solution. Samples and controls were
incubated over night at 4 °C in the anti SCF coated Elisa plate, washed four times, incubated with the biotinylated SCF detection antibody 1 h at RT, washed again, incubated with HRP streptavidin secondary antibody 45 min at RT, TMB was added, after 30 min stop solution was pipetted into the wells, and photometrically measured at $\lambda=450$ nm to determine the SCF concentration.

### 6.5.2 SCF Immobilization and its Kinetics

Many HSC influencing cytokines are presented in a membrane anchored fashion inside the BM to maintain stem cell properties, e.g. SCF and FL3 [111, 109]. Immobilization of these factors might prevent *in vitro* differentiation and allow for stem cell expansion, plus basic understanding of these mechanisms.

Therefore, SCF was immobilized either onto hydrophobic POMA-coated cover slips to achieve very strong and covalent attachment of SCF or to POMA/ PEG7 functionalized culture carriers for covalent binding but better accessibility for the HSCs due to the flexible PEG7 spacer (Fig. 7). For comparison, SCF was directly given into the cell media in the same concentration per cell culture well as for the immobilized cytokine. The surface stability, the availability in solution of SCF, and the according cell behavior (proliferation, c-kit surface expression and activation, and differentiation) were measured.

Elisa measurement of the SCF content in the media after 7 d of cell culture proved the cytokine to be stably immobilized on the POMA and POMA/ PEG7 surfaces. The concentration of SCF presented in solution slightly decreased over time but was still available after 7 d culture period (Fig. 38 A). However, HSC proliferation didn’t increase with increasing amount of immobilized SCF irrespective of the co-polymer coating compared to SCF presented in solution (Fig. 38 B). Thus, there is either not enough immobilized SCF or the active site of the cytokine is not detectable for the HSCs. As already mentioned in chapter 3.2, radioactive labeling of SCF proved the cytokine to be immobilized onto the POMA and POMA/ PEG7 surfaces [130]. Presumably the active side of the SCF which is recognized by its receptor c-kit is impeded by the covalent binding to the co-polymers, even though the PEG7 spacer was expected to allow better accessibility to the cytokine. The c-kit active core of the SCF dimer contains lysine residues which also bind to the anhydride moiety of the co-polymer [129] and thereby are probably blocked for receptor recognition.

![Figure 38 SCF Surface Stability/ Availability and SCF Dependent Proliferation.](image)

(A) Elisa of the amount of SCF in cell media after 10 min, 1 d, 3 d, and 7 d of cell culture proves the immobilized protein to be stable, compared to amount of SCF presented in solution, which is available during the whole...
culture period. (B) Proliferation of HSCs correlates with the solution concentration of SCF in the cell culture media. Immobilized SCF in contrast does not affect HSC expansion. Data are represented as mean ± SEM, pictures (right) are modified after [130].

No impact was seen for the surface expression of c-kit (Fig. 39 A) during culture of HSCs on immobilized SCF. In contrast to soluble SCF, that reduces c-kit surface expression from 80 % to 20 % within 10 min of culture (Fig. 39 B). Surface expression was recovered after 3 d. The c-kit receptor is not recycled after activation but requires new peptide synthesis [172] which explains the rather long time for surface reappearance in response to SCF stimulation. After 3 d the highest receptor activation was detected for HSC stimulation with soluble SCF and decreases by 40 % after 7 d (Fig. 39 C). As shown in Fig. 38 A, the amount of SCF decreases over time which then leads to less receptor activation although approximately 95 % of HSC exhibit surface c-kit after 7 d (Fig. 39 B). Thus HSCs express a certain amount of c-kit on their surface, once it gets activated and internalized it takes some time (3 d) until the cells adjust the amount of surface c-kit to the available SCF in solution. The same vice versa, when the available SCF gets less, it takes some time until the surface c-kit expression is adapted. No receptor activation could be measured for HSCs cultured on immobilized SCF (data not shown) which underlines the assumption of non-active SCF on the surface.

![Figure 39 c-kit Surface Expression and Activation.](image)

Thus SCF in solution positively affects HSC proliferation and effectively leads to receptor internalization, activation and re-expression. In contrast covalent immobilization of SCF to POMA or POMA/PEG7, although detectable and stable, prevents receptor recognition and is therefore not sufficient for the maintenance of human HSCs in vitro. The accessibility to the active core of SCF ought to be provided by a biomaterial for successful cell culture.

### 6.5.3 c-kit Expression Kinetics and HSC Differentiation

Surface c-kit expression of HSC is reduced from approximately 90 % to ~ 10 % after 10 min of stimulation with soluble SCF. After 1 d receptor expression recovers up to 50 % and is fully available on the surface 3 d after stimulation (Fig 40 A).
Appendices

Figure 40  Surface Expression Kinetics of c-kit and HSC Differentiation. (A) Surface c-kit was reduced from approximately 90 % to approximately 10 % after 10 min. After 1 day in culture HSCs recovered their surface c-kit expression up to 50 %, and up to 80 % after 3 days. (B) CD 34 and CD 133 expression profile after 7 days of HSC culture on immobilized SCF or with SCF in solution (control). With increasing SCF amount in solution higher number of CD negative cells was observed compared to cells cultured on immobilized SCF. No differences were seen between POMA and POMA/ PEG7. Data are presented as mean ± SEM.

The analysis of CD marker expression after 7 d in culture upon stimulation with different SCF concentrations in solution (ctrl) or immobilized shows increasing CD negative cells with increasing SCF amount in solution (from 3 % to 10 %); in contrast to surface immobilized SCF with only ~4 % CD negative cells. Whereby, no difference was observed for the different co-polymer coatings (POMA or POMA/ PEG7) (Fig. 40 B).

Short Discussion on the Growth Factor Immobilization

For better understanding of the various cytokine actions within the BM, immobilization of these onto biomaterials may exhibit insights into their in vivo kinetics.

The kinetic of action of soluble SCF on HSCs revealed c-kit internalization within 10 min of stimulation with maximum receptor activation after 3 days in culture. In contrast, immobilized SCF didn’t impact cell behavior, which is suggests the covalent binding of the active core of the cytokine to the co-polymer and thereby prevented the recognition by its receptor c-kit. Thus other immobilization strategies need to be applied. Adsorption of SCF to surfaces might lead to better accessibility for the HSC [173] but is not stably bound to the surface; it might get ripped off the surface by the HSCs and get internalized displaying same actions upon cell behavior as the soluble isoform. Therefore, a covalent anchorage of the non-active site of the SCF to a biomaterial is necessary. Lutolf et al used the coupling of Fc-chimeric cytokines to ProteinA adsorbed hydrogels for functionalization [136] which he proved functional for mouse HSCs. Another possibility for active immobilization of POMA or PEMA co-polymer-coated cell culture carriers would be the application commercially available polyhistidine (his) tagged SCF. The his-tag (typically 6 x his) is fused to the c-terminus of the SCF and can be coupled to the anhydride moiety of the co-polymer. Such his-tag approach will be part of future experiments on immobilized growth factors for stem cell maintenance and proliferation. In combination with the microcavity culture carrier and/ or immobilized ECM such strategy of growth factor presentation is proposed to disclose further insights into stem cell fate decisions.
Publications

Posters


Kurth, I.; Pompe, T.; Franke, M.; Bornhäuser, M.; Werner, C.: Cycling and Differentiation of Hematopoietic Stem Cells within Extracelular Matrix Microcavities 8th World Biomaterials Congress, Amsterdam, The Netherlands, 28.05. – 01.06.2008

Kurth, I.; Pompe, T.; Franke, M.; Bornhäuser, M.; Werner, C.: Cycling and Differentiation of Hematopoietic Stem Cells within Extracelular Matrix Microcavities 2nd CRTD Summer Conference on Regenerative Medicine, Dresden, 06.06.2008


Proceedings

Talks

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Papers


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Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt. Bei der Auswahl und Auswertung des Materials habe ich Unterstützungsleistungen von folgenden Personen erhalten:

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