Mobilisation, Isolation and Coculture of Haematopoietic Stem Cells

D i s s e r t a t i o n s s c h r i f t

zur Erlangung eines doctor rerum medicinalium (Dr. rer. medic.)

der Medizinischen Fakultät Carl Gustav Carus

der Technischen Universität Dresden

vorgelegt von

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Dresden 2009

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Tag der mündlichen Prüfung: 10. August 2010

gez: Prof. Dr. med. G. Wozel

Vorsitzender der Promotionskommission

DECLARATION

I herewith declare that I have produced this paper without the prohibited assistance of third parties and without making use of aids other than those specified; notions taken over directly or indirectly from other sources have been identified as such. This thesis has not previously been presented in identical or similar form to any other German or foreign examination board. The thesis was conducted from October 2006 to October 2009 under the direct supervision of Dr Rainer Ordemann, Medical Faculty, Technical University Dresden.

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CONTENTS

SUMMARY

Since decades, hematopoietic stem cell transplantation (HSCT) has become a well established treatment modality for hematological malignancies and non-malignant disorders. Autologous and allogeneic hematopoietic stem cells (HSCs) mobilized into the peripheral blood (PB) have been used as a preferred source of transplantable stem cells[1-3]. And umbilical cord blood (UCB) has been introduced as a more attractive HSC source for HSCT, because fetal stem cells in UCB are speculated to be more primitive in comparison to adult stem cells. However the limited amount of HSCs is limiting their application for stem cell therapy in clinic. Therefore, people started to utilize extra-embryonic tissue to harvest more fetal stem cells, while people also tried to optimize the clinical protocol to mobilize more adult stem cells out of adult bone marrow. The innovative strategies and feasible procedures were discussed in this thesis.

The axis of the chemokine receptor CXCR4 and its ligand SDF-1 is important for trafficking and homing of HSCs. It has already been demonstrated that the bicyclam AMD3100, a CXCR4 antagonist, in combination with G-CSF is able to induce a significant mobilization of CD34+ cells[4]. And human placenta is a potent hematopoietic niche containing hematopoietic stem and progenitor cells throughout development[5]. The homing of HSCs to the placenta is probably also mediated by the expression of SDF-1 as demonstrated for the bone marrow niche. In this study (part 1 of the chapter "Results and discussions"), we utilized AMD3100 to mobilize HSCs from placenta. And we can demonstrate that the CXCR4 antagonist AMD3100 mobilise placenta derived CD34+ cells ex utero already after 30 min of incubation and may further enhance the efficacy of harvesting placenta-derived HSC.

The alpha4 integrin CD49d is involved in migration and homing of hematopoietic stem cells (HSC). Therapeutic application of natalizumab, an anti-CD49d antibody, in patients with multiple sclerosis (MS) has been associated with increased levels of circulating CD34+ progenitors. In our study (part 2 of the chapter "Results and discussions"), we compared circulating HSCs from MS patients after natalizumab treatment and HSCs mobilized by G-CSF in healthy volunteers, with regard to their migratory potential, clonogenicity and gene expression. CD34+ cells in the blood and marrow of natalizumab-treated patients expressed less of the stem cell marker CD133, were enriched for erythroid progenitors (CFU-E) and expressed lower levels of adhesion molecules. The level of surface CXCR-4 expression on CD34+ cells from patients treated with natalizumab was higher compared to that of CD34+ cells mobilized by granulocyte-colony stimulating factor (G-CSF) (median 43.9% vs. 15.1%). This was associated with a more than doubled migration capacity towards a chemokine stimulus. Furthermore, CD34+ cells mobilized by natalizumab contained more m-RNA for p21 and less MMP9 compared to G-CSF mobilised HSC. Our data

indicate that G-CSF and CD49d blockade mobilize different HSC subsets and suggest that both strategies may be differentially applied in specific cell therapy approaches.

In order to further improve the clinical outcome of HSC transplantation, many groups are focusing on ex vivo maintain or expand HSC. Unfortunately, the maintenance of HSC in vitro is difficult to achieve because of their differentiation. This is presumably caused by a lack of appropriate cues that are provided in vivo by the microenvironment. Indeed, HSCs located in the bone marrow are interacting with a specific microenvironment referred to as the stem cell niche, which regulates their fate in terms of quiescence, self-renewal and differentiation. An orchestra of signals mediated by soluble factors and/or cell-to-cell contact keeps the balance and homeostasis of self-renewal, proliferation and differentiation in vivo. To investigate the communication between HSCs and the niche, coculture assays with mesenchymal stromal cells (MSCs) were performed in vitro. Here, we can demonstrate that cell-to-cell contact has a significant impact on hematopoietic stem cells expansion, migratory potential and stemness. In this study (part 3 of the chapter "Results and discussions"), we investigated in more detail the spatial relationship between hematopoietic stem cells and mesenchymal stromal cells during ex-vivo expansion. And we defined three distinct localizations of HSCs relative to MSC layer: (i) those in supernatant (non-adherent cells); (ii) cells adhering on the surface of mesenchymal stromal cells (phase-bright cells) and (iii) cells beneath the mesenchymal stromal cells (phase-dim cells). Our data suggest that the mesenchymal stromal cell surface is the dominant location where hematopoietic stem cells proliferate, whereas the compartment beneath the mesenchymal stromal cell layer seems to be mimicking the stem cell niche for more immature cells. Our data provide novel insight into the construction and function of three-dimensional HSC–MSC microenvironments.

In summary, we provided a new method to isolate fetal stem cells from extra-embryonic tissue (i.e. placenta) in the first part, then we discussed an innovative strategy with CD49d blockade to improve clinical modality for adult stem cell mobilization in the second part, and finally we investigated HSC maintenance and expansion in vitro and provided feasible way to mimic HSC niche in vitro in the last part.

This thesis contributes to HSC-based stem cell therapy in two aspects, i.e. 1) fetal and adult stem cell isolation holding great therapeutic potential for blood diseases; 2) ex vivo stem cell manipulation providing a valuable platform to model HSC niche regulation.

INTRODUCTION

1. Isolation of primary human Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) are defined by their ability to give rise to all types of blood cells [6-8]. During the last three decades hematopoietic stem cell transplantation (HSCT) has become a well established treatment for hematological malignancies and non-malignant disorders. Lately HSCs are attracting more and more attention for their potential use in regenerative medicine and tissue engineering [9]. In clinic, human primary HSCs can be harvested from healthy donors either by bone marrow aspiration, peripheral stem cell mobilization or from cord blood. In this study, we firstly investigated two alternative ways for HSC mobilisation.

1.1 HSC mobilization out of Placenta after Cord Blood collection

Allogeneic bone marrow and peripheral blood stem cell transplantation is the treatment of choice for some malignant hematologic diseases, marrow failure syndromes, and severe congenital immunodeficiency states. Since Gluckman et al reported in 1988 the first successful transplantation of human umbilical cord blood (UCB), UCB has been used increasingly in both pediatric and adult patients. However the limiting factor of UCB transplantation is the low amount of hematopoietic stem cells (HSC) [10]. Therefore many groups are working on an optimized collection procedure. A Spanish group could demonstrate that a modified in utero / ex utero two-step collection method in which a cord blood fraction obtained by umbilical venal puncture was combined with a second fraction harvested after placental perfusion with 50 ml heparinised 0.9% saline could significantly increase the amount of nucleated cells [11].

The axis of the chemokine receptor CXCR4 and its ligand SDF-1 is important for trafficking and homing of HSC. AMD3100 is able to mobilize CD34+ HSC in mouse and men. Especially in so called poor mobilizer AMD3100 in combination with G-CSF is able to induce a sufficient mobilisation of CD34+ cells[12]. Dipersio et al have already shown that AMD3100 may provide an alternative method of HSC mobilisation of allogeneic donors[13]. We were interested to investigate if AMD3100, a CXCR4 antagonist, is able to mobilize HSC from the placenta.

1.2 HSC mobilization out of Bone Marrow

Autologous and allogeneic hematopoietic stem cells (HSC) mobilized into the peripheral blood (PB) have been used as a preferred source of transplantable stem cells[1-3]. In order to facilitate a rapid and efficient mobilization of HSC, donors or patients are treated with granulocyte colony stimulating factor (G-CSF) which causes a transient release of HSC out of the bone marrow (BM)[14-16]. However, the number of HSC mobilized by G-CSF shows a broad interindividual variation[17]. Since as many as 14% of autologous donors demonstrate a poor response to G-CSF[18], more efficient strategies for HSC mobilization have to be developed. One strategy which has already been introduced in clinical trials is the combination of G-CSF with the CXCR-4 antagonist Plerixafor allowing a significantly better mobilization especially in heavily pretreated patients with NHL or Multiple Myeloma[19].

Moreover, antibodies against the Very-late antigen 4 (VLA-4) have shown some promise to mobilize long-term repopulating hematopoietic progenitors in preclinical models[20,21]. Natalizumab, a human monoclonal antibody against integrin α4 (CD49d), is used in the clinic to treat Multiple Sclerosis (MS). Recently, it has been reported that a marked increase in the number of circulating HSC can be observed during natalizumab treatment in MS patients[22,23]. These reports have raised great interest since they have opened the perspective to explore CD49d blockade as a new mobilization strategy. Nevertheless the phenotypes and functions of HSC mobilized with natalizumab and the impact on steady-state hematopoiesis in the bone marrow have not been characterized in detail. Therefore, in our study, BM, PB, and purified CD34+ cells of MS patients after Natalizumab treatment were evaluated in comparison to BM and G-CSF mobilized PB from healthy donors as well as to PB of MS patient without natalizumab treatment. Our data for the first time show that chronic administration of Natalizumab leads to an increase in BM CD34+ cells and that CD34+ cells mobilized into the peripheral blood have a different phenotype and migratory potential compared to G-CSF mobilized CD34+ HSC.

2. Hematopoietic Stem Cells in Coculture with Mesenchymal Stromal Cells

To improve the clinical outcome of autologous and allogeneic HSC transplantation many groups are focusing on ex vivo expansion of HSCs particularly in cases with a limited graft size. Unfortunately, the expansion of HSC in vitro is difficult to achieve because their proliferation is accompanied by differentiation [24]. This is presumably caused by a lack of appropriate cues that are provided in vivo by the microenvironment. Indeed, HSCs are located mainly in the bone marrow where they

interact within a specific microenvironment called the stem cell niche, which regulates their fate in terms of quiescence, self-renewal and differentiation [25-27]. Recent data suggest that quiescent HSCs are primarily located in the trabecular endosteum (i.e. osteoblastic niche) whereas dividing ones reside in sinusoidal perivascular areas (i.e. vascular niche) of the bone marrow [28,29]. In response to stress or injury, HSCs can be released from the vascular niche into the circulation [30]. An orchestra of signals mediated by soluble factors and/or cell-to-cell contact keeps the balance and homeostasis of self-renewal, proliferation and differentiation in vivo [31]. Though important regulatory components of the stem cell niche in vivo have been identified, this has not been translated into improved ex vivo expansion protocols for clinical applications. The most excellent defined culture medium for HSC expansion is supplemented with cytokines such as fetal liver tyrosine kinase-3 ligand (FLT3-L), stem cell factor (SCF), interleukin-3 (IL-3) and thrombopoietin (TPO) [32,33]. Interestingly, mesenchymal stromal cells (MSCs), which are characterized by multi-differentiation potential [34,35], are important players of the bone marrow HSC niche [36]. In recent years, MSCs have been shown to support HSC maintenance and engraftment [37,38]. In addition, an immuno-modulatory capacity of MSCs has been described [39].

According to several recent studies including those from our laboratory, MSCs facilitate HSC maintenance in a coculture system in vitro via the secretion of soluble factors and cell-cell contact [40-43]. In addition evidence is emerging that a three-dimensional architecture is important to mimic physiologic conditions ex vivo [30,44]. Therefore we were prompted to investigate how HSCs interact with MSCs in different spatial compartments over time in vitro. Usually HSCs in the coculture were considered as a single population, and their localization relative to the MSC layer has not been investigated intensively. In this study, MSCs served as a physical boundary of distinct compartments, and the properties and features of HSCs in different localizations were evaluated at various times. Our data provides novel insight into the construction and function of a three-dimensional HSC–MSC coculture microenvironment in vitro.

DESIGN AND METHODS

1. Stem Cell Isolation and manipulation

Samples

Peripheral blood (PB) and bone marrow (BM) of healthy volunteers and some patients were investigated in this study. Briefly, mobilized PB was collected from healthy donors after treatment with 7.5 µg/kg granulocyte colony-stimulating factor (G-CSF) for 5 days. And PB was also collected from MS patients who had not responded to other immunomodulatory treatment, received 300 mg natalizumab IV at monthly intervals (Tysabri, Biogen Idec, Cambridge, MA, USA). BM was aspirated from healthy donors and the patients. Written informed consent was obtained in all cases.

Purification of CD34+ cells

CD34+ HSC were purified from leukapheresis samples by attachment to magnetic beads conjugated with CD34 antibody, according to the manufacturer's instructions (MACS, Miltenyi Biotec). The purities for the HSC fraction derived from samples mobilized by G-CSF were 95.6±0.9% and for natalizumab 91.2±0.6%as assessed by flow cytometry analysis using PE conjugated CD34 antibody (Miltenyi Biotec, Germany). Trypan blue exclusion confirmed >96% vitality in every sample.

Isolation of mesenchymal stromal cells

MSCs were isolated from bone marrow aspirates after informed consent and approval by the local ethics committee and cultured as described previously[45]. The phenotype of all MSC batches was tested by fluorescence-activated cell sorting (FACS): The presence of CDw90, CD105, CD166, and CD73, and absence of CD34 and CD45 were required. MSC batches used in the cocultures had been tested for their potential to differentiate along the osteogenic and adipogenic lineages using standard commercial differentiation media. MSCs of passage two were then seeded in 12-well plates or 24-well plates at a density of $1 \times 10^4/cm^2$ with MSC medium. The medium was changed every 3 days until the MSC feeder layer reached confluence.

Mobilize HSCs from Placenta

The first blood fraction was obtained from the placenta ex utero after delivery of the placenta. After the blood flow ceased the placental vessels were flushed with 30 ml 0.9% saline supplemented with 10µg/ml AMD3100. Then 30 min of incubation and collected the fraction were performed. HSCs were identified.

Coculture of hematopoietic stem cells with mesenchymal stromal cells layer

CD34+ HSCs were suspended in CellGro® SCGM medium (CellGenix, Freiburg, Germany) with 10% FCS, 150 ng/ml Fetal Liver Tyrosine Kinase-3 ligand (FLT3-L), 150 ng/ml stem cell factor (SCF) (both Biosource, Camarillo, CA, USA) and 50 ng/ml

IL-3 (Miltenyi Biotec, Germany). HSC suspensions were plated at a density of 1×10^4 cells/cm^2 on confluent MSC layer at 37°C in normaxia or hypoxia condition (lower than 1% O2 tension).

In some experiments HSCs were cultured without MSCs in a cytokine-driven assay. After 4 days the cell suspension containing around 50% CD34+CD38- cells was seeded on an MSC layer for 5 hours for further analysis.

2. Analyses on stem cells before and after manipulation in vitro

Flow cytometric immunophenotyping

Immunophenotyping was by four-color lyse-wash flow cytometry. Progenitor cells were identified using CD34-APC and CD45-PerCP5.5 (BD Biosciences, Heidelberg, Germany). Two further antibodies, labeled with FITC or PE, were added for characterizing expression of the following adhesion molecules: CD11a/CD18, which is a beta2 integrin, also called lymphocyte function-associated antigen-1 (LFA 1; BD Biosciences), CD62L L-selectin, and CD31, the platelet endothelial cell adhesion molecule PECAM (both from Beckman-Coulter, Krefeld, Germany), plus heparan sulphate proteoglycan CD44 and integrin a4 (CD49d) (Biozol, Esching, Germany). The chemokine receptor CXCR-4/CD184 (BD Biosciences) and the stem cell antigen CD133 (Miltenyi Biotec, Bergisch Gladbach, Germany) were also evaluated. Stem cells were selected by gating as CD34+CD45^{dim} cells with low side scatter. Whenever possible, 2000 stem cells with about 500,000 events overall were registered. Fluorescence-minus-one controls for FITC and PE provided the threshold setting for adhesion molecule expression. All measurements were performed on a FACS Canto II flow cytometer equipped with three lasers (488nm, 633nm, and 405nm) using FACS DiVa software. Percentages and mean fluorescence intensity (MFI) were assessed. Appropriate murine antibodies served as a negative isotype.

Proliferation assay

Purified CD34+ cells were suspended in CellGro® SCGM medium (CellGenix, Freiburg, Germany) with 10% FCS, 150 ng/ml fetal liver tyrosine kinase-3 ligand (FLT3-L), 150 ng/ml stem cell factor (SCF) (both from Biosource, Camarillo, CA, USA), and 50 ng/ml interleukin-3 (IL-3) (Miltenyi Biotec). HSC suspensions were plated at 4×10^4 cells/well in 6-well plates at 37°C and 5% CO_2. The number of total nucleated cells (TNC) was counted on days 1, 3, 5 and 7 by Trypan Blue (vitality more than 95%).

CFU-GM assay in unmanipulated leukapheresis samples

1×10^5 cells from each leukapheresis sample were plated in 35-mm Petri dishes (Greiner, Frickenhausen, Germany) in a total volume of 1ml standard methylcellulose medium with recombinant cytokines (MethoCult GF+ H4435; Stem Cell Technologies, Vancouver, Canada) in triplicate. All cultures were incubated at 37°C in fully humidified air with 5% CO_2. After 14 days, erythroid bursts (BFU-E), myeloid

colonies (CFU-GM), and mixed-cell colonies (CFU-GEMM) were scored with an inverted light microscope (Carl Zeiss, Jena, Germany). Colonies were defined as clusters consisting of ≥40 cells. The frequency of colonies is provided per 1000 CD34+ cells inserted.

Immunofluorescence microscopy
After coculture the supernatant was discarded and the MSC layer was washed twice gently. Next the remaining cells including phase-bright and phase-dim cells along with the MSC layer were fixed using 3.8% formaldehyde in PBS for 15min and permeabilized using 0.1% Triton X-100 in PBS for 3-4min. After blocking, cells were incubated with CD45-PE (1:10; Miltenyi Biotec, Germany) for 1h and then incubated with anti-mouse-TRITC (1:400; Sigma, USA) and phaloidin-ALEXA488 (1:500; Invitrogen, UK) for 1h in RT. Then nuclei were labelled with DAPI (Sigma, USA). Finally fluorescence imaging was performed by confocal microscopy (Zeiss LSM 510, Germany).

Scanning electron microscopy
Samples for the scanning electron microscopy analysis were prepared as previously described [46]. Briefly, the co-cultured cells growing on fibronectin-coated coverslips were fixed in 2% glutaraldehyde for 1 h at RT and then overnight at 4°C. After being subjected to dehydration in an acetone gradient (30-100%), cells were critical point-dried in a CO_2 system (Critical Point Dryer CPD 030, BAL-TEC GmbH, Germany). Samples were then sputter-coated with gold (Sputter Coating Device SCD 050, BAL-TEC GmbH) and examined at 10 kV accelerating voltage in an environmental scanning electron microscope (PHILIPS XL 30 ESEM FEG, Philips, The Netherlands).

Time-lapse video microscopy
Freshly isolated CD34+ HSCs were cultured on MSCs growing on 24 x 60 mm fibronectin-coated coverslips − attached to a silicone reusable chamber. During the time-lapse recording, cells were kept in a 37°C chamber with a 5% CO2 atmosphere. Serial phase contrast images were captured with an inverted microscope (Zeiss Axiovert 200M; 20X objective) at 30 sec intervals. The images were built into a movie using the Metamorph software.

Cell collection from three distinct localizations
HSCs from 3 distinct compartments in coculture were collected separately as described HSC/MSC cocultures were prepared as described above. On each day during the first week HSCs from 3 distinct localizations in coculture were collected separately. Briefly, the supernatant of the coculture was aspirated and the cells in the supernatant (non-adherent cells) were collected. The MSC layer was gently washed twice with PBS to remove the remaining non-adherent cells. After washing, the cells remaining on the MSC layer (phase-bright cells) were collected by further intensive washing steps with PBS. When no phase-bright cells could be observed under

phase-contrast microscopy, the MSC layer with the cells underneath the layer (phase-dim cells) was trypsinized and collected as well. To exclude a trypsin effect, non-adherent cells and phase-bright cells were also trypsinized for 5 min. No notable change in the mean intensity of CD38 was observed after trypsinization, showing CD38 antigen is relatively resistant to digestion by trypsin[47]. Finally the three cell fractions were counted by Trypan Blue (vitality more than 95%) and measured as described below.

Cell cycle analysis using Propidium Iodide (PI)
After cell collections each time, cells were fixed at 4°C overnight with cold 70% ethanol. Then the cells were washed and incubated in PBS with 50μg/ml PI and 1mg/ml RNase for 30 min in RT. After washing, the cells were measured by flow cytometry (FACScalibur; BD, Germany). Finally, the FACS data were analyzed using Modifit software (Becton Dickinson), and the proportions of G0/G1 phase, S phase and G2/M phase of cell cycle were calculated automatically.

PCR analysis
After 5 days coculture, cells from the three compartments were labeled with antihuman CD45-FITC and CD166-PE. Then, cells were sorted and MSCs were discarded based on forward-scatter (FSC), side-scatter (SSC), and CD45+CD166-staining using BD FACSAria and software B&D FACSDiva. Total RNAs isolated by Trizol reagent (Invitrogen, USA) were reverse transcribed to total cDNAs with oligo dT. And a quantitative TaqMan real-time polymerase chain reaction (RT-PCR) was performed for Cyclin-dependent kinase inhibitor 1A (CDKN1A, p21) according to the Taqman manufacturer's instructions (Applied Biosystems, USA). The housekeeping gene GAPDH was used as a control. All primers were purchased from Applied Biosystems (USA). The relative gene expression of p21 was calculated by normalization to the expression level in phase-bright cells which was considered as 1.

Cell generation tracking with CFSE staining
Generations of HSCs were identified using CellTrace[TM] CFSE Cell Proliferation Kit (Invitrogen, UK). Briefly, CD34+ HSCs were labelled by CFSE according to the manufacturer's instructions and placed in coculture with MSCs as described above. At fixed time points, the cells from the respective compartment were collected and analyzed using FACS. The number of cell divisions was quantified according to CFSE signal intensity using CellQuest software. As control CD34+ HSCs were labelled with CFSE and treated with 50μg/ml Mitomycin (Santa Cruz) to arrest the cell population at generation 0.
The CD34 expression pattern throughout subsequent cell generations was evaluated by CD34-APC and CFSE double staining.

Transwell migration assay
A 600μl aliquot of conditional medium or serum-free medium containing 300 ng/ml stromal cell-derived factor-1 (SDF-1; R&D Systems, Minneapolis, MN, USA) was

added to the lower chamber of a 5-μm 24-well polycarbonate transwell culture dish (Corning Costar Corporation, Cambridge, MA, USA). Subsequently, 2 x 10^5 fresh HSCs or expanded HSCs were suspended in 0.1 ml medium and added to the upper chamber to migrate for 4 hours at 37°C. Cells that had migrated into the lower chamber were collected and counted. Spontaneous migration towards serum-free medium without SDF-1 addition was estimated as a control.

Inhibition of hematopoietic stem cell migration under mesenchymal stromal cells by blocking Integrin ß1 and CXCR4

HSCs were cultured in suspension without a MSC layer for 2 days. These primed HSCs started to migrate under the MSC layer within 5 hours. In addition, the primed HSCs were preincubated for 1h in normal culture medium (CM) supplemented with 10μg/ml P-selectin antibody (BD, Germany), or with 10μg/ml Integrin ß1 antibody (BD, Germany) [48], or with 500ng/ml AMD3100 (a nonpeptide antagonist of CXCR4; Sigma, USA) [43], or with both 500ng/ml AMD3100 and 10μg/ml Integrin ß1 antibody. After blocking, 1×10^5/ml cells were plated on the MSC layer for 5 hours. Finally HSCs from distinct localizations were counted using trypan blue as mentioned before.

Statistics of cell division to time and cell passage differentiation

All data were derived from at least three independent experiments. Data were represented as "mean ± standard error of the mean (SEM)" and analyzed with the paired student's test. Observed differences were regarded as significant if the calculated two-sided *p*-value was below 0.05.

RESULTS AND DISCUSSIONS

Part 1: AMD3100 Mobilization of Human Hematopoietic Progenitor

Cells from the Placenta *(Bone Marrow Transplantation, 2010 Feb 22.)*

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Umbilical cord blood (UCB) has been introduced as an alternative graft source for allogeneic hematopoietic stem cell transplantation (HSCT) in both pediatric and adult patients. However the limited amount of HSC leads to delayed engraftment especially in adult recipients[10]. Therefore, several groups are working on an optimized collection procedure in order to increase the amount of transplantable progenitors. Bornstein and colleagues have demonstrated recently that a modified in utero / ex utero two-step collection procedure can lead to a significant increase of the amount of harvested progenitor cells[11].

Especially in patients with a limited hematopoietic capacity the bicyclam AMD3100, a CXCR4 antagonist, in combination with G-CSF is able to induce a significant mobilisation of CD34+ cells[49]. Dipersio et al have already shown that AMD3100 may provide an alternative method of HSC mobilisation even in allogeneic donors[13]. Gene expression data indicate that AMD3100 mobilized HSCs may represent a functionally different subset of HSCs[50].

As demonstrated for the bone marrow niche, the homing of HSC to the placenta is probably also mediated by the expression of SDF-1[51]. Recently Serikov et al. could demonstrate AMD3100 is able to mobilize additional HSC from the placenta by perfusion of the placenta for up to six hours[52]. Within a pilot-study we could confirm Serikovs data indicating that AMD3100 is able to mobilize hematopoetic progenitor cells from the placenta.

First we obtained cord blood samples using standard techniques ex utero after delivery of the placenta. After the blood flow had ceased we flushed the placental vessels with 30 ml 0.9% saline and 10% ACD to collect the remaining cord blood (saline wash). The proportions of CD34+ and CD133+ cells in "saline wash" (CD34+%: 0.13% ± 0.03%; CD133+%: 0.05% ± 0.02%) were not significantly different to the primary cord blood samples. Then we flushed the placental vessels again with 30 ml 0.9% saline supplemented with 10 µg/ml AMD3100. After 30 min of incubation the instilled solution was eluted. No clotting was detected. Despite the

lower cell concentration in the recollected elution, fluorescence-activated cell sorting (FACS) revealed a significant increase (P = 0.01) of the proportion of CD34+ and CD133+ cells comparing to cord blood samples in 9 consecutive placenta studies, as shown in the Table. The total number of CD34+ cells recovered in 28 ml of rinsing solution containing AMD3100 was $0.13 \pm 0.034 \times 10^6$. Whether this amount of transplantable HSC is clinically meaningful, remains to be studied. As control the placenta was incubated with 0.9% saline without AMD3100 for 30 min. No increase of the CD34+ or CD133+ cell count was detectable (Table).

In summary our preliminary data demonstrate that the CXCR4 antagonist AMD3100 mobilise placenta derived CD34+ cells ex utero already after 30 min of incubation and may further enhance the efficacy of harvesting placenta-derived HSC.

Table: Comparison of the primary cord blood collection, AMD-Mobilized Cells and control (Incubation with saline without AMD3100 for 30 min).

	Cord Blood	AMD Mobilization	Control
Volume (ml)	50 ± 8.4	28 ± 2.0	30 ± 2.0
Cell Count ($\times 10^8$)	6.20 ± 1.12	0.45 ± 0.12	0.34 ± 0.05
CD34+%	0.14% ± 0.03%	0.28% ± 0.07%	0.10% ± 0.01%
CD133+%	0.09% ± 0.02%	0.17% ± 0.04%	0.08% ± 0.01%
N (Placenta number)	9	9	4

Part 2: CD49d blockade by natalizumab in patients with multiple sclerosis affects steady state hematopoiesis and mobilizes progenitors with a distinct phenotype and function *(Bone Marrow Transplantation, 2010 Jan 25)*

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RESULTS

Frequency of CD34+ progenitor cells. The CD34+ cell concentration was significantly higher in the BM of MS patients receiving natalizumab compared to values measured in healthy volunteers donating bone marrow donors during the same period of time (BM-donor; p=0.005). Interestingly, the frequency of CD34+ cells in the BM was directly correlated with the number of natalizumab infusions with 617/μl after 15 infusions, 1342/μl after 21 infusions, 1350/μl after 23 infusions and 2250/μl after 32 cycles, respectively. As previously shown, an elevated CD34+ count was also detectable in PB from MS patients after repetitive natalizumab therapy (PB-nat) v.s. PB from MS patients without natalizumab, whereas the CD34+ counts of the latter group were comparable to those of steady-state PB in unstimulated healthy donors (Figure 2-1).

Repeated measurements, done before and up to 4 days after the first administration of natalizumab in two patients, revealed the maximum CD34+ absolute counts on day 4 (4/μl and 8/μl, respectively). Almost all adhesion molecules were down-regulated after the first natalizumab administration. The blocking of VLA-4 via natalizumab was reflected as a decrease in mean VLA-4 mean fluorescence intensity (MFI) from 4837 before treatment to 3089 after 72h.

Differences in adhesion molecule and CD133 expression. The expression (percentage and/or MFI) of most of the tested antigens (L-selectin, Pecam, CD44, LFA-1) was lower in both BM and PB of MS patients treated with natalizumab compared to healthy donors (Figure 2-2 a-c). Remarkably, CD133 expression on CD34+ cells was also clearly lower in the BM as well as in PB of natalizumab treated patients compared to healthy donors (BM-donor and PB-donor) (Figure 2-2 a-c).

a) Peripheral blood

CD34/µl	56	8	1
(range)	(15 – 158)	(4 – 15)	(0.5 – 1.5)
no. of pts.	11	6	6

b) Bone Marrow

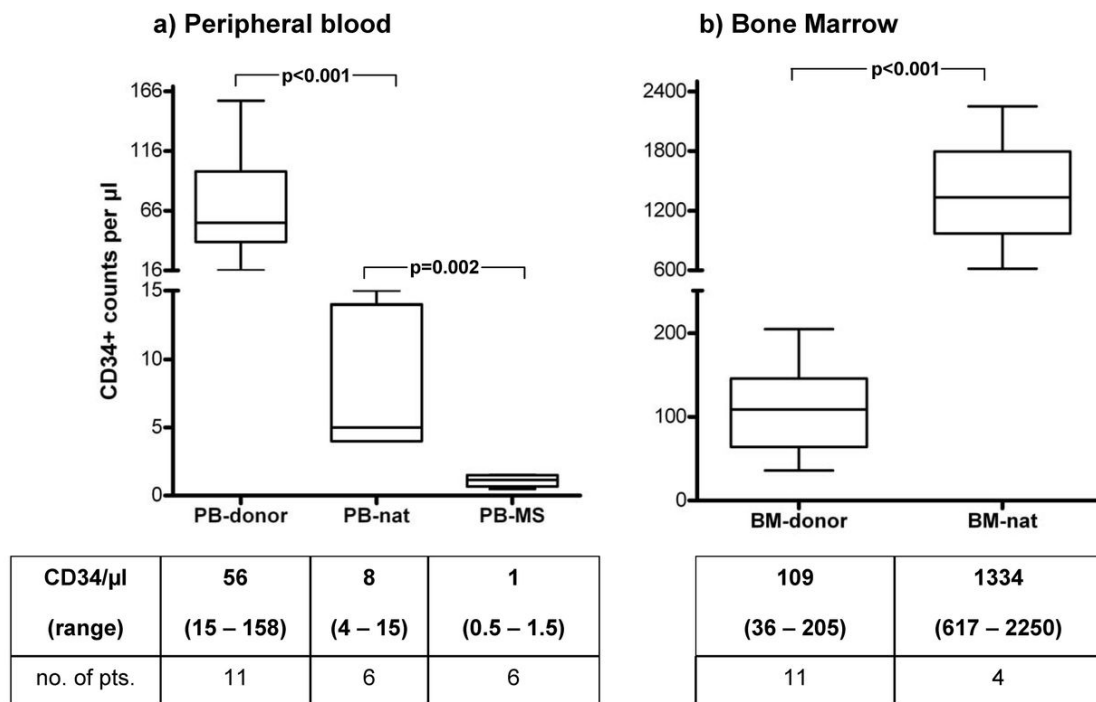| 109	1334
(36 – 205)	(617 – 2250)
11	4

Figure 2-1: CD34+ cell counts in peripheral blood a) and bone marrow b) Box and whisker plots for the CD34+ counts (median and range) in MS patients with or without natalizumab and in healthy controls. The boxes represent the lower and upper quartiles, whiskers depict maximum and minimum. a) PB-donor, peripheral blood of healthy donors after G-CSF treatment; PB-nat, peripheral blood of MS patients after natalizumab therapy; PB-MS, peripheral blood of MS patients without natalizumab therapy. b) BM-donor, bone marrow of healthy donors; BM-nat, bone marrow of MS patients receiving natalizumab. Tables provide median and ranges and number of patients studied.
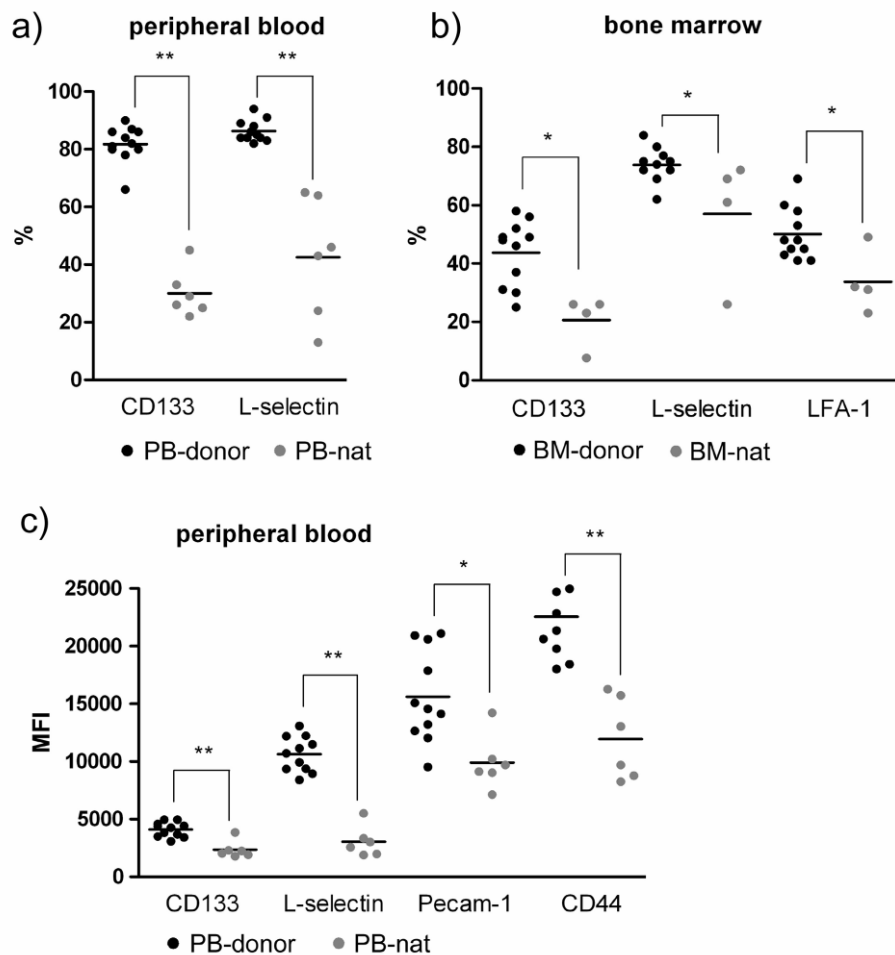
Figure 2-2: Phenotype of CD34+ cells in natalizumab treated patients and controls Graphs a) and b) summarize the percentage of cells being positive for the respective markers as measured by fluorescence activated cell sorting (FACS). Black and grey dots represent values from G-CSF mobilized healthy donors and MS patients, respectively. In graph c) the mean fluorescence intensity (MFI) is provided to quantify the expression of adhesion molecules on peripheral blood cells. PB-donor, peripheral blood of healthy donors after G-CSF treatment; PB-nat, peripheral blood of MS patients after natalizumab therapy; BM-donor, bone marrow of healthy donors; BM-nat, bone marrow of MS patients receiving natalizumab. *p< 0.05; ** p< 0.01.

Stem cell subpopulations in purified CD34+ cells. Besides BM and mobilized PB from MS patients after natalizumab, purified CD34+ cells from apheresis samples (HSC-nat) also expressed the primitive hematopoietic antigen CD133 less than the G-CSF mobilized control cells (G-CSF HSC) did. However, no significant differences were observed in the CD34+CD38- compartment (data not shown). The expression of CXCR-4 (CD184) on CD34+ cells from natalizumab-treated patients was significantly higher than on G-CSF mobilized CD34+ cells from healthy controls (43.9% (range, 42.3–52.9%) vs 15.1% (range, 7.8–17.8%), p < 0.05, Figure 2-3b). Representative contour plots are shown in Figure 2-3a.

Transwell migration assay. The increased expression of CXCR-4, as described above, was associated with a significantly better migration of CD34+ cells from patients receiving natalizumab compared to G-CSF mobilized CD34+ cells measured 4 h after establishing a chemotactic stimulus (SDF-1) (13.4% (range, 12.2–17.1%) vs 6.1% (range, 4.9–7.4%), p < 0.05, Figure 2-3c).

Gene expression and cell cycle analysis. In our study, MMP9 mRNA was significantly diminished in natalizumab mobilized CD34+ cells whereas p21, a negative regulator of cell cycle, was significantly upregulated in CD34+ cells mobilized by natalizumab (2.15 fold (range, 1.62-2.74 folds)). Correspondingly, the mRNA level of G0S2, a gene involved in cell cycle progression was decreased (Figure 2-4).

Ex-vivo expansion and clonogenicity. The proliferation capacity of CD34+ selected cells derived from leukapheresis products after 7 days of in vitro culture is shown in Figure 2-5a. The absolute cell numbers were not significantly different after 7 days of culture in vitro. Consistent with the lower frequency of CD34+ cells in the peripheral blood of natalizumab treated patients (Figure 2-1), the absolute number of BFU-E and CFU-GM contained in leukapheresis samples was lower than in G-CSF mobilized products. But still, the frequency of colonies per 1000 CD34+ cells was comparable (Figure 2-5b). Consistent with previous reports, BFU-E prevailed in HSC-nat (89.2% (range, 81.1-90.9%)) whereas the percentage of BFU-E (46.7% (range, 35.5-54.3%)) was similar to the granulocyte-macrophage compartment (52.7% (range, 44-64.5%)) in G-CSF HSC (Figure 2-5b). This strongly suggests that the precursor cells mobilized by natalizumab are different from those mobilized by G-CSF.

DISCUSSION
This is the first study investigating the bone marrow of patients receiving natalizumab for high-risk multiple sclerosis (MS). Our findings suggest that continued administration of the VLA-4 blocking antibody natalizumab stimulates hematopoiesis with ongoing increased numbers of CD34+ HSC in the BM and (as previously shown) potential mobilisation of clonogenic HSC into the peripheral blood[22,23].
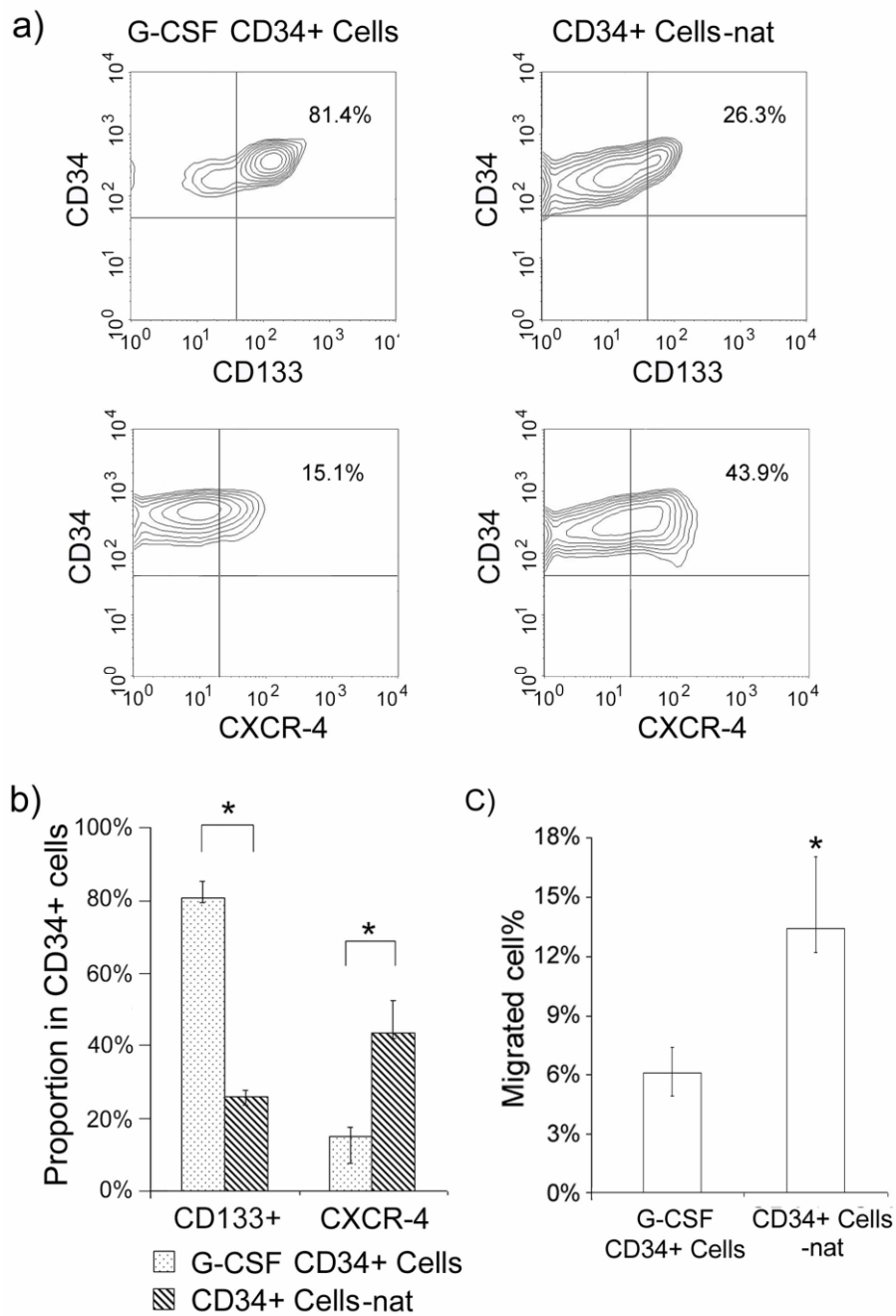
Figure 2-3: Phenotype and migratory capacity of CD34+ cells mobilized by G-CSF or CD49d blockade a) CD133 and CD184 (CXCR-4) expression of purified CD34+ cells from an apheresis of a healthy donor after G-CSF treatment (G-CSF CD34+ Cells) and a MS patient on natalizumab therapy (CD34+ Cells-nat). b) Median expression and ranges of the respective surface markers on G-CSF mobilized CD34+ cells compared to CD34+ cells-nat (n=3). c) CD34+ selected cells from leukaphereses derived from patients on natalizumab showed a significantly higher migration capacity in a transwell assay compared to G-CSF CD34+ cells. (n=3, *p < 0.05)
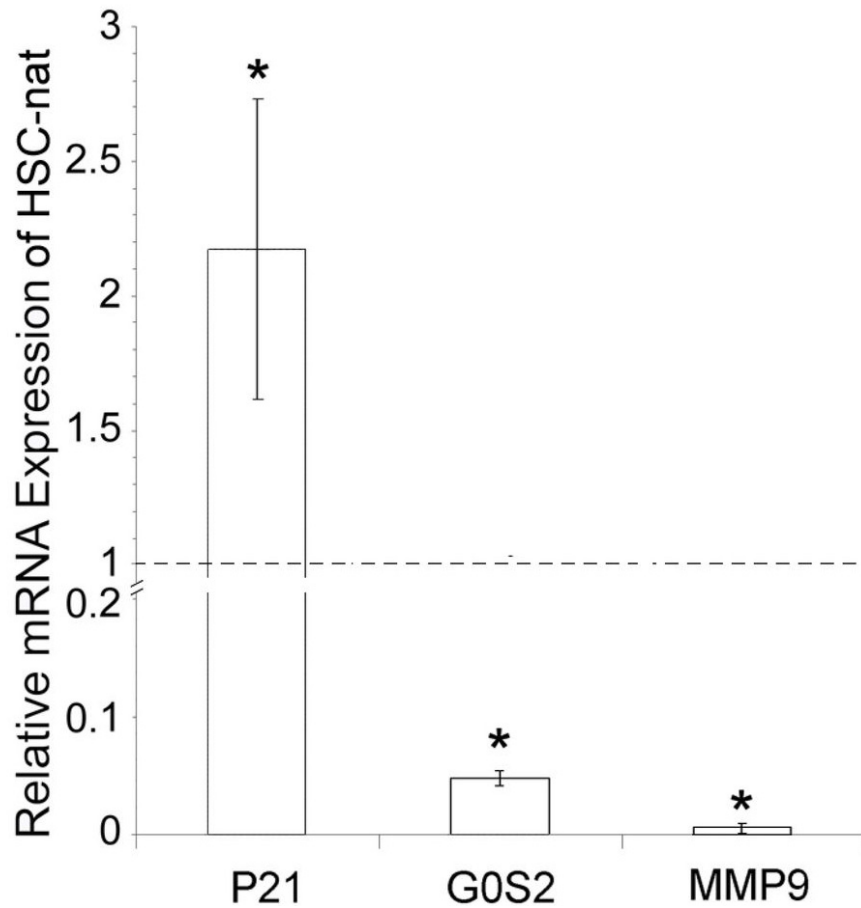
Figure 2-4: Gene expression profile of CD34+ cells mobilized by CD49d blockade Relative expression of p21, G0S2 and MMP9 in CD34+ selected HSC mobilized by natalizumab (HSC-nat) (n=3, *p < 0.05). Expression levels were normalized to mRNA levels in G-CSF mobilized CD34+ selected HSC. Dashed line= mRNA level of CD34+ cells mobilized by G-CSF set as 1.
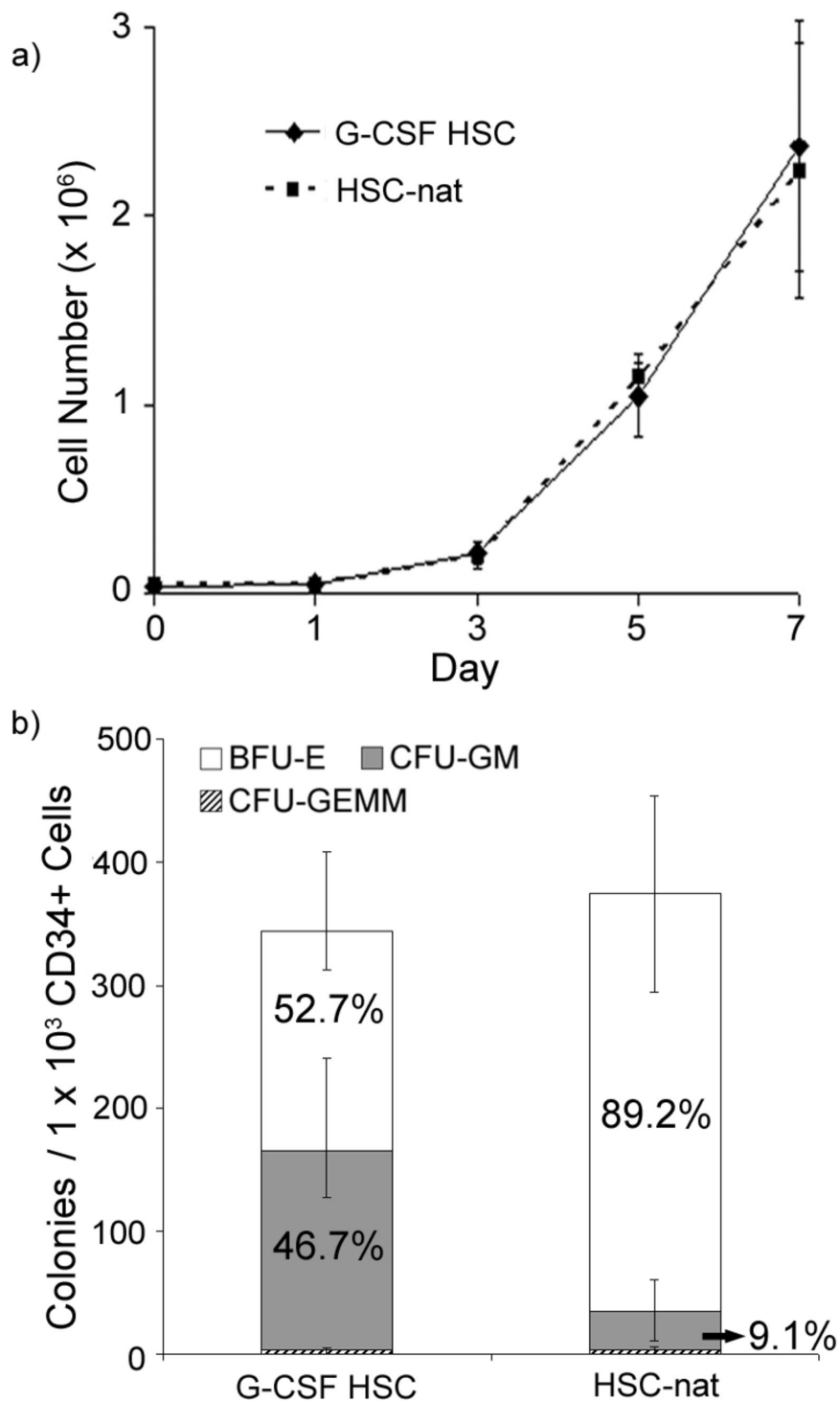
Figure 2-5: Proliferation and clonogenicity (a) The graph depicts the cell numbers measured during cytokine-driven in-vitro expansion of G-CSF HSC and HSC-nat. (b) Clonogenicity quantified by the number of colonies per 1000 CD34+ cells detectable after 14 days in semi-solid medium (G-CSF HSC, n=10) and from MS patients after natalizumab therapy (HSC-nat, n=3).

The argument that many patients with MS might have elevated levels of circulating CD34+ cells due to the chronic inflammatory condition is invalidated by the negligible CD34+ counts in MS patients not treated with natalizumab, who served as a control in our series[53]. At present we would not wish to exclude the possibility that the downregulation of adhesion molecules on CD34+ cells, as confirmed in the phenotypic analysis, makes more CD34+ cells occur in the aspirate simply because the HSCs are less adhesive and physically easier to dislocate. The high absolute levels of CD34+ cells in the BM aspirates argues nevertheless that natalizumab induces a compensatory increase in hematopoietic activity. Histological analysis of BM biopsies do not suggest a significant increase in overall BM cellularity (n=2, data not shown).

The initial homing of HSC into bone marrow depends on the SDF-1/CXCR4 chemokine axis[54], where after the interaction between HSC and stromal cells via the various integrins keeps HSCs in the BM stem cell niche[30]. In theory, both pathways contribute to HSC mobilization in parallel. Therefore, by disrupting HSC retention, both G-CSF and natalizumab promote egress of HSC into the peripheral blood. Our present results show that natalizumab mobilizes HSC with a higher CXCR4 expression but less expression of adhesion molecules (L-selectin, Pecam-1, LFA-1 and CD44) when compared to CD34+ cells mobilized by G-CSF. This is in line with recent data suggesting that CXCR4 expression on HSC and SDF1 levels in BM are reduced after G-CSF administration[55].

The differences in surface molecule expression also reflect the alternative mechanisms involved in HSC mobilization. Since the SDF-1/CXCR4 axis plays a separate essential role in HSC survival and repopulation in vivo[56-58] and natalizumab induces HSC mobilization without interacting with SDF1 signalling[59], CD49d blockade could be effective when used in combination with G-CSF.

The lower expression of MMP-9 in CD34+ cells derived from apheresis products mobilized by natalizumab fits the induction of MMP-9 and the subsequent cleavage and reduction of SDF-1 described after G-CSF administration. Consistent with the upregulation of CXCR4, HSC mobilized by natalizumab were better at migration in a chemokine-driven transwell assay, which would predict better primary engraftment in vivo. This contrasts with a recent report of poor migration of CFU-C in the PB of patients receiving natalizumab. The reported difference may be because we investigated CD34+ selected cells derived from leukapheresis samples whereas Bonig et al[22] analyzed unmanipulated peripheral blood.

The downregulation of prominin-1/CD133, a ubiquitous marker of both neural and hematopoietic stem/progenitor cells, argues that natalizumab mobilizes a compartment that is beyond multipotential commitment, allowing that CD133+ expression has been linked with long-term repopulating potential in vivo[60,61]. The predominance of erythroid progenitors in the leukapheresis samples that we

mobilized with natalizumab is in line with our own evidence that the CD34+CD133- fraction contains a significantly higher proportion of erythroid BFU-E, whereas most of the leukocyte CFU-GM are concentrated in the CD34+CD133+ cells[62]. A predominance of erythroid progenitors after VLA-4 blockade[20] in mice has been described in the literature .

We could not observe a difference in the percentage of CD34+/CD38- cells between natalizumab and G-CSF mobilized samples. Since this phenotype has been associated with stemness and repopulation potential in several reports[63,64], this finding suggests that the original repopulating HSC pool is still preserved after natalizumab mobilization of blood stem cells.

Although cell cycle analysis revealed no difference in cell-cycle status between CD34+ cells after G-CSF and natalizumab, the higher expression of p21 suggests that disrupting the CD49d–stroma/matrix interaction might induce a compensatory decrease in cell cycle activity. This conjecture is supported by the lower expression of a gene involved in cell cycle progression, G0/S2.

In summary, disrupting the integrin-based interaction between HSC and their niche may provide a complementary strategy for their mobilization. Although the long half-life of natalizumab presently precludes its use in patients scheduled for autologous transplantation or healthy donors, selective antagonists of CD49d could be developed. Our data suggest that CD49d blockade does not affect the SDF-1/CXCR-4 axis and could therefore improve conventional mobilization. Further in-vivo studies and clinical trials to evaluate the repopulating capacity of HSC mobilized by combined administration of G-CSF and CD49d antagonists are recommended.

Part 3: Hematopoietic Stem Cells in Coculture with Mesenchymal Stromal Cells- Modelling the Niche Compartments in-vitro

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RESULTS

Identification of hematopoietic stem cell localization. To localize HSCs cocultured with MSCs, we have performed scanning electron microscopy. Interestingly, two distinct fractions of cells were observed (Figure 3-1A, B). HSCs were located either on the surface of the MSC layer (Figure 3-1A, black asterisks) or beneath (Figure 3-1A, arrow). HSCs migrating underneath of MSCs were also detected (Figure 3-1A, B, outlined white asterisks). The time-lapse video microscopy analysis depicted the movement of an individual HSC migrating either above (Figure 3-1C, black arrow) or beneath (Figure 3-1C, white arrow) the MSC layer. By phase contrast microscopy, the HSC population underneath the MSC layer appeared as phase-dim cells whereas those located above as phase-bright cells (Figure 3-1D). The hematopoietic origin of these two cell fractions was confirmed by immunolabelling for CD45 (Figure 3-1E). Indeed, three optical confocal x-y–sections showed that both phase-bright and phase-dim cells were positive (Figure 3-1F, H, respectively) in contrast to MSCs (Figure 3-1G). Upon gently washings (for technical details see Design and Methods), the only remaining cells as those underneath MSC layer, i.e phase-dim cells (Figure 3-1I, J).

Mesenchymal stromal cells surface is the dominant place for hematopoietic stem cells proliferation. To determine the influence of cellular localization on HSC expansion, we counted cells in their separated environments. Until day 5 of coculture, the cell number of the non-adherent and the phase-bright cells increased similarly and much faster than phase-dim cells (Figure 3-2A). After day 5 the number of non-adherent cells increased further, the number of phase-bright cells and phase-dim cells however remained constant (Figure 3-2A). Interestingly though non-adherent cells showed highest cell counts these cells were not in the G2/M phase shown by the propidium iodide (PI) staining. As shown in (Figure 3-2 B, C), the phase-bright cells (PB) contain a higher proportion in G2/M phase compared to non-adherent cells (NA) and phase-dim cells (PD) throughout the whole week of coculture, e.g. on day 3: PB $11.9 \pm 2.7\%$ vs NA $0 \pm 0\%$ ($p < 0.001$) and PD $3.6 \pm 2.4\%$ ($p < 0.001$) and on day 7: PB $13.9 \pm 1.0\%$ vs NA $1.3 \pm 1.2\%$ ($p < 0.001$) and PD $2.7 \pm 2.0\%$ ($p < 0.001$). This is consistent with the expression of p21 which is an essential regulator for the quiescence of HSCs [65]. P21 expression was significantly diminished in phase-bright
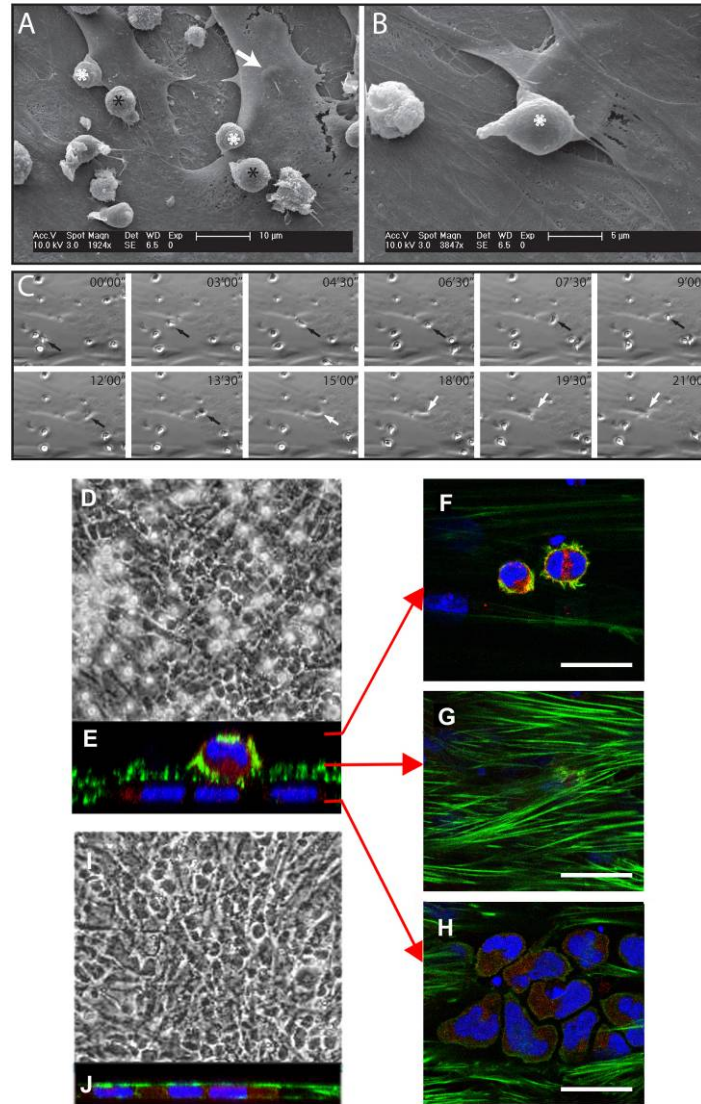
Figure 1

Figure 3-1. Distinct compartmentalization of HSCs cultured on MSCs. (A, B) Scanning electron microscopy analysis reveals that HSCs are either over the MSC layer (black asterisks) or beneath (white arrow). HSCs migrating underneath the MSC layer were also observed (outlined white asterisks). (C) Individual frames taken from time-lapse video depict of an individual HSC moving above (black arrow) and below (white arrow) the MSC layer for a period of 54 min. M indicates MSCs. (D) Phase-contrast microscopy analysis shows that HSCs located either above or beneath the MSC layer appeared as phase-bright and -dim cells, respectively. (E) Confocal laser scanning microscopy upon immunolabeling for CD45. (F) phase-bright cells on the surface of MSC layer. (G) MSC layer. (H phase-dim cells beneath MSC layer. (I) After removing phase-bright cells MSC layer with the cells beneath the layer (phase-dim cells) performing phase contrast microscopy. (J) A cross-section is shown using immunofluorescence imaging (actin, green; nucleus, blue; CD45, red). Scale bar: 20 μm.
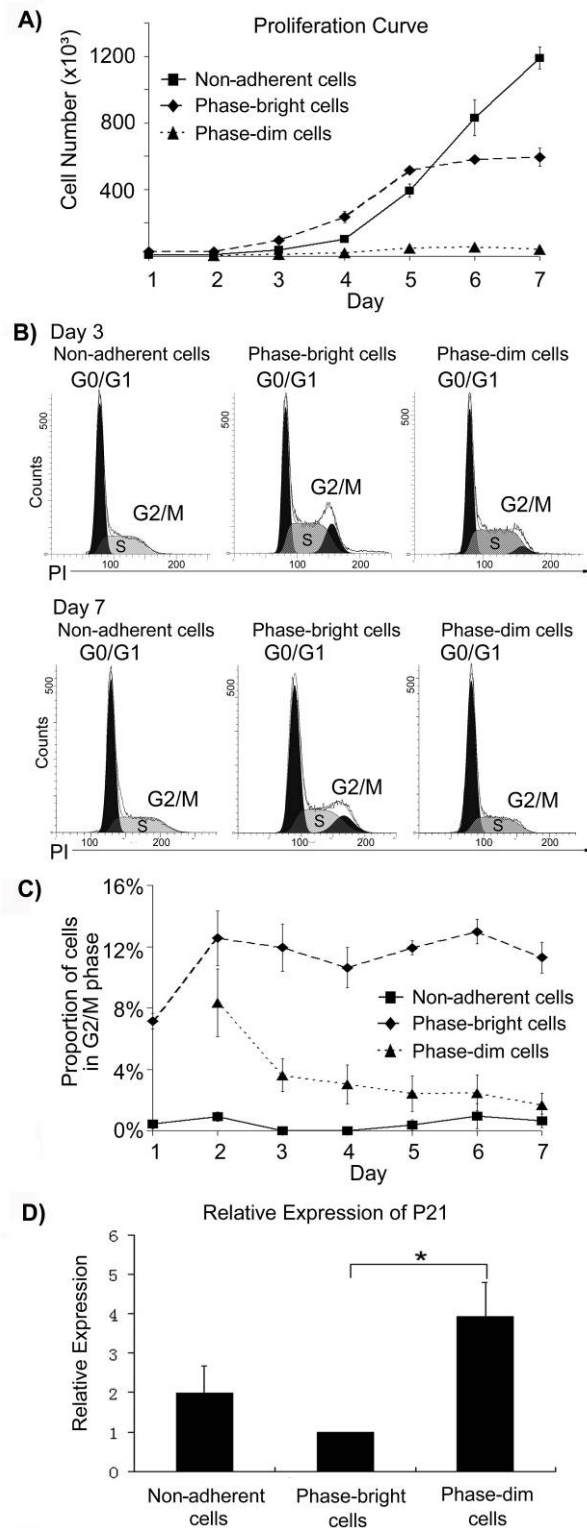
Figure 3-2. Cell proliferation and cell cycle status. (A) Proliferation kinetics of the three cell fractions (N = 4). (B) Representative PI staining of cells from three distinct compartments at day 3 and 7. (C) Dynamics of the three cell fractions in G2/M phase during one week coculture (N = 3). (D) Relative expression of p21 in the three compartments at day 5 (N = 3, *p < 0.05). The data were normalized to the p21 expression in phase-bright cells, which was as arbitrarily set to 1.

cells at day 5 (Figure 3-2D). These data indicate that in vitro MSC surface is enriched for proliferating progeny and phase-bright cells may detach from the MSC surface on reaching a non-adherent status.

Hematopoietic stem and progenitor cells beneath the mesenchymal stromal cells layer remain immature. To investigate the impact of the localization on HSC differentiation, HSC phenotypes were measured by FACS analysis. CD34 is a classical HSC marker [66], and CD34+CD38- cells are usually considered as a more primitive HSC population [67]. After apheresis we performed MACS purification of CD34+ cells. We could show in particular that both CD34+ and CD34+CD38- cells are enriched in the phase-dim fraction in comparison to the phase-bright cells and the non-adherent cells (Figure 3-3). At day 2, almost 100% of phase-dim cells were CD34+CD38-, while the percentage of CD34+CD38- cells within the non-adherent and phase-bright fraction was below 75%. In the following days, CD34+ and CD34+CD38- cells in the phase-dim fraction decreased as well, but the drop was significantly delayed in comparison to the other cell fractions. Shown within the circles in Figure 3-3A, CD38 was highly expressed in non-adherent cells and phase-bright cells compared to phase-dim cells at day 4. In addition, we were able to show a higher concentration of clonogenic CFU-C within phase-dim cells compared to non-adherent cells and phase-bright cells (PD 150 ± 20 colonies vs NA 119 ± 11 colonies (N = 6, p = 0.07) and PB 125 ± 20 colonies (N = 6, p < 0.05)).

Cell divisions in distinct localizations. To determine the impact of localization on cell division, HSC generations were tracked by CFSE staining. Generation 0 was represented by the control, which was treated with Mitomycin. Two representative experiments are shown in (Figure 3-4A). At day 2, approximately 50% of phase-dim cells had not divided (generation 0), while generation 0 cells of the other two cell fractions were less than 20%. In the following days, phase-dim cells exhibited a higher proportion of cells having undergone less divisions compared to the other two cell fractions. Comparing the distribution of cell generations in the three compartments at day 2, 3 and 4, the data indicate the down regulating impact of the compartment beneath the MSC on cell division (Figure 3-4B).

Immunophenotypes after each cell division. In order to determine the retention of the progenitor phenotype after each cell division, CFSE and CD34 double staining was performed in the three separated groups. After the first two cell divisions (in HSC generation 0, 1 and 2), almost all cells in all three localizations were CD34 positive (Figure 3-4C). However in generation 3 and 4 only the phase-dim population retained high percentage of CD34 expressing cells, which indicates their delayed differentiation compared to the other two cell fractions. In the following cell divisions, as shown in (Figure 3-4D), there were always more phase-dim cells that retained CD34 expression compared to the same HSC generation of the other two cell fractions. Taken together, the outcome of HSC division differed according to their localizations,
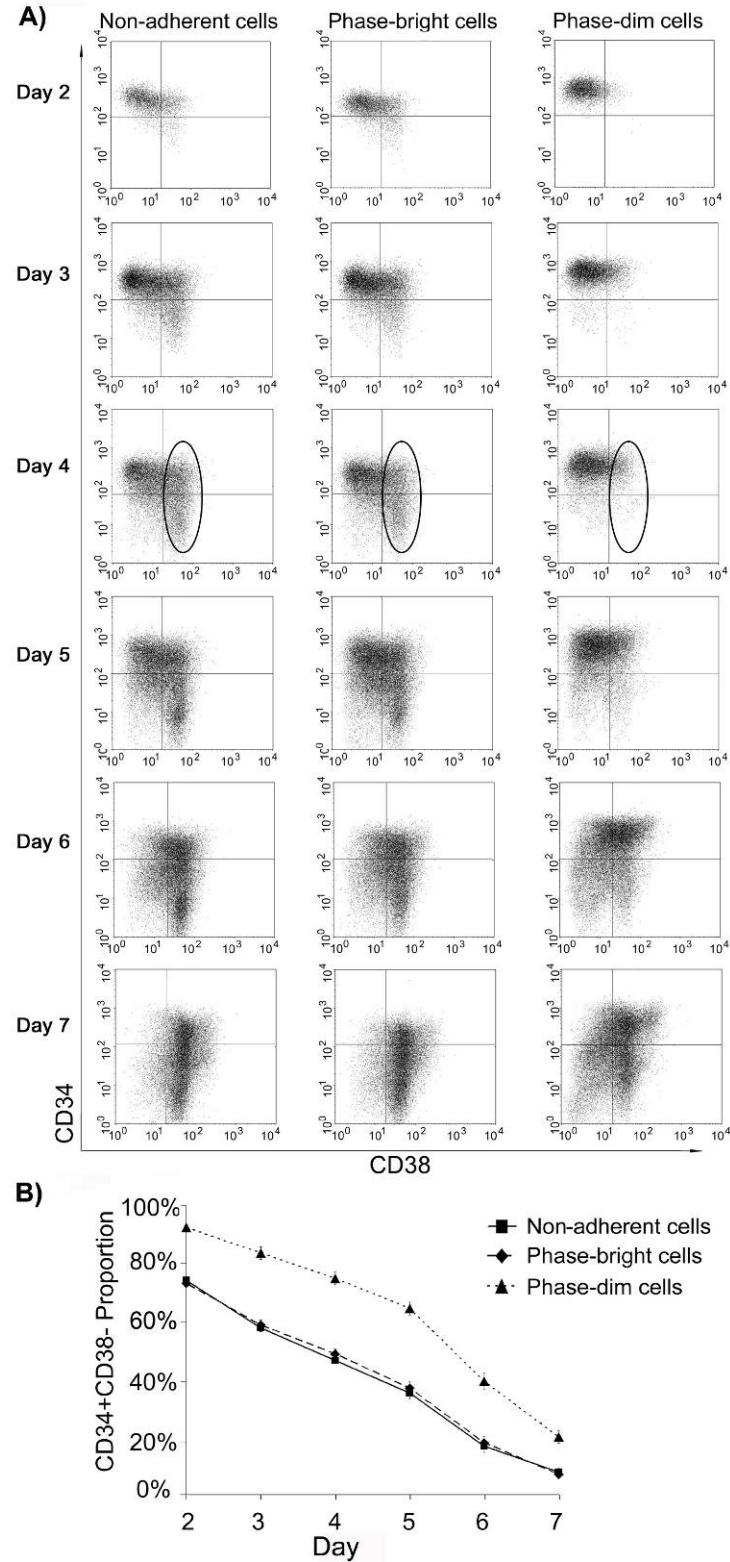
Figure 3

Figure 3. Immunophenotype of the HSCs in distinct compartments. (A) Representative FACS analysis of the three cell fractions from day 2 to 7. CD38+ cell fraction at day 4 was marked within circle. (B) CD34+CD38- proportion of the three cell fractions during one week coculture (N = 7).
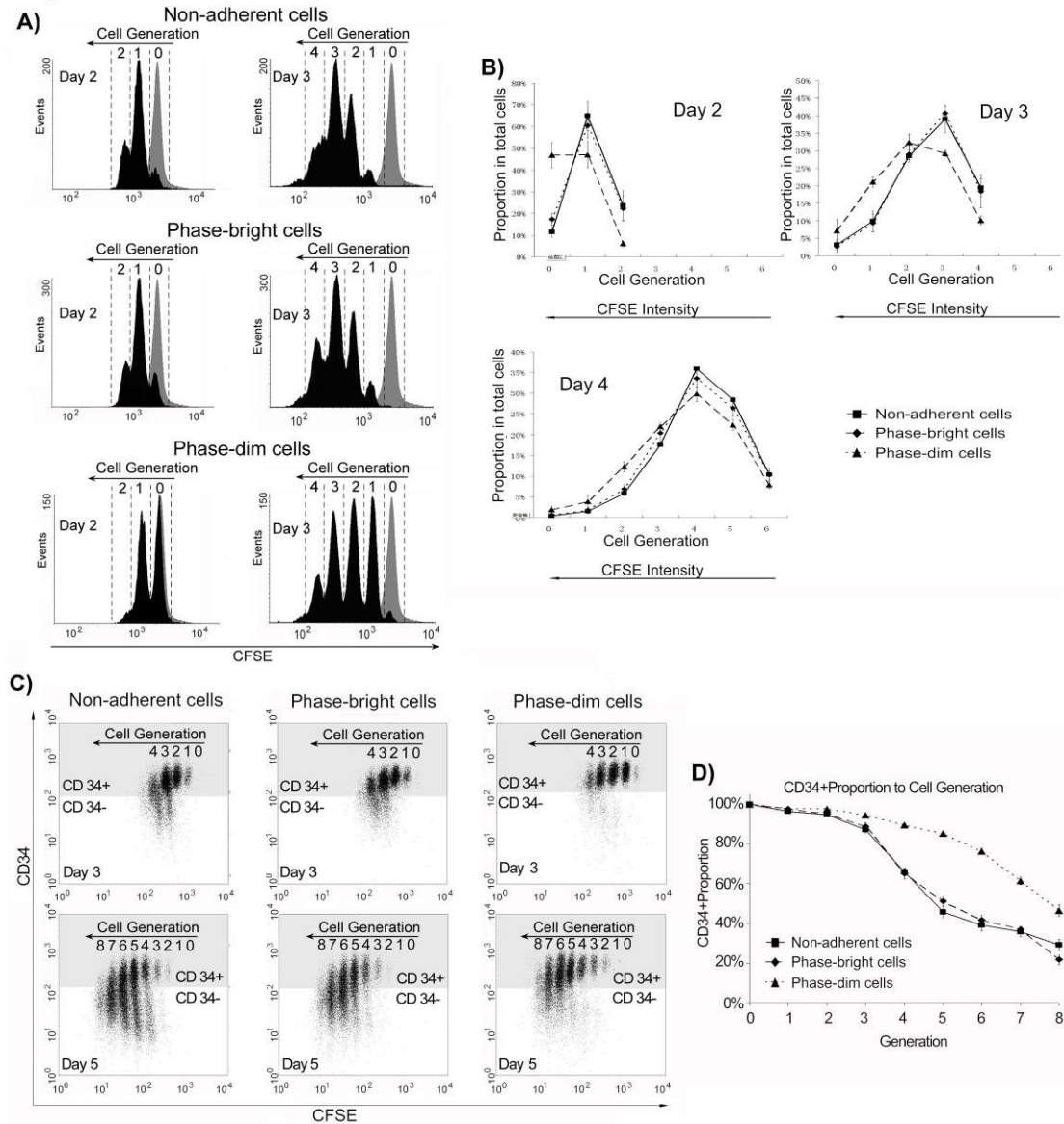
Figure 4

Figure 4. Tracking cell division in distinct compartments using CFSE staining. (A) Representative FACS analysis of CFSE staining at day 2 and 3. Cell generations were identified according to the control cells which were treated with Mitomycin at day 0 (gray peak). (B) Distributions of cell generations of the three cell fractions at day 2, 3 and 4 (N = 3). Then the pattern of CD34 expression in different cell generations was studied. (C) Representative FACS analysis of CD34 and CFSE double staining at day 3 and 5. The dots in gray region are positive for CD34, the dots in white region are negative for CD34. Cell generations were identified according to the control cells which were treated with Mitomycin at day 0. (D) CD34+ expression over eight cell generations within the three cell fractions (N = 8).

suggesting that the more slowly proliferating HSCs grown beneath MSCs retain their stem cell characteristics during cell division.

Immature hematopoietic stem cells migrate beneath the mesenchymal stromal cell layer. Next we ask whether more immature cells prefer the compartment beneath the MSC layer, migrating directly to this area in vitro. As already mentioned we detected phase-dim cells in newly established cocultures only after day 2. However when purified HSCs were cultured without MSCs in a cytokine supplemented medium for four days and then seeded on a MSC layer in a second culture as described in the method part we could see up to 6% phase-dim cells already after 5 hrs of coculture, indicating an increased capacity for migration beneath the MSC layer for primed HSCs. As shown in (Figure 3-5A) HSCs with 54.67% ± 2.55% CD34+CD38- expressing phenotype were plated on the MSC layer. After 5 hours those CD34+CD38- cells (78.23% ± 7.65%) were enriched in the phase-dim fraction, suggesting that more immature HSCs preferentially home beneath the MSC layer.

CXCR4 and Integrins influence phase-dim cell formation. Recently we demonstrated that CXCR4 is upregulated in the adherent-cell fraction in comparison to the non-adherent cell fraction [43]. Other adhesion molecules such as integrins is also important for homing and migration [48,68,69]. The assay described above allowed us to investigate the impact of the surface molecules on the primary attachment and migration of HSC underneath the MSC layer. First we blocked integrin ß1 as described in the methods section. As shown in Figure 5B phase-bright cells were reduced from 69.89% ± 4.08% to 40.56% ± 12.45% ($p < 0.01$). Phase-dim cells were reduced from 7.18% ± 1.50% to 3.38% ± 1.19% ($p < 0.01$) indicating that at least integrin ß1 takes part in the process of HSCs adhesion on the MSC surface and migration beneath the MSC layer. Next we blocked CXCR4 by adding AMD3100 (Figure 3-5B). Although the count of phase-bright cells was not reduced (69.89% ± 4.08% vs 75.33% ± 8.42%), phase-dim cells were diminished from 7.18% ± 1.50% to 3.19% ± 0.77% ($p < 0.01$), indicating that in our assay CXCR4 plays a role in HSC migration beneath the MSC layer. Finally by blocking both CXCR4 and Integrin ß1, the count of phase-dim cells was further reduced to 1.97% ± 0.18% in comparison to individual blocking of integrin ß1 or CXCR4 (3.38% ± 1.19% and 3.19% ± 0.77%) indicating that both integrins and CXCR4 are involved in the migration of HSCs beneath the MSC feeder layer. Our data confirms also that CXCR4 has no impact on the adhesiveness as already shown in our paper published recently [43]. Blocking P-selectin as control showed not significant decrease of phase-bright and phase-dim cells.

DISCUSSION
Many studies have demonstrated that HSCs can be expanded in cytokine-driven culture. However, this kind of expansion is accompanied by concomitant differentiation and gradual loss of stemness. In recent years, it has been shown that stromal feeder layers can be used to support HSC expansion. It has become evident
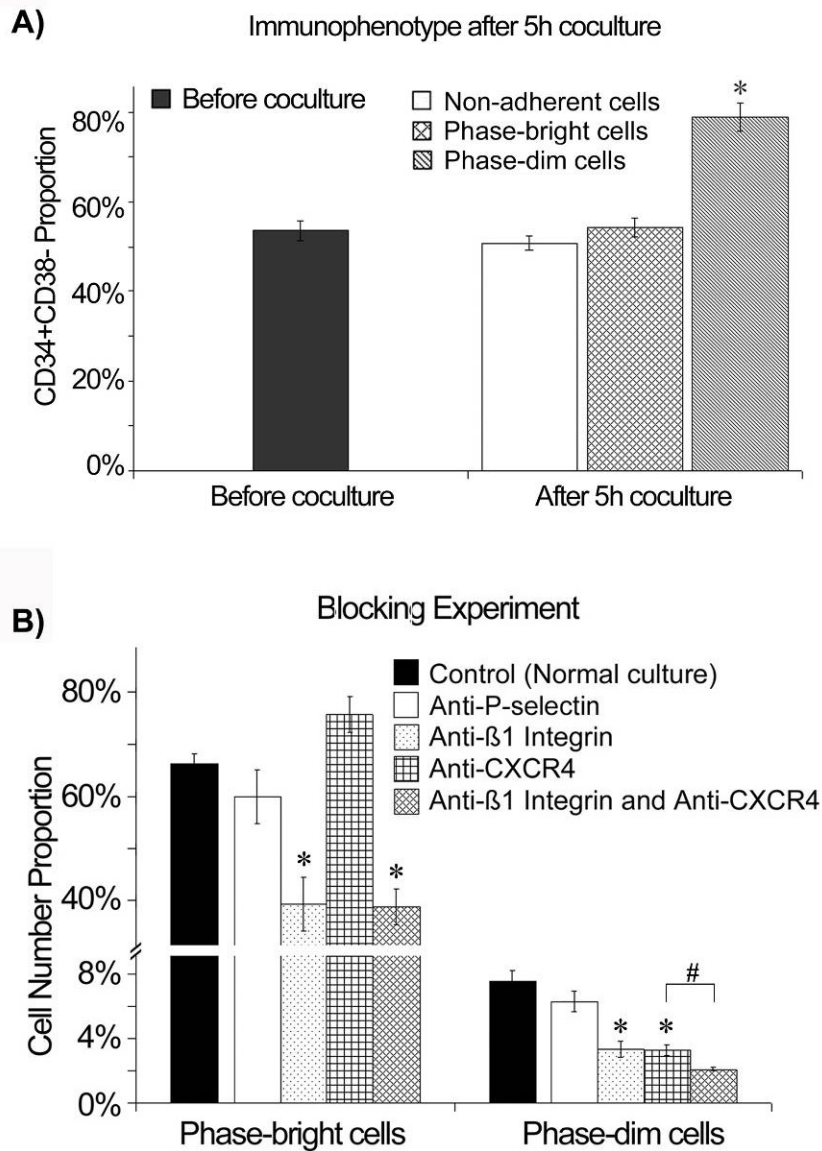
Figure 5

Figure 3-5. (A) Enrichment of CD34+CD38- cells in phase-dim fraction. Cells containing around 50% CD34+CD38- cells were plated on MSC layer. After 5 hours CD34+CD38- proportion in phase-dim cells was enriched up to 80% ($N = 4$, $*p < 0.01$). (B) Blocking experiments for P-selectin, Integrin ß1 and CXCR4. By blocking Integrin ß1 or CXCR4 or both, percentages of cell numbers of phase-bright or phase-dim cells were down-regulated ($N = 4$, $*p < 0.01$ (relative to control); $\#p < 0.05$ (relative to individual blocking for Integrin ß1 or CXCR4)).

that the interaction between HSCs and MSCs is an important issue for keeping HSCs quiescent both in vivo and in vitro [30]. We and others have shown that cell-to-cell contact in vitro has a significant impact on HSCs in terms of functional, phenotypic, and clonogenic parameters. We have also demonstrated that direct contact with MSCs affects the migratory behavior and gene expression profile of CD133+ HSCs during ex vivo expansion [43]. In the present report, the distribution of HSCs in the coculture assay has been investigated in more detail. We have observed that three different localizations in coculture system represent distinct microenvironments for HSCs having a significant impact on their fate in vitro. By performing phase-contrast, confocal and electron microscopy, we separated HSCs which were non-adherent, cells adherent to the surface of the MSC layer, and cells which had migrated beneath the feeder layer. A confluent MSC layer may serve as a boundary between two distinct compartments. These spatial constraints influence the proliferation and differentiation of HSCs.

One remarkable difference is the cell cycle status of cultured HSC. It is known that immediately after HSCs are isolated from peripheral blood (PB), they usually are in G0/G1 [70]. Their status however changes if the cells are plated out in expansion assays with stimulating factors[32]. In our study phase-bright cells significantly and consistently showed an increase of G2/M cells (over 10%) throughout the whole culture period. In contrast among the non-adherent cells and the phase-dim cells the G2/M phase was almost completely lacking. Interestingly, G2/M phases happen mainly on the surface and not in a non-adherent status. On the other hand, the number of non-adherent cells increased continuously, suggesting that the adherent fraction supplies the non-adherent fraction with detaching cells. We have evidence that the cell-to-cell contact on the surface of the MSC layer promotes the cell cycle, however the mechanism has still not been clarified in detail.

Interestingly we found that in our coculture system the translocation between HSCs on the surface and those beneath the MSC layer was between two distinct compartments. Performing serial studies we found that phase-dim cells retained a more immature phenotype in comparison to the HSCs on the surface of the MSC layer and had a significantly delayed rate of cell division in comparison to the other two cell fractions. Therefore we speculate that the MSC boundary layer has a significant effect on HSC fate. Admittedly, HSCs are highly motile and able to move between the compartments. Our daily measurements still represent snap-shots taken at defined time-points, which may not represent the whole complex dynamics of the process.

It is still not clear whether the environment beneath the MSC layer actively keeps HSCs in an immature state or creates a 'niche' atmosphere which specifically attracts HSCs. We speculate that both mechanisms are involved. We could at least demonstrate that CD34+CD38- HSCs prefentially migrate through the MSC layer.
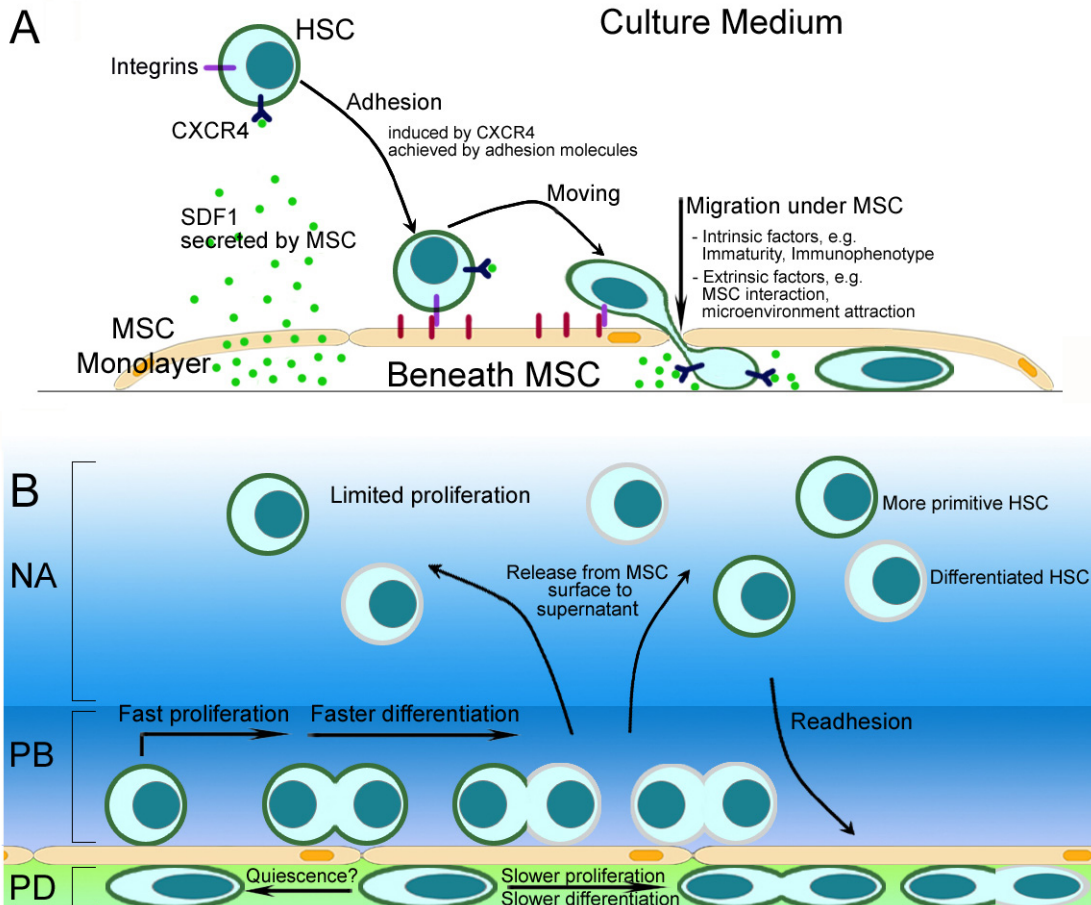
Figure 6

Figure 3-6. Graphic of the HSC/MSC coculture system in vitro. (A) Migration towards and retention of HSCs to MSCs is mediated by SDF1/CXCR4 and adhesion molecules such as Integrins. (B) The MSC layer serves in vitro as a boundary of two distinct compartments, i.e. the MSC surface mediating cell proliferation and a niche-like compartment beneath the layer. Here beneath the layer cells (PD) show a delayed cell-cycle activity and a more immature phenotype. In opposite HSCs on the MSC surface (PB) revealed significantly more proliferation activity. We assume that cells from the MSC surface are released into the supernatant, the third microenvironment (NA) in the coculture system. The graphic demonstrates a dynamic interplay of HSCs within the three compartments.

In addition, the interaction between HSCs and MSCs via soluble factors (SDF1/CXCR4) or adhesion molecules (integrins) has an impact on HSC migration between the two microenvironments. Phase-dim cell formation could be reduced by blocking integrins and CXCR4. This effect could be enhanced even further by combined blockade, indicating that ß1-integrins and the SDF1/CXCR4 axis play synergistic roles in phase-dim cell formation. Interestingly the count of phase-bright cells was not reduced by AMD3100, indicating that CXCR4 is not relevant in adhesiveness confirming our recently published data [43].

In summary, we can distinguish three different compartments in our coculture system; i) supernatant, i.e. the milieu where HSCs are growing without direct contact with MSCs; ii) surface of MSCs and iii) the environment beneath the MSC layer. All three locations are dynamically linked with each other, and are characterized by special features. Specifically, the niche-like microenvironment (i.e. beneath MSCs) probably recruits and retains HSCs with more primitive properties whereas the other favors the active expansion of HSC (MSC surface).

Interestingly, in vivo there is a similar but certainly even more complex system of niches. It has been reported that quiescent HSCs are preferentially located in osteoblastic niches, while more actively cycling and self-renewing HSCs are kept in the perivascular niche [30,71]. The three-compartment coculture system probably mimics the cooperation of stem cell niches in vivo.

The model of the HSC "in vitro niche" is demonstrated in (Figure 3-6), showing the MSC surface as the major site of HSC proliferation and the microenvironment beneath the MSC layer as the storage site of more primitive cells, all affected by an orchestra of various soluble and non-soluble signals. Further investigations are needed to study the complex mechanism of hematopoietic stem cell expansion in vitro, including investigation of the repopulating potential of the different cell fractions by performing in-vivo repopulation experiments.

REFERENCES

1. Hansen, J. A. *et al.* Transplantation of hematopoietic stem cells (HSC). *Br. Med. Bull.* **43**, 203-216 (1987).
2. Thomas, E. D. Landmarks in the development of hematopoietic cell transplantation. *World J. Surg.* **24**, 815-818 (2000).
3. Bornhauser, M. *et al.* Rapid reconstitution of dendritic cells after allogeneic transplantation of CD133+ selected hematopoietic stem cells. *Leukemia* **19**, 161-165 (2005).
4. Broxmeyer, H. E. *et al.* Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. *J. Exp. Med.* **201**, 1307-1318 (2005).
5. Robin, C. *et al.* Human placenta is a potent hematopoietic niche containing hematopoietic stem and progenitor cells throughout development. *Cell Stem Cell* **5**, 385-395 (2009).
6. Osawa, M., Hanada, K., Hamada, H. & Nakauchi, H. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science* **273**, 242-245 (1996).
7. Akashi, K., Traver, D., Miyamoto, T. & Weissman, I. L. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* **404**, 193-197 (2000).
8. Metcalf, D. On hematopoietic stem cell fate. *Immunity.* **26**, 669-673 (2007).
9. Gordon, M. Y. Stem cells for regenerative medicine--biological attributes and clinical application. *Exp. Hematol.* **36**, 726-732 (2008).
10. Rocha, V. *et al.* Transplants of umbilical-cord blood or bone marrow from unrelated donors in adults with acute leukemia. *N. Engl. J. Med.* **351**, 2276-2285 (2004).
11. Bornstein, R. *et al.* A modified cord blood collection method achieves sufficient cell levels for transplantation in most adult patients. *Stem Cells* **23**, 324-334 (2005).
12. Broxmeyer, H. E. *et al.* Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. *J. Exp. Med.* **201**, 1307-1318 (2005).
13. Devine, S. M. *et al.* Rapid mobilization of functional donor hematopoietic cells without G-CSF using AMD3100, an antagonist of the CXCR4/SDF-1 interaction. *Blood* **112**, 990-998 (2008).
14. Sheridan, W. P. *et al.* Effect of peripheral-blood progenitor cells mobilised by filgrastim (G-CSF) on platelet recovery after high-dose chemotherapy. *Lancet* **339**, 640-644 (1992).
15. Schmitz, N. *et al.* Primary transplantation of allogeneic peripheral blood progenitor cells mobilized by filgrastim (granulocyte colony-stimulating factor). *Blood* **85**, 1666-1672 (1995).
16. Lane, T. A. *et al.* Harvesting and enrichment of hematopoietic progenitor cells mobilized into the peripheral blood of normal donors by granulocyte-macrophage colony-stimulating factor (GM-CSF) or G-CSF: potential role in allogeneic marrow transplantation. *Blood* **85**, 275-282 (1995).

17. Roberts, A. W. *et al.* Broad inter-individual variations in circulating progenitor cell numbers induced by granulocyte colony-stimulating factor therapy. *Stem Cells* **13**, 512-516 (1995).

18. Moncada, V., Bolan, C., Yau, Y. Y. & Leitman, S. F. Analysis of PBPC cell yields during large-volume leukapheresis of subjects with a poor mobilization response to filgrastim. *Transfusion* **43**, 495-501 (2003).

19. Dipersio, J. F. *et al.* Plerixafor and G-CSF versus placebo and G-CSF to mobilize hematopoietic stem cells for autologous stem cell transplantation in patients with multiple myeloma. *Blood* (2009).

20. Papayannopoulou, T. & Nakamoto, B. Peripheralization of hemopoietic progenitors in primates treated with anti-VLA4 integrin. *Proc. Natl. Acad. Sci. U. S. A* **90**, 9374-9378 (1993).

21. Craddock, C. F., Nakamoto, B., Andrews, R. G., Priestley, G. V. & Papayannopoulou, T. Antibodies to VLA4 integrin mobilize long-term repopulating cells and augment cytokine-induced mobilization in primates and mice. *Blood* **90**, 4779-4788 (1997).

22. Bonig, H., Wundes, A., Chang, K. H., Lucas, S. & Papayannopoulou, T. Increased numbers of circulating hematopoietic stem/progenitor cells are chronically maintained in patients treated with the CD49d blocking antibody natalizumab. *Blood* **111**, 3439-3441 (2008).

23. Zohren, F. *et al.* The monoclonal anti-VLA-4 antibody natalizumab mobilizes CD34+ hematopoietic progenitor cells in humans. *Blood* **111**, 3893-3895 (2008).

24. Sorrentino, B. P. Clinical strategies for expansion of haematopoietic stem cells. *Nat. Rev. Immunol.* **4**, 878-888 (2004).

25. Calvi, L. M. *et al.* Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* **425**, 841-846 (2003).

26. Zhang, J. *et al.* Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* **425**, 836-841 (2003).

27. Scadden, D. T. The stem-cell niche as an entity of action. *Nature* **441**, 1075-1079 (2006).

28. Kiel, M. J. *et al.* SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* **121**, 1109-1121 (2005).

29. Kiel, M. J. & Morrison, S. J. Maintaining hematopoietic stem cells in the vascular niche. *Immunity.* **25**, 862-864 (2006).

30. Wilson, A. & Trumpp, A. Bone-marrow haematopoietic-stem-cell niches. *Nat. Rev. Immunol.* **6**, 93-106 (2006).

31. Blank, U., Karlsson, G. & Karlsson, S. Signaling pathways governing stem-cell fate. *Blood* **111**, 492-503 (2008).

32. Heike, T. & Nakahata, T. Ex vivo expansion of hematopoietic stem cells by cytokines. *Biochim. Biophys. Acta* **1592**, 313-321 (2002).

33. Ueda, T. *et al.* Expansion of human NOD/SCID-repopulating cells by stem cell factor, Flk2/Flt3 ligand, thrombopoietin, IL-6, and soluble IL-6 receptor. *J. Clin. Invest* **105**, 1013-1021 (2000).

34. Sorrentino, A. *et al.* Isolation and characterization of CD146+ multipotent

mesenchymal stromal cells. *Exp. Hematol.* **36**, 1035-1046 (2008).

35. Crisan, M. *et al.* A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* **3**, 301-313 (2008).

36. Sacchetti, B. *et al.* Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* **131**, 324-336 (2007).

37. Majumdar, M. K., Thiede, M. A., Haynesworth, S. E., Bruder, S. P. & Gerson, S. L. Human marrow-derived mesenchymal stem cells (MSCs) express hematopoietic cytokines and support long-term hematopoiesis when differentiated toward stromal and osteogenic lineages. *J. Hematother. Stem Cell Res.* **9**, 841-848 (2000).

38. Noort, W. A. *et al.* Mesenchymal stem cells promote engraftment of human umbilical cord blood-derived CD34(+) cells in NOD/SCID mice. *Exp. Hematol.* **30**, 870-878 (2002).

39. Aggarwal, S. & Pittenger, M. F. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* **105**, 1815-1822 (2005).

40. Breems, D. A. *et al.* Stroma-contact prevents loss of hematopoietic stem cell quality during ex vivo expansion of CD34+ mobilized peripheral blood stem cells. *Blood* **91**, 111-117 (1998).

41. Gottschling, S. *et al.* Human mesenchymal stromal cells regulate initial self-renewing divisions of hematopoietic progenitor cells by a beta1-integrin-dependent mechanism. *Stem Cells* **25**, 798-806 (2007).

42. Wagner, W. *et al.* Molecular and secretory profiles of human mesenchymal stromal cells and their abilities to maintain primitive hematopoietic progenitors. *Stem Cells* **25**, 2638-2647 (2007).

43. Alakel, N. *et al.* Direct contact with mesenchymal stromal cells affects migratory behavior and gene expression profile of CD133+ hematopoietic stem cells during ex vivo expansion. *Exp. Hematol.* **37**, 504-513 (2009).

44. Fuchs, E., Tumbar, T. & Guasch, G. Socializing with the neighbors: stem cells and their niche. *Cell* **116**, 769-778 (2004).

45. Oswald, J. *et al.* Mesenchymal stem cells can be differentiated into endothelial cells in vitro. *Stem Cells* **22**, 377-384 (2004).

46. Freund, D. *et al.* Polarization of human hematopoietic progenitors during contact with multipotent mesenchymal stromal cells: effects on proliferation and clonogenicity. *Stem Cells Dev.* **15**, 815-829 (2006).

47. Barcena, A. *et al.* The human placenta is a hematopoietic organ during the embryonic and fetal periods of development. *Dev. Biol.* **327**, 24-33 (2009).

48. Fierro, F. A. *et al.* BCR/ABL expression of myeloid progenitors increases beta1-integrin mediated adhesion to stromal cells. *J. Mol. Biol.* **377**, 1082-1093 (2008).

49. Broxmeyer, H. E. *et al.* Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. *J. Exp. Med.* **201**, 1307-1318 (2005).

50. Fruehauf, S. *et al.* The CXCR4 antagonist AMD3100 releases a subset of G-CSF-primed peripheral blood progenitor cells with specific gene expression characteristics. *Exp. Hematol.* **34**, 1052-1059 (2006).

51. Faye, A. *et al.* Evaluation of the placental environment with a new in vitro model of histocultures of early and term placentae: determination of cytokine and chemokine expression profiles. *Placenta* **26**, 262-267 (2005).

52. Serikov, V. *et al.* A BRIEF COMMUNICATION: Human Term Placenta as a Source of Hematopoietic Cells. *Experimental Biology and Medicine* **234**, 813-823 (2009).

53. Vuaillat, C., Androdias, G., Davoust, N. & Nataf, S. About multiple sclerosis, natalizumab, and CD34+ hematopoietic progenitors. *Blood* **112**, 208-209 (2008).

54. Lapidot, T., Dar, A. & Kollet, O. How do stem cells find their way home? *Blood* **106**, 1901-1910 (2005).

55. Levesque, J. P., Hendy, J., Takamatsu, Y., Simmons, P. J. & Bendall, L. J. Disruption of the CXCR4/CXCL12 chemotactic interaction during hematopoietic stem cell mobilization induced by GCSF or cyclophosphamide. *J. Clin. Invest* **111**, 187-196 (2003).

56. Lapidot, T. & Kollet, O. The essential roles of the chemokine SDF-1 and its receptor CXCR4 in human stem cell homing and repopulation of transplanted immune-deficient NOD/SCID and NOD/SCID/B2m(null) mice. *Leukemia* **16**, 1992-2003 (2002).

57. Li, K. *et al.* Small peptide analogue of SDF-1alpha supports survival of cord blood CD34+ cells in synergy with other cytokines and enhances their ex vivo expansion and engraftment into nonobese diabetic/severe combined immunodeficient mice. *Stem Cells* **24**, 55-64 (2006).

58. Faber, A. *et al.* The Many Facets of SDF-1alpha, CXCR4 Agonists and Antagonists on Hematopoietic Progenitor Cells. *J. Biomed. Biotechnol.* **2007**, 26065 (2007).

59. Christopher, M. J., Liu, F., Hilton, M. J., Long, F. & Link, D. C. Suppression of CXCL12 production by bone marrow osteoblasts is a common and critical pathway for cytokine-induced mobilization. *Blood* (2009).

60. Fargeas, C. A., Corbeil, D. & Huttner, W. B. AC133 antigen, CD133, prominin-1, prominin-2, etc.: prominin family gene products in need of a rational nomenclature. *Stem Cells* **21**, 506-508 (2003).

61. Hess, D. A. *et al.* Selection based on CD133 and high aldehyde dehydrogenase activity isolates long-term reconstituting human hematopoietic stem cells. *Blood* **107**, 2162-2169 (2006).

62. Freund, D. *et al.* Comparative analysis of proliferative potential and clonogenicity of MACS-immunomagnetic isolated CD34+ and CD133+ blood stem cells derived from a single donor. *Cell Prolif.* **39**, 325-332 (2006).

63. Prosper, F., Stroncek, D. & Verfaillie, C. M. Phenotypic and functional characterization of long-term culture-initiating cells present in peripheral blood progenitor collections of normal donors treated with granulocyte colony-stimulating factor. *Blood* **88**, 2033-2042 (1996).

64. Hao, Q. L., Thiemann, F. T., Petersen, D., Smogorzewska, E. M. & Crooks, G. M. Extended long-term culture reveals a highly quiescent and primitive human hematopoietic progenitor population. *Blood* **88**, 3306-3313 (1996).

65. Cheng, T. *et al.* Hematopoietic stem cell quiescence maintained by p21cip1/waf1. *Science* **287**, 1804-1808 (2000).

66. Krause, D. S., Fackler, M. J., Civin, C. I. & May, W. S. CD34: structure, biology, and clinical utility. *Blood* **87**, 1-13 (1996).

67. Petzer, A. L., Hogge, D. E., Landsdorp, P. M., Reid, D. S. & Eaves, C. J. Self-renewal of primitive human hematopoietic cells (long-term-culture-initiating cells) in vitro and their expansion in defined medium. *Proc. Natl. Acad. Sci. U. S. A* **93**, 1470-1474 (1996).

68. Lapidot, T., Dar, A. & Kollet, O. How do stem cells find their way home? *Blood* **106**, 1901-1910 (2005).

69. Peled, A. *et al.* The chemokine SDF-1 activates the integrins LFA-1, VLA-4, and VLA-5 on immature human CD34(+) cells: role in transendothelial/stromal migration and engraftment of NOD/SCID mice. *Blood* **95**, 3289-3296 (2000).

70. Uchida, N. *et al.* The unexpected G0/G1 cell cycle status of mobilized hematopoietic stem cells from peripheral blood. *Blood* **89**, 465-472 (1997).

71. Xie, Y. *et al.* Detection of functional haematopoietic stem cell niche using real-time imaging. *Nature* **457**, 97-101 (2009).

ABBREVIATIONS

- ALL: Acute lymphoblastic leukemia
- AML: Acute myeloid leukemia
- BFU-E: Burst-forming unit-erythroid
- BM: Bone marrow
- CFU-C: Colony-forming units in culture
- CFU-GEMM: Colony-forming unit - granulocyte, erythroid, macrophage, megakaryocyte
- CFU-GM: Colony forming unit-granulocyte and macrophage
- CFU-M: Colony forming unit-macrophage
- CLL: Chronic lymphocytic leukemia
- CML: Chronic myeloid leukemia
- CXCR4: CXC chemokine receptor 4
- DMSO: Dimethyl sulfoxide
- FACS: Fluorescence activated cell sorting
- FCS: Fetal cow serum
- FLT3-L: Fetal liver tyrosine kinase-3 ligand
- GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
- h: hours
- HSA: Human serum albumin
- HSC: Hematopoietic stem cells
- ICAM-1: Intercellular adhesion molecule-1
- IL-3: Interleukin-3
- LTC-IC: Human long-term culture-initiating cell
- MACS: Magnetic activated cell sorting
- MSC: Mesenchymal stromal cells
- P21: Cyclin-dependent kinase inhibitor 1A
- PB: Peripheral blood
- PBS: Phosphate buffered saline
- RT-PCR: Real-time reverse transcription-polymerase chain reaction
- SCF: Stem cell factor
- SDF-1: Stromal cell–derived factor–1
- TNC: Total nucleated cells
- TPO: Thrombopoietin
- UCB: Umbilical cord blood
- VCAM-1: Vascular cell adhesion molecule-1
- VEGF: Vascular endothelial growth factor
- VLA: Very late antigen

THESES

Hematopoietic stem cells (HSCs) are defined by their ability to give rise to all types of blood cells. Hematopoietic stem cell transplantation (HSCT) has become a well established treatment modality for hematological malignancies and non-malignant disorders.

Part 1: HSC isolation from extra-embryonic tissue.
1. Fetal stem cells in Umbilical cord blood (UCB) are speculated to be more primitive in comparison to adult stem cells. Hereby, UCB was introduced as an attractive HSC source for HSCT. In order to increase amount of fetal HSCs, recently extra-embryonic tissue was used as a complemental source to UCB.
2. The axis of the chemokine receptor CXCR4 and its ligand SDF-1 is important for trafficking and homing of HSC. Bicyclam AMD3100, a CXCR4 antagonist, in combination with G-CSF is able to induce a significant mobilization of CD34+ cells.
3. Human placenta is a potent hematopoietic niche containing hematopoietic stem and progenitor cells throughout development We can demonstrate that the CXCR4 antagonist AMD3100 mobilise placenta derived CD34+ cells ex utero already after 30 min of incubation and may further enhance the efficacy of harvesting placenta-derived HSC.

Part 2: CD49d blockade to mobilize a subset of HSCs.
1. In clinic, HSCs of adult healthy donor were mobilized out of bone marrow into the peripheral blood (PB) after G-CSF treatment. The HSCs were then collected and used in HSCT.
2. The alpha4 integrin CD49d is involved in migration and homing of hematopoietic stem cells (HSC). Therapeutic application of natalizumab, an anti-CD49d antibody, in patients with multiple sclerosis (MS) has been associated with increased levels of circulating CD34+ progenitors.
3. We compared circulating HSCs from MS patients after natalizumab treatment and HSCs mobilized by G-CSF in healthy volunteers, with regard to their migratory potential, clonogenicity and gene expression. Our data indicate that G-CSF and CD49d blockade mobilize different HSC subsets and suggest that both strategies may be differentially applied in specific cell therapy approaches.

Part 3: Modelling the Niche Compartments in-vitro.
1. In order to further improve the clinical outcome of HSC transplantation, many groups are focusing on ex vivo maintain or expand HSC.
2. HSCs located in the bone marrow are interacting with a specific microenvironment referred to as the stem cell niche, which regulates their fate in terms of quiescence, self-renewal and differentiation. An orchestra of signals

mediated by soluble factors and/or cell-to-cell contact keeps the balance and homeostasis of self-renewal, proliferation and differentiation in vivo.

3. To investigate the communication between HSCs and the niche, coculture assays with mesenchymal stromal cells (MSCs) were performed in vitro.

4. Cell-to-cell contact has a significant impact on hematopoietic stem cells expansion, migratory potential and stemness. The spatial relationship between hematopoietic stem cells and mesenchymal stromal cells is critical for HSC maintenance during ex-vivo expansion.

5. We defined three distinct localizations of HSCs relative to MSC layer: (i) those in supernatant (non-adherent cells); (ii) cells adhering on the surface of mesenchymal stromal cells (phase-bright cells) and (iii) cells beneath the mesenchymal stromal cells (phase-dim cells).

6. Our data suggest that the mesenchymal stromal cell surface is the dominant location where hematopoietic stem cells proliferate, whereas the compartment beneath the mesenchymal stromal cell layer seems to be mimicking the stem cell niche for more immature cells. Our data provide novel insight into the construction and function of three-dimensional HSC–MSC microenvironments.

ACKNOWLEDGEMENTS

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Education

2006 ~ 10   **Dr. rer. Medicine (PhD) (expected)**
University Hospital Carl-Gustav-Carus
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2000 ~ 04   **Bachelor of Science**
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Research Experience

2005 ~ 10   **Master and PhD student**
Supervisor: Prof. Dr. Martin Bornhäuser and Dr. Rainer Ordemann
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1. Took part in a project to evaluate the responses of human mesenchymal stem cells (MSCs) to distinct chemokines, such as BMP and Imatinib. This holds a great promise in tissue engineering for bone and bone marrow reconstruction.

2. Initiated a project to investigate the regulation of human hematopoietic stem cells (HSCs) by the niche in vitro which is constructed by cytokines and MSCs. For the first time showed distinct microenvironments in HSC/MSC coculture in vitro and their distinct regulations to HSCs. This provides a novel insight into the construction and function of three-dimensional stem cell niche. (Main topic)

3. Contributed to clinical study in many aspects from the stem cell therapy to the clinical open-questions, e.g. natalizumab induced HSC increase in patients with multiple sclerosis.

4. To isolate HSCs from human placenta by chemokine attraction. After collecting cord blood from the placenta ex utero, AMD3100 was used to mobilize HSCs out of the placenta. This provides a possibility to utilize stem cell pool in the placenta.

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Objective: biocompatible chitosan tubes as the potential nerve guides that could bridge proximal nerve stumps and synaptic target region after peripheral nerve defects. We isolated Schwann cells from the preinjured sciatic nerve of adult rat, and test their adhesion, spreading and proliferation capacities on chitosan films in vitro. In vivo study of Chitosan tubes was performed in goat.

Publications

1. **Jing D**, Bornhäuser M, Ehninger G, Ordemann R. Oxygen tension regulates hematopoietic stem cell migration into an in-vitro niche beneath mesenchymal stromal cells. Manuscript in Preparation.
2. **Jing D**, Alakel N, Bornhäuser M et al. SDF-1/CXCR4 blockade to mobilise Hematopoietic Progenitor Cells from the Placenta. Bone Marrow Transplantation. 2010.
3. **Jing D**, Fonseca AV, Alakel N et al. Hematopoietic Stem Cells in Coculture with Mesenchymal Stromal Cells- Modelling the Niche Compartments in-vitro. Haematologica. 2010;95:542-550.
4. **Jing D**, Oelschlaegel U, Ordemann R et al. CD49d blockade by natalizumab in patients with multiple sclerosis affects steady state hematopoiesis and mobilizes progenitors with a distinct phenotype and function. Bone Marrow Transplantation. 2010.
5. Alakel N, **Jing D**, Muller K et al. Direct contact with mesenchymal stromal cells affects migratory behavior and gene expression profile of CD133+ hematopoietic stem cells during ex vivo expansion. Exp.Hematol. 2009;37:504-513.
6. Seib FP, Franke M, **Jing D**, Werner C, Bornhauser M. Endogenous bone morphogenetic proteins in human bone marrow-derived multipotent mesenchymal stromal cells. Eur.J.Cell Biol. 2009;88:257-271.
7. Fierro F, Illmer T, **Jing D** et al. Inhibition of platelet-derived growth factor receptorbeta by imatinib mesylate suppresses proliferation and alters differentiation of human mesenchymal stem cells in vitro. Cell Prolif. 2007;40:355-366.
8. Wenling C, **Duohui J**, Jiamou L et al. Effects of the degree of deacetylation on the physicochemical properties and Schwann cell affinity of chitosan films. J.Biomater.Appl. 2005;20:157-177.
9. Cao W, Wang A, **Jing D** et al. Novel biodegradable films and scaffolds of chitosan blended with poly(3-hydroxybutyrate). J.Biomater.Sci.Polym.Ed 2005;16:1379-1394.

Poster Presentation in International Congresses

1. 51th ASH Annual Meeting. 2009 in New Orleans, LA, USA.
2. DGHO Deutsche Gesellschaft für Hämatologie und Onkologie. 2009, in Mannheim, Germany.
3. DGHO Deutsche Gesellschaft für Hämatologie und Onkologie. 2008 in Vienna, Austria.
4. The 2nd International Congress on Stem Cells and Tissue Formation. 2008 in Dresden, Germany.
5. European Life Scientist Organization (ELSO) meeting, 2007 in Dresden, Germany.

FULL TEXT OF SELECTED PUBLICATIONS

1. **Jing D**, Alakel N, Bornhäuser M et al. SDF-1/CXCR4 blockade to mobilise Hematopoietic Progenitor Cells from the Placenta. Bone Marrow Transplantation. 2010.
2. **Jing D**, Oelschlaegel U, Ordemann R et al. CD49d blockade by natalizumab in patients with multiple sclerosis affects steady state hematopoiesis and mobilizes progenitors with a distinct phenotype and function. Bone Marrow Transplantation. 2010.
3. **Jing D**, Fonseca AV, Alakel N et al. Hematopoietic Stem Cells in Coculture with Mesenchymal Stromal Cells- Modelling the Niche Compartments in-vitro. Haematologica. 2010;95:542-550.
4. Alakel N, **Jing D**, Muller K et al. Direct contact with mesenchymal stromal cells affects migratory behavior and gene expression profile of CD133+ hematopoietic stem cells during ex vivo expansion. Exp.Hematol. 2009;37:504-513.