Bone marrow-on-a-chip: Long-term culture of human hematopoietic stem cells in a 3D microfluidic environment

Stefan Sieber[1,2], Lorenz Wirth[1], Nino Cavak[1], Marielle Koenigsmark[1], Uwe Marx[3], Roland Lauster[1], Mark Rosowski[1,*]

[1] Technische Universität Berlin, Institute of Biotechnology, Department Medical Biotechnology, Berlin DE

[2] Berlin-Brandenburg School for Regenerative Therapies, Charitè Campus Virchow Klinikum, Berlin, DE

[3] TissUse GmbH, Oudenarder Str. 16, Berlin, DE

*Senior author:

Mark Rosowski, Dr. (mark.rosowski@tu-berlin.de)

Technische Universität Berlin, Institute of Biotechnology, Department Medical Biotechnology

Sekr. TIB 4/4-2

Gustav-Meyer-Allee 25

13355 Berlin, Germany

Tel: +49-314-27922

Fax: +49-314-27914

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/term.2507

Abstract

Multipotent hematopoietic stem and progenitor cells (HSPCs) are the source for all blood cell types. The bone marrow stem cell niche in which the HSPCs are maintained is known to be vital for their maintenance. Unfortunately, to this date no *in vitro* model exists that truthfully mimics the aspects of the bone marrow niche and simultaneously allows the long-term culture of HSPCs. In this study, we present a novel 3D co-culture model, based on a hydroxyapatite coated zirconium oxide scaffold, comprising of human mesenchymal stromal cells (MSCs) and cord blood derived HSPCs, enabling successful HSPC culture for a time span of 28 days within the microfluidic Multi-Organ-Chip (MOC). The HSPCs were found to stay in their primitive state ($CD34^{+}CD38^{-}$) and capable of GEMM colony formation. Furthermore, a microenvironment was formed bearing molecular and structural similarity to the *in vivo* bone marrow niche containing ECM and signaling molecules known to play an important role in HSPC homeostasis. Here, we present for the first time a novel human *in vitro* bone marrow model, capable of long-term culture of primitive HSPCs in a microfluidic environment.

Keywords: Bone marrow on a chip, Stem cell niche, Ceramic scaffold, Hematopoietic stem cells, Mesenchymal stem cells, Alternative to animal testing

1. Introduction

Multipotent hematopoietic stem and progenitor cells (HSPCs) are the source for all blood cell types. HSPCs remain their stemness by residing in a specific microenvironment called stem cell niche located in the trabecular structures of the bone marrow. These niches play a key role in the determination of cell fate (Lilly et al. 2011, Nagasawa et al. 2011) and can be classified according to their structural and biological properties into the endosteal and perivascular niche (Wilson & Trumpp 2006, Levesque et al. 2010). In contrary to the classical view that the most primitive, slow proliferating, quiescent HSPCs are preserved by the microenvironment generated by the endosteal niche compartment, recent publications indicate that dormant HSCs are maintained in a perivascular niche, close to blood vessels (Oguro et al. 2013, Morrison & Scadden 2014). The function of individual niche compartments is the subject of intense discussions, currently.

The HSPC niche is a complex structure composed of various cell types, secreted factors and extracellular matrix (ECM) that promote HSPC localization, maintenance and differentiation induction (Lilly et al. 2011). Several studies have pointed out the importance of direct cell-to-cell contact by 'partner cells' for the maintenance of HSPCs within the niche (Arai & Suda 2007, Lilly et al. 2011). Specialized bone marrow stromal cells, besides endothelial cells, are thought to be the most important associated cells for HPSC preservation (Morrison & Scadden 2014). Mesenchymal stromal cells (MSCs) give rise to a variety of cell types in the bone marrow and niche structures. Providing the required partner cell subsets, signaling molecules and ECM components, such as Fibronectin that is known to mediate HSPC homing (Abdallah & Kassem 2008). The control of maintenance, guided differentiation and mobilization of HSPCs in the hematopoietic niche has been a subject of considerable interest in recent times.

To study the interplay of partner cells and signaling cues for HPSC maintenance, several *ex vivo* culture approaches have been developed. Unfortunately, until now the concepts were not successful in cultivating HSPCs over a prolonged period. However, several studies have revealed the importance of a 3D scaffold, which mimics the properties of the physiological niche and thereby improves HSPC culture (Lee et al. 2014, Choi & Mahadik 2015). Different approaches with culture times up to 14 days emphasize that 3D structure providing scaffolds like PEG hydrogels (Raic et al. 2013), collagen gels (Leisten et al. 2012), fibrin polymers (Ferreira et al. 2012) or fibronectin conjugated polyethylene terephthalate (PET) matrices (Feng et al. 2006) seeded with mesenchymal stromal cells support the expansion of HSPCs. In 2012, Sharma et al. presented a 3D hydrogel-based MSC HSPC co-culture system. They could show that the HSPCs stayed in a quiescent state for 7 days of culture and attributed this to an interaction with the MSCs and the formation of a hypoxia-gradient. Di Maggio et al. cultured freshly isolated human bone marrow nucleated cells on a hydroxyapatite scaffold in a perfused system for 3 weeks. Subsequently, HSPCs were successfully cultured in this scaffold for one week (Walasek et al. 2012, Di Maggio et al. 2011, Sharma et al. 2012).

Besides the individual approaches of *in vitro* culture systems, animal test systems are used since decades to elucidate scientific issues. Apart from ethical considerations, this practice contains multiple downsides. Among these are the unreliable results due to species-specific differences in rodent models. Due to the high rate of failure in clinical trials and the associated high costs, the development and the release of drugs with higher specificity and efficacy to treat various diseases is significantly delayed. Thus, innovative *in vitro* 3D culture models, simulating the human *in vivo* situation, would be of great value. The aspiring field of miniaturized organ (called organoids) culture technologies provide a promising tool to meet these requirements. The assembled organoids resemble their *in vivo* counterparts and are suited to address biological issues with high significance (Lancaster & Knoblich 2014, Yin et al. 2016). To increase the fidelity of the *in vitro* culture approaches particular preassembled organoid structures are combined in a microfluidic perfusion network, called microphysiological systems or organs on chips (Huh et al. 2011). Concerning the bone marrow niche, in 2014, Torisawa et al. introduced a bone marrow-on-a-chip which was engineered *in vivo* in a mouse employing mouse hematopoietic stem cells. The model was subsequently transferred to a microfluidic device and cultured for 7 days (Torisawa et al. 2014).

The aim of this study was the generation of a versatile, pure *in vitro* culture system imitating the human bone marrow and niche biology for HSPC sustainment and multiplication, suitable to addresses diverse biological issues. Here, we describe a novel 3D co-culture model, based on a hydroxyapatite coated zirconium oxide ceramic scaffold, enabling successful HSPC culture for up to 28 days in a microfluidic environment. The scaffold was engineered with the intention of mimicking the porous yet rigid properties of the cancellous bone microstructure. Primary MSCs isolated from bone marrow were employed to generate a niche-like microenvironment for successful HSPC cultivation within the scaffold. The niche generation could be achieved by the pre-culture of MSCs on the ceramic scaffold inducing the deposition of ECM and the secretion of various factors. As medium, a special HSPC culture medium with the addition of a minimal basic combination of cytokines was chosen.

The here described bone marrow model was built up on the Multi-Organ-Chip (MOC), a microfluidic device, developed in our group (Wagner et al. 2013). It consists of a circular channel system, which connects tissue culture compartments for the culture of different organoids. Thus, enabling the culture of the bone marrow model within a dynamic environment and offering the possibility of culturing it in concert with another organoid.

Besides the applications as a model to study specific niche interactions, the development of this bone marrow model could help to reduce animal testing and provide the prerequisite for the expansion of HSPCs prior to transplantation.

2. Material & Methods

2.1 Isolation and expansion of MSC

Human MSCs were isolated from the bone marrow of femoral heads, which were obtained after hip replacement surgery, with written consent of the donating patients as per the guidelines of the Ethics board of the *Charité* – Universitätsmedizin Berlin. The cells were washed out with PBS and separated by density gradient centrifugation. The cells were characterized by staining of specific MSC markers (CD105, CD73, CD90, CD106 and CD146) and by performing osteogenic, adipogenic and chondrogenic differentiation assays as described in (Pittenger et al. 1999). The cells were expanded in Dulbecco's modified Eagle's medium (DMEM) (Corning Inc., USA) + 10% fetal calf serum (FCS) (Biochrom, Germany) + 1% Penicillin-Streptomycin (P/S) (Biowest, France). The cells were used until passage 7.

2.2 Isolation of HSPCs

Human HSPCs were isolated from umbilical cord blood that was obtained directly after birth, with written consent of the donating mother as per the guidelines of the Ethics board of the *Charité* – Universitätsmedizin Berlin. The blood was diluted with PBS + 3% BSA + 5mM EDTA (PBE)and separated by density gradient centrifugation. The HSPCs were then segregated from the other cells by use of immunomagnetic separation, using the MACS CD34+ isolation kit (Miltenyi Biotec, Germany). Cell number, phenotype and purity were evaluated by flow cytometry according to CD34 and CD38 expression. The freshly isolated cells were directly added onto the scaffold.

2.3 Cell culture systems

Hydroxyapatite coated zirconium oxide based Sponceram® 3D ceramic scaffolds (Zellwerk GmbH, Germany) of 5.8 mm in height and diameter were used as a scaffold. The ceramic was seeded with MSCs in an ultra low attachment 96 well plate (Corning Inc., USA) at a density of 500,000 cells per ceramic. After 7 hours, the ceramics were transferred to an ultra low attachment 24 well plate (Corning Inc., USA). DMEM + 10% FCS + 1% P/S was used as medium and was exchanged every 48 hours. The MSCs were cultured in the ceramic for 7 days prior to the seeding of HSPCs.

The ceramic was transferred to a 96 well ultra low attachment plate and the medium was changed to Stemspan-ACF (Stemcell Technologies, Canada) + 25 ng/ml Fms-related tyrosine kinase 3 ligand (FLT3-L) + 10 ng/ml Thrombopoietin (TPO) (both PeproTech, USA) + 1% P/S. Concurrently, 1,000 - 5,000 $CD34^{+}CD38^{-}$HSPCs were added and allowed to adhere overnight. Next, the ceramics were transferred to the MOC (see below).The medium was exchanged every 48 hours. Cell culture took place at 37°C and 5% CO_2.

Experiments were performed for 1, 2, 3 and 4 weeks. Afterwards, HSPCs were extracted from the ceramic by incubating it for 10 min in PBE. The ceramics were subsequently flushed with the PBE and centrifuged for 5 min at 300xg. Four independent MSC and HSPC donors were used for this study. The cells were analyzed by flow cytometry, immunofluorescent staining, qPCR and CFU-GEMM assays.

2.4 Microfluidic system

In order to simulate a microfluidic environment, the ceramic was integrated into the multi-organ-chip platform, developed in our institute.The MOC used in this study consists of two separate independent circular channel systems. Each circuit hosting two culture compartments interconnected by a channel system. A peristaltic on-chip micro pump integrated into each circuit controls the flow rate of the medium. The pump provides a pulsatile medium flow through 500 µm wide and 100 µm high channels. The pumping volume ranges 5-70 µl $min^{-1}$and the frequency 0.2-2.5 Hz. (Sonntag et al. 2010, Marx et al. 2012, Ataç et al. 2013, Maschmeyer et al. 2015a). We used a pump frequency of 2 Hz for the continuous dynamic operation at a flow rate of 5 µl min^{-1}(Fig. 2B+C).

The microfluidic device was manufactured as described by Wagner et al.(Wagner et al. 2013).In brief, a silicon rubber additive (WACKER PRIMER G 790; Wacker Chemie, Munich, Germany) was applied to the cover-plate and incubated for 20 min at 80°C. Next, a casting chamber was prepared consisting of the prepared cover-plate, a master mold and a casting frame. PDMS was injected into this casting station and the whole setup was incubated for 60 min at 80°C. The resulting 2 mm thick PDMS layer containing the imprint of the channels and pumps was permanently bonded by low pressure plasma oxidation (Femto; Diener, Ebhausen, Germany) to a glass slide with a footprint of 75 x 25 mm (Menzel, Braunschweig, Germany), thereby forming the enclosed microfluidic channel system.

The ceramic was placed in one culture compartment of each circuit, hosting the ceramic holder that was specially designed for the cultivation of the bone marrow model on the MOC. The ceramic holder has the same diameter as a 96 well plate well. It can hold 400µl of medium when loaded with a ceramic. The channel itself contains a volume of 10 µl. Only one of the compartments of the system was used for the ceramic while the other served as a medium reservoir. 400µl of medium was added to each compartment of each circuit of the MOC. The circular medium flow was directed away from the ceramic thereby first passing through the medium reservoir.

2.5 Immunofluorescence and 2-photon microscopy

After the extraction of the HSPCs, the ceramics were fixed in 4% PFA for 3 hours at RT. Afterwards, the scaffolds were rinsed in PBS and cut by using a scalpel. The pieces were transferred into 1.5ml reaction tubes and stained with primary antibodies for Stem cell factor (Abcam, UK) and Fibronectin (eBioscience, USA). Samples were washed and then stained with goat anti-rabbit and goat anti-mouse secondary antibodies coupled with Alexa-488 or Alexa-594 (both Invitrogen, USA), respectively. Nuclei were counterstained with DAPI (Sigma, USA). Both steps were performed over night at 4°C. Subsequently, samples were washed and visualized using a 2-photon microscope (Trimscope II, LaVision BioTec, Germany) and processed by employing Imaris version 7.5 (Bitplane Scientific Software, Switzerland).

2.6 Flow cytometry

After 1, 2, 3 and 4 weeks of culture, the HSPCs were extracted from the ceramic and subsequently analyzed by flow cytometry. Cells were stained with CD34-PE, CD38-APC, CD49f-FITC, CD90-FITC and CD133-FITC (all Miltenyi Biotec, Germany) for 10 min on ice. Samples were washed and analysis was carried out using a MACSQuant® Analyzer (Miltenyi, Germany) flow cytometer. Here, the percentage of HSPCs that maintained their primitive phenotype ($CD34^{+}CD38^{-}$) was investigated. Data was processed using FlowJo 10 (Tree Star inc., USA).

2.7 CFU-GEMM assay

The myeloid differentiation potential of the HSPCs extracted from the ceramic was assessed by colony forming unit - granulocyte, erythrocyte, macrophage, megakaryocyte (CFU-GEMM)assay (Miltenyi Biotec, Germany). The assay was performed according to the manufacturer's protocol.

2.8 qPCR

Isolation of RNA was carried out using NucleoSpin RNA II kit (Macherey-Nagel, Germany), following the instructions provided. Mesenchymal stromal cells were cultured in a monolayer or in the ceramic for one week in DMEM + 10% FCS + 1% P/S before switching the medium to Stemspan-ACF + 25 ng/ml FLT3-L + 10 ng/ml TPO + 1% P/S and culturing the cells for an additional 1, 2, 3 or 4 weeks. The ceramics were transferred onto the MOC after the first week of static pre culture. After the respective culture time, the cells were lysed in the lysis buffer supplied by the manufacturer. MSCs isolated from three different donors were used. Reverse Transcription of mRNA was performed by using TaqMan Reverse Transcription Reagents cDNA kit (Applied Biosystems, USA), as per manufacturers' instructions. Real time PCR was performed using 1 µl cDNA with 1µl primer mix and SensiFAST Sybr No-ROX kit (Bioline, Germany), in 96-well PCR plates (Biozym Scientific, Germany), and were read with Stratagene MX 3005P Multiplex Quantitative PCR System (Agilent Technologies, USA). Ubiquitin-conjugating enzyme E2 D2 (UBE2D2) was used as housekeeping gene. Four and three different donors were used for the graph in figure 3 and 4, respectively.

Primers used were as follows:

Target	Primer	Sequence
Angiopoietin 1	forward	GAAGGGAACCGAGCCTATTCAC
	reverse	CATCAAACCACCATCCTCCTG
Fibronectin	forward	CAGACCTATCCAAGCTCAAGTGG
	reverse	TGGGTGGGATACTCACAGGTC
ICAM-1	forward	CCGACTGGACGAGAGGGATT
	reverse	TCGGCCCGACAGAGGTAGGT
Jagged1	forward	ATGGGAACCCGATCAAGGAA
	reverse	TCCGCAGGCACCAGTAGAAG
SCF	forward	TTCAAGAAGTGTAATTGTGGCTTGT
	reverse	TGGGTAGCAAGAACAGATAAAGATG
Nestin	forward	AGCGTTGGAACAGAGGTTGG
	reverse	AGGCTGAGGGACATCTTGAGG
Pleiotrophin	forward	GTGGAGAATGGCAGTGGAGTGT
	reverse	CAGGGCTCGCTTCAGACTTC
Osteopontin	forward	CACTGATTTTCCCACGGACCT
	reverse	CCATTCAACTCCTCGCTTTCC
CXCL12	forward	GAGCTACAGATGCCCATGCC
	reverse	AGCTTCGGGTCAATGCACAC
VEGF	forward	AGCCTTGCCTTGCTGCTCTA
	reverse	GTGCTGGCCTTGGTGAGG
UBE2D2	forward	GACTTGGGTGACTCTAGGGCA
	reverse	CTGCGACGGAACTAGCTGTG

2.9 Scanning electron microscopy (SEM)

Electron microscopy was performed in the department for electron microscopy (ZELMI) of the TU Berlin. The bone marrow model was cultured for four weeks on the MOC. Medium was removed, the ceramics were carefully washed with PBS and afterwards fixed in 4% PFA for three hours. They were then stored in PBS at 4°C. The samples were cut by using tweezers and a scalpel. Subsequently, they were mounted on a support using silver. Afterwards, the samples were transferred to a vacuum chamber and sputter-coated with gold. Next, the support with the mounted samples was transferred through an air lock into the electron microscope where the pictures were taken.

2.10 Statistical analysis

Unpaired t-test was applied to the data sets, using GraphPad Prism software version 6.04 (GraphPad Software Inc., USA). P values smaller than or equal to 0.05 were considered significant.

3. Results & Discussion

The bone marrow stem cell niche that maintains HSPCs is a highly complex environment in which various cell types and physical conditions play a role. Of all the cell types MSCs, besides endothelial cells, are thought to be the most important (Morrison & Scadden 2014). They can differentiate into various cell types and further contribute to niche regulation by secreting signaling molecules, direct cell-to-cell contact and production of ECM **(**Lilly et al. 2011)**.**

Although most important cell types, signal molecules and physical aspects that make up the bone marrow niche are known, their exact interaction to form a functioning surrounding for the preservation of HSPCs remains unclear. Unfortunately, to this date no *in vitro* model exists that truthfully mimics the aspects of the bone marrow niche and simultaneously allows the long-term culture of HSPCs. The here presented model lives up to the *in vivo* situation and enables us to culture HSPCs for at least four weeks. It is relevant for a variety of applications, including the study of certain cell interactions as well as for drug testing.

The establishment of the bone marrow model is comprised of several sequential steps depicted schematically in figure 1, combining the scaffold and microfluidic microphysiological system presented in figure 2. Mesenchymal stromal cells isolated from femoral heads were cultivated on cancellous bone like hydroxyapatite coated scaffolds. After one week of ECM deposition and microenvironment adjustment by the MSCs, HSPC were added and cultured under dynamic conditions for up to 4 weeks.

3.1 MSCs build up a suitable environment for HSPC culture within 7 days

Several studies have revealed the importance of stromal support cells cultured on 3D scaffolds for HSPC culture. Hence, we employed primary human bone marrow derived MSCs, which have been described as being particularly efficient in the long-term culture of primitive HSPCs (Tan et al. 2010, Takizawa et al. 2011, Di Maggio et al. 2011, Leisten et al. 2012, Cook et al. 2012, Ferreira et al. 2012) and cultured them on the hydroxyapatite-coated Sponceram 3D ceramic scaffold. The scaffold was chosen since it mimics the bone marrow as close as possible. Hydroxyapatite is a close analogue of bone-apatite that is the foundation of the hard tissue in all vertebrates and is produced by bio-mineralization in the body. The pore size and structure of the scaffold is comparable to human bone marrow cavities (Murphy et al. 2010) Therefore, the structure should mimic the cancellous (spongy) bone (Fig. 2A).

Within 7 days of culture, the MSCs generate a suitable environment for HSPC culture in the 3D scaffold. Part of this environment is the ECM that is vital for the securement of HSPCs in the bone marrow. Upon scaffold cultivation, the MSCs produce a web like network of ECM composed of fibronectin as shown by immunofluorescent staining and qPCR. Fibronectin is an important part of the ECM in the bone marrow and known to mediate HSPC homing by adhering to surface receptors and trapping secreted factors (Abdallah & Kassem 2008). The fibronectin deposition could be observed throughout the ceramic. It was higher expressed in the ceramic scaffold in comparison to the 2D culture (Fig. 4B). The MSCs were located within this dense network of arranged matrix molecules (Fig. 3A).

Furthermore, the presence of stem cell factor (SCF) which exists as a membrane-bound as well as a secreted cytokine was investigated. This signaling molecule plays an important role in the preservation and self-renewal of HSPCs *in vivo* (Broudy 1997, Oguro et al. 2013). The expression of SCF as part of HSC niche generation by the mesenchymal stromal cells could be shown by immunofluorescent staining and qPCR (Fig.3).

QPCR results revealed the expression of various other important bone marrow associated genes (Fig. 3B). These markers which are also found in the *in vivo* bone marrow niche, have been described as essential for the long-term sustainment of HSPCs, most notably Nestin and Osteopontin (Aggarwal et al. 2012, Sharma et al. 2012, Morrison & Scadden 2014). Quiescent HSCs have been described to reside in close proximity of Nestin+ MSCs within the niche (Kunisaki et al., 2013). Higher levels of Osteopontin as an early marker for osteoblast differentiation indicate the starting differentiation of MSCs to osteoblast in the ceramic culture. As stated in previous work, scaffolds with a rigid surface promote partial spontaneous osteogenic differentiation of MSCs (Diederichs et al. 2009). Osteoblasts are responsible for the generation of bone and have been described numerous times as vital part of the HSC niche (Calvi et al. 2003). Additionally, genes known to play a putative role in the HSC niche for adhesion (ICAM-1, Angiopoietin 1, Fibronectin), vascular development (VEGF), HSPC chemotaxis (CXCL12) and HSPC maintenance (Jagged1, Pleiotrophin) were being expressed by the MSCs (Balduino et al., 2012; Calvi, 2006). Interestingly, all genes are expressed in MSC monolayer expansion cultures in comparable levels, emphasizing the suitability of this cell type to maintain the HSC phenotype in co-culture systems, especially in a 3D microenvironment (Raic et al. 2013; Leisten et al. 2012; Ferreira; et al. 2012; Feng et al. 2006) . Thus, the maintained expression of the various bone marrow niche associated genes suggests that the MSCs generate a microenvironment, when cultured within the ceramic scaffold, which is conducive to HSC maintenance.

3.2. The bone marrow niche stays intact over the course of 28 days

In order to further characterize the niche, MSCs were cultured for 1, 2, 3 and 4 weeks in the serum-free HSPC culture medium on the MOC and the expression rate of numerous bone marrow associated genes was measured by the use of qPCR. The transition to serum-free condition was mandatory, since serum components foster the differentiation induction of the HSPCs and limit the spectrum of therapeutically application of the stem cells. In order to bypass these difficulties several approaches for HSC culture and expansion *in vitro* has been developed (Walasek et al. 2012).

Mesenchymal stromal cells were cultured in a 2D monolayer or the ceramic for one week in DMEM + 10% FCS + 1% P/S before switching the medium to Stemspan-ACF + 25 ng/ml FLT3-L + 10 ng/ml TPO + 1% P/S and culturing the cells for an additional 1, 2, 3 or 4 weeks. MSCs cultured in a monolayer did not tolerate the switch in medium very well (Fig. 4A). While the cells of one donor died, the remaining donors exhibited a poor phenotype and low mRNA levels (Fig S1) compared to the ceramic counterpart after 4 weeks of culture in the serum-free medium. RNA yield reflect the cell amount and/or the metabolic activity of a cell culture system. The increasing RNA concentrations yielded in the DMEM monolayer culture indicate the proliferative activity in this expansion culture. Accordingly to the differentiation induction in the ceramic scaffolds, the cells down-modulate their proliferative activity mirrored by the moderate RNA levels. Nevertheless, compared to the serum-free monolayer condition the RNA yield was 3 to 4 times higher in the serum free scaffold culture reflecting the superior general state of the cells. Interestingly, the expansion medium seems to have an adverse impact on the 3D culture, since the RNA yield was rather lowered compared to the serum free counterpart.

The different behavior in a 2D or 3D environment might be ascribed to the ceramic allowing the cells to interact in an additional dimension (Fig S2) and promoting the differentiation potential of MSCs which has also been described elsewhere (Diederichs et al. 2009, Sharma et al. 2012). Nevertheless, cells in both, 2D and 3D, culture conditions were lysed and qPCR was performed.

Nestin, Osteopontin, VEGF, Angiopoietin 1 and Fibronectin were up regulated in comparison to the monolayer. Nestin and Osteopontin, have been described as essential for the long-term sustainment of HSPCs while VEGF and Angiopoietin 1 are important angiogenesis promoting factors (Aggarwal et al. 2012, Sharma et al. 2012, Morrison & Scadden 2014). Especially Nestin-expressing MSCs have been described to reside in close proximity to quiescent HSCs within the *in vivo* bone marrow niche (Kunisaki et al., 2013). The ostensible up regulation of the two angiogenesis promoting proteins, Angiopoietin 1 and VEGF, could be ascribed to the hypoxic conditions assumed to prevail within the ceramic. Jagged1, SCF, CXCL12 and ICAM-1 were evenly expressed in both cultures. Solely Pleiotrophin which is a novel niche factor was higher expressed in the 2D culture (Morrison & Scadden 2014).

The expression levels stayed relatively stable over the course of the 4 weeks indicating a robust environment for HSPC culture. As mentioned above, the difference in expression levels can most likely be ascribed to the different environment which is present in the scaffold. The tendency of relative higher expression of some marker genes in the 3D culture compared to the 2D counterpart can be explained by the poor general state of the monolayer MSC culture in the serum-free setting. Thus, the expression of the niche supporting genes is maintained by the 3D culture in serum-free conditions, enabling the long-term cultivation of HSCs in the well-conditioned but not differentiation inducing environment.

The bone marrow mimicking 3D environment allows the cells to grow and interact in an additional dimension while the rigid surface among other factors promotes the differentiation of the MSCs (Diederichs et al. 2009). The cellular spread within the ceramic cavities was enabled by an active remodeling of the structural environment by ECM secretion. The deposition of matrix molecules interconnected cavity surfaces by bridge like structures (Fig S2).

To determine the robustness of the niche, the expression of fibronectin and SCF were again visualized after 4 weeks of culture. After HSPC extraction, the ceramics were fixed in 4% PFA and immunofluorescent stainings for fibronectin and SCF were performed. As illustrated in figure 5, the ceramics were still densely populated after four weeks of culture in the specific serum-free HSPC culture medium. The MSCs still expressed SCF on their surface and a web-like fibronectin structure was present. Thus, after 4 weeks a suitable niche with two key factors, SCF and fibronectin, for HSPC culture was still existent (Abdallah & Kassem 2008,Broudy 1997, Oguro et al. 2013).

In conclusion, it can be asserted that all relevant bone marrow niche genes were stably expressed over the course of the four weeks after the transition to serum free conditions. It is uncertain if up regulation of certain genes would yield a better result since the simplicity of the model reduces the comparability to the *in vivo* situation.

3.3 HSPCs remain their native state after 4 weeks of culture in dynamic conditions

To approximate to the *in vivo* situation, we build up the bone marrow model on the perfused MOC. This microfluidic device presents the possibility to build up a second niche in the insert that is presently solely used as a medium reservoir. The utilization of the MOC will further allow the addition of the human endothelial cell component in the microfluidic channels and the integration of the bone marrow model into a systemic setup of interconnected organoids (Schimek et al. 2013**,**Wagner et al. 2013).

As mentioned above, after one week of static culture, a microenvironment was formed by the MSCs bearing molecular and structural similarity to the *in vivo* bone marrow niche. Allowing us to reduce the cytokines added to the HSPC culture medium to only two, TPO and FLT3-L. Subsequently, $CD34^+$ HSPCs were added to further complement the artificial niche. The ceramic containing MSCs and HSPCs was transferred to the MOC and cultured for up to four weeks.

Using flow cytometry we were able to demonstrate the presence of $CD34^{+}CD38^{-}$ HSPCs within the ceramic after up to four weeks of culture (Fig. 6A).Although the percentage of $CD34^{+}CD38^{-}$HPSCs decreased over time, a significant proportion of the regained cells, on average 31.71%, retained their primitive phenotype after four weeks of culture(Fig. 6B).Smaller proportions of the population were also positive for the native HSC markers CD90, CD133 and CD49f. Thus, indicating their potential of repopulating the *in vivo* bone marrow niche (Song Chou & Pat Chu 2010, Notta et al. 2011). The proportion of these native HSC markers was very similar to the one in the starting population directly after isolation from umbilical cord blood (Fig. 6E). The $CD34^{-}CD38^{+}$ population stayed stable around 10% (Fig.6C). The $CD34^{+}CD38^{+}$ population decreased after week 2 to5% and remained at this level for the rest of the culture time (Fig. 6D).

The percentage decrease of $CD34^{+}CD38^{-}$ HSPCs over the course of the four weeks might be attributed to a part of the cells differentiating. This assumption is backed up by the fact that total cell numbers were increasing while the percentage of primitive HSPCs declined (Tab.1). In fact, total cell count increases from week 1 to week 2, remained more or less stable until week 3 and dropped again after 4 weeks. Nevertheless, a higher total cell count was determined after 4 weeks compared to 1-week cultures. Still, after four weeks, roughly one third of the overall cell population consisted of primitive HSPCs. It might also be possible that the decrease in $CD34^{+}CD38^{-}$ HSPCs was part of niche adjustments taking place since the amount of HSCs in the human bone marrow is less than 1% (Song Chou & Pat Chu 2010, Notta et al. 2011).

3.4 HSPCs extracted from the ceramic retain their characteristic multi-lineage differentiation potential

After the confirmation that the HSPCs maintained their primitive phenotype by flow cytometry, we tested whether these cells kept their ability to differentiate. For this, the $CD34^+$cells were separated from the other cells by immunomagnetic separation and a CFU-GEMM assay was performed.

$CD34^+$ cells isolated from the 3D co-culture system 1, 2, 3 or 4 weeks after seeding yielded burst-forming-unit erythrocyte (BFU-E), colony-forming-unit erythrocyte (CFU-E), granulocyte (CFU-G), macrophage (CFU-M), granulocyte, macrophage (CFU-GM) and granulocyte, erythrocyte, macrophage, megakaryocyte (CFU-GEMM) colonies (representative data shown in Fig. 6F). BFU-E, CFU-E and CFU-GM numbers were similar to the ones formed from freshly isolated HSPCs. More CFU-G and CFU-M and fewer CFU-GEMM colonies were found in the CFU-GEMM assay performed with the HSPCs isolated from the co-culture system in comparison to freshly isolated HSPCs (Fig. 6G).

Thus, demonstrating that the cells were still functional and capable of differentiating into their various progenies. Indicating a stable maintenance of functional HSPCs within the here described model.

The bone marrow model is suitable for various applications. It can be used as a tool to study human niche specific interactions due to its in vivo like microenvironment and as an *ex vivo* $CD34^+$ HSPC expansion system since the numbers of primitive HSPCs increased drastically over the course of the four weeks (Tab.1).

Only Torisawa et al. introduced a bone marrow-on-a-chip so far. It was engineered *in vivo* in a mouse employing mouse hematopoietic stem cells and then transferred to a microfluidic device. The mouse HSPCs were subsequently cultured for one week on their chip (Torisawa et al. 2014, Kim et al. 2015). In contrast to the bone marrow model of Torisawa et al., only primary human cells were employed in the here presented work, thus, mimicking the human *in vivo* situation as close as possible. Additionally, we were able to expand the culture time of primitive HSPCs from the commonly presented one week to up to four weeks. Thereby, generating a sufficient time span for extensive drug testing. Even though the bone marrow model consists of only two different cell types right now, the amount of cytokines being added could be reduced to two. This simplicity of the medium could lead to less unwanted effects on other putative organoids present on the MOC. In the future, with the addition of further cell types it is planned to use cytokine-free medium.

The simple manufacturing process of the MOC offers the possibility to run large-scale substance tests with more than 40 replicates while still making it easy to extract samples for analysis. This was successfully proven for the MOC-platform by Wagner et al. by testing troglitazone on a liver skin co-culture (Wagner et al. 2013). Furthermore, the MOC offers the possibility of vascularizing its channels that will lead to a vascularized bone marrow model (Schimek et al. 2013). Jusoh et al. showed that hydroxyapatite has a positive effect on sprouting angiogenesis which might be beneficial for our model (Jusoh et al. 2015).

In summary, the here described 3D co-culture system combines a rigid and well-defined scaffold with bone marrow derived MSCs, which build up a suitable environment for the successful long-term culture of primitive HSCs in a dynamic condition. This is the first time a bone marrow model was successfully build up and cultured for a time span of four weeks in a microfluidic micro physiological environment.

4. Conclusion

Here, we present for the first time a novel *in vitro* bone marrow model, capable of long-term culture of primitive HSPCs. The utilization of a scaffold that mimics the human *in vivo* bone marrow structure and the co-culture of MSCs allows us to come closer to the *in vivo* bone marrow niche environment. Combining it with another organoid on the MOC or even with three other organoids on the four-organ-chip may also be possible, leading to the generation of a functioning multi-organ-chip (Maschmeyer et al. 2015b, Materne et al. 2015). Besides this application, it could also be used to investigate interactions within the bone marrow niche or as the basis for an effective HSPCs expansion system prior to transplantations.

Acknowledgments

The authors sincerely thank the Vivantes Hospital in Friedrichshain, Berlin for providing umbilical cord blood samples and the Immanuel Hospital in Berlin for providing bone marrow samples. We also thank Dr. Ilka Maschmeyer and Dr. Eva-Maria Dehne for helping in the editing process of the manuscript and Alexandra Lorenz for designing the bone marrow culture compartments. Contributions by Stefan Sieber were made possible by DFG funding through the Berlin-Brandenburg School for Regenerative Therapies GSC 203. The work has been funded by the German Federal Ministry for Education and Research, ERA-Net "EuroTransBio", Grant No.031A597B.

References

Abdallah BM, Kassem M. 2008, Human mesenchymal stem cells: from basic biology to clinical applications,*Gene therapy*,**15**: 109–16

Aggarwal R, Lu J, Pompili VJ, Das H. 2012, Hematopoietic stem cells: transcriptional regulation, ex vivo expansion and clinical application,*Current molecular medicine*,**12**: 34–49

Arai F, Hirao A, Ohmura M, Sato H, Matsuoka S, Takubo K et al. . 2004,Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche,*Cell*,**118**: 149-161

Arai F, Suda T. 2007, Maintenance of quiescent hematopoietic stem cells in the osteoblastic niche,*Annals of the New York Academy of Sciences*,**1106**: 41–53

Ataç B, Wagner I, Horland R, Lauster R, Marx U, Tonevitsky AG, Azar RP, Lindner G. 2013, Skin and hair on-a-chip: in vitro skin models versus ex vivo tissue maintenance with dynamic perfusion,*Lab on a chip*,**13**: 3555–61

Balduino A, Mello-Coelho V, Wang Z, Taichman RS, Krebsbach PH, Weeraratna AT et al. 2012, Molecular signature and in vivo behavior of bone marrow endosteal and subendosteal stromal cell populations and their relevance to hematopoiesis,*Experimental cell research*,**318**: 2427–37

Boitano AE, Wang J, Romeo R, Bouchez LC, Parker AE, Sutton SE et al.2011, NIH *Public Access*,**329**: 1345–1348

Broudy VC. 1997, Stem Cell Factor and Hematopoiesis,*The Journal of The American Society of Hematology*,**93**: 2143–2148

Calvi LM, Adams GB, Weibrecht KW, Weber JM, Olson DP, Knight MC et al. 2003, Osteoblastic cells regulate the haematopoietic stem cell niche, *Nature*, **425**: 841–846

Calvi LM. 2006, Osteoblastic activation in the hematopoietic stem cell niche,*Annals of the New York Academy of Sciences*,**1068**: 477–88

Cook MM, Futrega K, Osiecki M, Kabiri M, Kul B, Rice A, Atkinson K, Brooke G, Doran M. 2012, Micromarrows--three-dimensional coculture of hematopoietic stem cells and mesenchymal stromal cells,*Tissue engineering. Part C, Methods*,**18**: 319–28

Didwania M, Didwania A, Mehta G, Basak GW, Yasukawa S, Takayama S, de Necochea-Campion R, Srivastava A, Carrier E. 2011, Artificial hematopoietic stem cell niche: bioscaffolds to microfluidics to mathematical simulations,*Current topics in medicinal chemistry*,**11**: 1599–605

Diederichs S, Ro S, Marten D, Scheper T, Kasper C. 2009, Dynamic Cultivation of Human Mesenchymal Stem Cells in a Rotating Bed Bioreactor System Based on the Z V RP Platform,*Biotechnology Progress,***25**: 1762-1771

Di Maggio N, Piccinini E, Jaworski M, Trumpp A, Wendt DJ, Martin I. 2011, Toward modeling the bone marrow niche using scaffold-based 3D culture systems,*Biomaterials*,**32**: 321–329

Ehring B, Biber K, Upton TM, Plosky D, Pykett M, Rosenzweig M. 2003, Expansion of HPCs from cord blood in a novel 3D matrix,*Cytotherapy*,**5**: 490–499

Feng Q, Chai C, Jiang X-S, Leong KW, Mao H-Q. 2006, Expansion of engrafting human hematopoietic stem/progenitor cells in three-dimensional scaffolds with surface-immobilized fibronectin,*Journal of Biomedical Materials Research Part A,* **78A**: 781–791.

Ferreira MSV, Jahnen-Dechent W, Labude N, Bovi M, Hieronymus T, Zenke M, Schneider RK, Neurs S. 2012, Cord blood-hematopoietic stem cell expansion in 3D fibrin scaffolds with stromal support,*Biomaterials* ,**33**: 6987–97

Hackney J a, Charbord P, Brunk BP, Stoeckert CJ, Lemischka IR, Moore K a. 2002, A molecular profile of a hematopoietic stem cell niche,*Proceedings of the National Academy of Sciences of the United States of America*,**99**: 13061–6

Huh D, Hamilton GA, Ingber DE, Program B. 2011, From Three-Dimensional Cell Culture to Organs-on-Chips Dongeun,*Trends Cell Biol.*,**21**: 745–754

Ji Sun Choi, Bhushan P. Mahadik BACH. 2015, Engineering the hematopoietic stem cell niche: Frontiers in biomaterial science,*Biotechnol J.*,**10**: 1529–1545

Jusoh N, Oh S, Kim S, Kim J, Jeon NL. 2015, Microfluidic vascularized bone tissue model with hydroxyapatite-incorporated extracellular matrix,*Lab Chip*,**15**: 3984-3988

Kiel MJ, Yilmaz ÖH, Iwashita T, Yilmaz OH, Terhorst C, Morrison SJ. 2005, SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells,*Cell*,**121**: 1109–1121

Kim J, Lee H, Selimović Š, Gauvin R, Bae H. 2015, Organ-On-A-Chip: Development and Clinical Prospects Toward Toxicity Assessment with an Emphasis on Bone Marrow,*Drug Safety*,**38**:409-18

Kunisaki, Y., Bruns, I., Scheiermann, C., Ahmed, J., Pinho, S., Zhang, D., Miz- oguchi, T., Wei, Q., Lucas, D., Ito, K. et al.2013, Arteriolar niches maintain haematopoietic stem cell quiescence,*Nature*,**502**: 637–643

Lancaster M a., Knoblich J a. 2014, Organogenesis in a dish: modeling development and disease using organoid technologies,*Science*,**345**: 1247125

Lee E, Han SY, Choi HS, Chun B, Hwang B, Baek EJ. 2014, Red Blood Cell Generation by Three-Dimensional Aggregate Cultivation of Late Erythroblasts,*Tissue engineering. Part A*,**21**: 1–30

Lee J, Kotov NA. 2009, Notch Ligand Presenting Acellular 3D Microenvironments for ex vivo Human Hematopoietic Stem-Cell Culture made by Layer-By-Layer Assembly,*Small*,**5**: 1008–1013

Leisten I, Kramann R, Ventura Ferreira MS, Bovi M, Neuss S, Ziegler P, Wagner W, Knüchel R, Schneider RK. 2012, 3D co-culture of hematopoietic stem and progenitor cells and mesenchymal stem cells in collagen scaffolds as a model of the hematopoietic niche,*Biomaterials,***33**: 1736–47

Levesque JP, Helwani FM, Winkler IG. 2010, The endosteal "osteoblastic" niche and its role in hematopoietic stem cell homing and mobilization,*Leukemia*,**24**: 1979–1992

Lilly AJ, Johnson WE, Bunce CM. 2011, The haematopoietic stem cell niche: new insights into the mechanisms regulating haematopoietic stem cell behaviour,*Stem cells international*,**2011**: 274564

Marx U, Walles H, Hoffmann S, Lindner G, Horland R, Sonntag F et al.2012, "Human-on-a-chip" developments: a translational cutting-edge alternative to systemic safety assessment and efficiency evaluation of substances in laboratory animals and man?,*Alternatives to laboratory animals,* **40**: 235–57

Maschmeyer I, Hasenberg T, Jaenicke A, Lindner M, Lorenz AK, Zech J et al. 2015a, Chip-based human liver–intestine and liver–skin co-cultures – A first step toward systemic repeated dose substance testing in vitro,*European Journal of Pharmaceutics and Biopharmaceutics,***95(Pt A)**:77-87

Maschmeyer I, Lorenz AK, Schimek K, Hasenberg T, Ramme AP, Hübner J et al. 2015b, A four-organ-chip for interconnected long-term co-culture of human intestine, liver, skin and kidney equivalents,*Lab on a Chip*,**15**: 2688–2699

Materne E, Maschmeyer I, Lorenz AK, Horland R, Schimek KMS, Busek M, Sonntag F, Lauster R, Marx U. 2015, The Multi-organ Chip - A Microfluidic Platform for Long-term Multi-tissue Coculture,*Jove*,**98**: 1–11

Méndez-Ferrer S, Michurina TV, Ferraro F, Mazloom AR, Macarthur BD LS. 2010, Mesenchymal and haematopoietic stem cells form a unique bone marrow niche,*Nature*,**466**: 829–34

Mercier FE, Ragu C, Scadden DT. 2012, The bone marrow at the crossroads of blood and immunity,*Nature reviews. Immunology*,**12**: 49–60

Morrison SJ, Scadden DT. 2014, The bone marrow niche for haematopoietic stem cells,*Nature*,**505**: 327–34

Murphy CM, Haugh MG, Brien FJO. 2010, Biomaterials The effect of mean pore size on cell attachment , proliferation and migration in collagen – glycosaminoglycan scaffolds for bone tissue engineering,*Biomaterials*,**31**: 461–466

Nagasawa T, Omatsu Y, Sugiyama T. 2011, Control of hematopoietic stem cells by the bone marrow stromal niche: the role of reticular cells,*Trends in immunology*,**32**: 315–20

Notta, F., Doulatov, S., Laurenti, E., Poeppl, A., Jurisica, I., and Dick JE. 2011, Isolation of Single Human Hematopoietic Stem Cells Capable of Long-Term Multilineage Engraftment, *Science*,**333**: 218–221

Oguro H, Ding L, Morrison SJ. 2013, SLAM family markers resolve functionally distinct subpopulations of hematopoietic stem cells and multipotent progenitors,*Cell Stem Cell*,**13**: 102–116

Owen M. 1978, Histogenesis of bone cells,*Calcified tissue research*,**25**: 205–207

Pittenger MF, Mackay AM, Beck S, Jaiswal RK, Douglas R, Mosca JD et al. 1999, Multilineage Potential of Adult Human Mesenchymal Stem Cells,*Science*,**284**: 143–147

Raic A, Rödling L, Kalbacher H, Lee-Thedieck C. 2014, Biomimetic macroporous PEG hydrogels as 3D scaffolds for the multiplication of human hematopoietic stem and progenitor cells,*Biomaterials*,**35**: 929–940

Schimek K, Busek M, Brincker S, Groth B, Hoffmann S, Lauster R et al. 2013, Integrating biological vasculature into a multi-organ-chip microsystem,*Lab on a chip*,**13**: 3588–98

Schofield R. 1978, The relationship between the spleen colony-forming cell and the haemopoietic stem cell,*Blood cells*,**4**: 7–25

Sharma MB, Limaye LS, Kale VP. 2012, Mimicking the functional hematopoietic stem cell niche in vitro: Recapitulation of marrow physiology by hydrogel-based three-dimensional cultures of mesenchymal stromal cells,*Haematologica*,**97**: 651–660

Song Chou, Pat Chu WH and HL. 2010, Expansion of Human Cord Blood Hematopoietic Stem Cells for Transplantation,*Cell Stem Cell*,**7**: 427–428

Sonntag F, Schilling N, Mader K, Gruchow M, Klotzbach U, Lindner G et al. 2010, Design and prototyping of a chip-based multi-micro-organoid culture system for substance testing, predictive to human (substance) exposure,*Journal of Biotechnology*,**148**: 70–75

Sugiyama T, Kohara H, Noda M, Nagasawa T. 2006, Maintenance of the Hematopoietic Stem Cell Pool by CXCL12-CXCR4 Chemokine Signaling in Bone Marrow Stromal Cell Niches,*Immunity*,**25**: 977–988

Takizawa H, Schanz U, Manz MG. 2011, Ex vivo expansion of hematopoietic stem cells : mission accomplished ?, *Swiss Med Wkly*,**141**: 1–9

Tan J, Liu T, Hou L, Meng W. 2010, Maintenance and expansion of hematopoietic stem / progenitor cells in biomimetic osteoblast niche,*Cytotechnology*, **62**:439-48

Ting S, Lecina M, Chan Y-C, Tse HF, Reuveny S, Oh SK. 2013, Nutrient supplemented serum-free medium increases cardiomyogenesis efficiency of human pluripotent stem cells,*World journal of stem cells*,**5**: 86–97

Torisawa Y-S, Spina CS, Mammoto T, Mammoto A, Weaver JC, Tat T, Collins JJ, Ingber DE. 2014, Bone marrow-on-a-chip replicates hematopoietic niche physiology in vitro,*Nature methods*,**11**: 663–9

Ventura Ferreira MS, Jahnen-Dechent W, Labude N, Bovi M, Hieronymus T, Zenke M, Schneider RK, Neurs S. 2012, Cord blood-hematopoietic stem cell expansion in 3D fibrin scaffolds with stromal support,*Biomaterials*,**33**: 6987–6997

Wagner I, Materne E-M, Brincker S, Süssbier U, Frädrich C, Busek M et al. 2013, A dynamic multi-organ-chip for long-term cultivation and substance testing proven by 3D human liver and skin tissue co-culture,*Lab on a chip*,**13**: 3538–47

Walasek M a., van Os R, de Haan G. 2012, Hematopoietic stem cell expansion: Challenges and opportunities,*Annals of the New York Academy of Sciences*,**1266**: 138–150

Wilson A, Trumpp A. 2006, Bone-marrow haematopoietic-stem-cell niches. *Nature reviews, Immunology*,**6**: 93–106

Yin X, Mead BE, Safaee H, Langer R, Karp JM, Levy O. 2016, Engineering Stem Cell Organoids,*Cell Stem Cell*,**18**: 25–38

Yu VWC, Scadden DT. 2016, Hematopoietic Stem Cell and Its Bone Marrow Niche,*Current Topics in Developmental Biology*,**118**: 21–44

Zhang J, Niu C, Ye L, Huang H, He X, Tong W-G et al. 2003, Identification of the haematopoietic stem cell niche and control of the niche size,*Nature*,**425**: 836–41

Zhang Y, Chai C, Jiang X-S, Teoh S-H LK. 2006, Co-culture of Umbilical Cord Blood

CD34+ Cells with Human Mesenchymal Stem Cells,*Tissue engineering*,**12**: 2161–70

Tab. 1: Mean absolute cell numbers after 1, 2, 3 and 4 weeks of culture.

	1W	2W	3W	4W
$CD34^-CD38^+$	9905 ±5426	22930 ±20175	25167 ±32645	16600 ±11106
$CD34^+CD38^+$	18248 ±10765	9281 ±7465	9267 ±12616	13928 ±11890
$CD34^+CD38^-$	52940 ±31855	67335 ±70929	47858 ±62649	39366 ±24583
$CD34^-CD38^-$	13347 ±7403	79339 ±54401	88400 ±91368	48953 ±41184

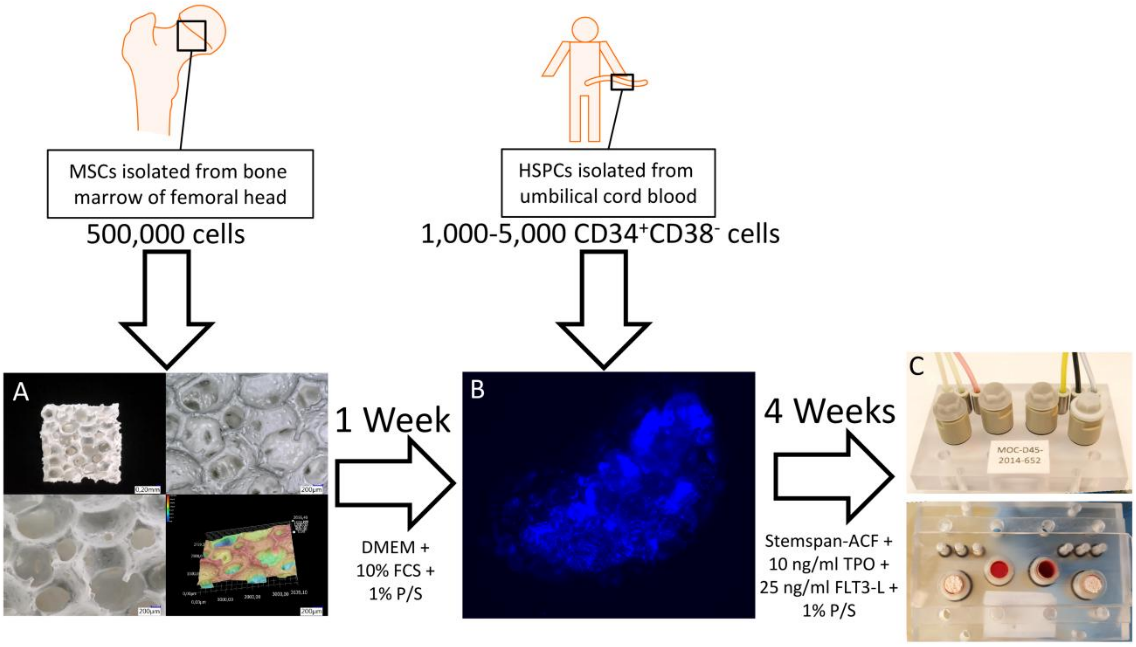

Fig. 1: Scheme for the generation of the bone marrow model A. MSCs, originally isolated from human bone marrow of the femoral head, were seeded onto the hydroxyapatite coated ceramic scaffold and cultured in DMEM + 10% FCS + 1% P/S in a static environment for one week. B. HSPCs isolated from human umbilical cord blood were added to the prepared ceramics. The picture shows a DAPI staining of MSCs cultured on the ceramic for 7 days. C. The whole model was then transferred onto the MOC. The medium was changed to Stempan-ACF + 10 ng/ml Thrombopoietin + 25 ng/ml Fms-related tyrosine kinase 3 ligand + 1% P/S. The bone marrow model was inserted into one of the culture compartments of the MOC. The other culture compartment within the circuit was used as a medium reservoir. The model can be cultured for up to 4 weeks in this dynamic environment.

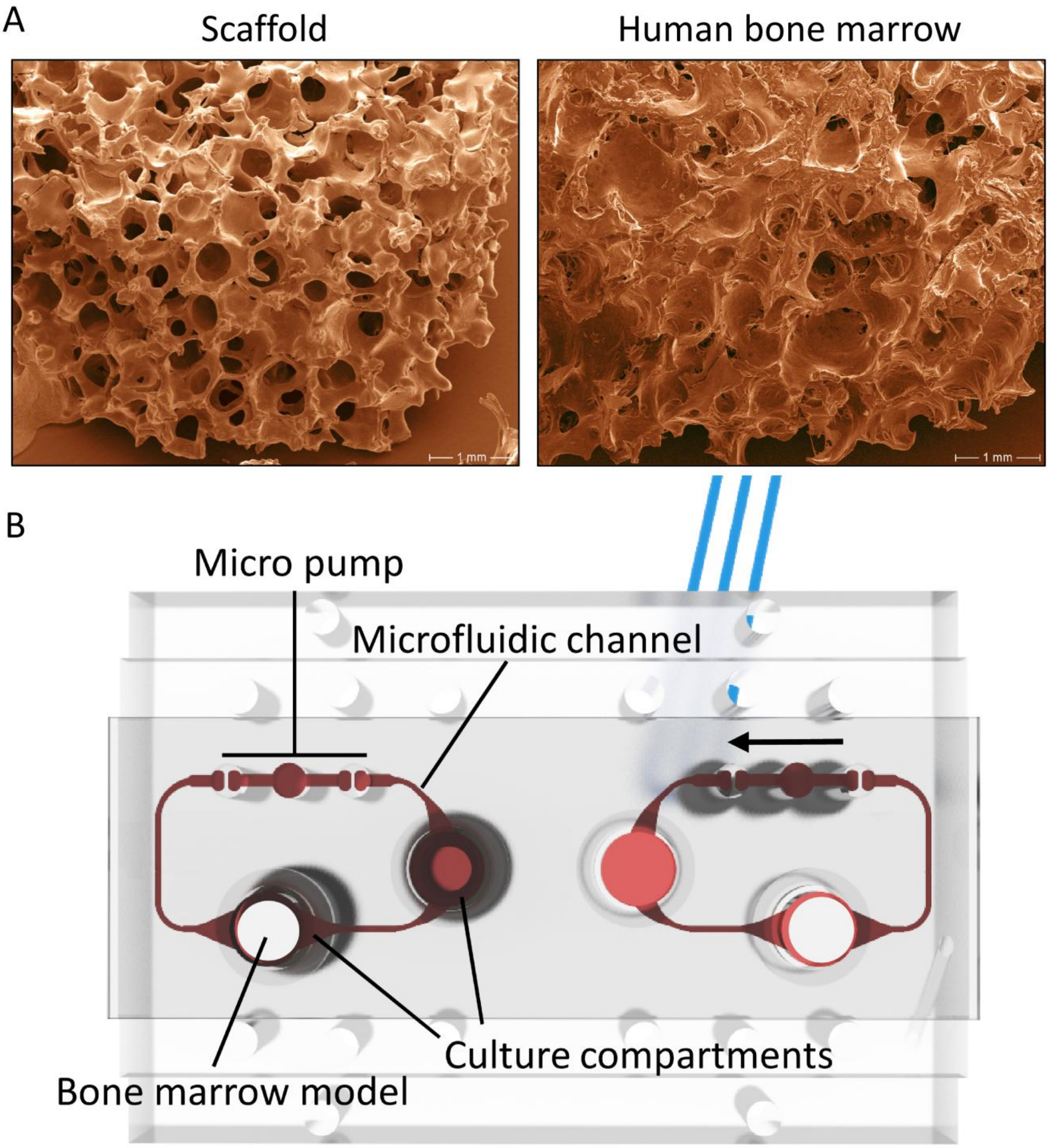

Fig. 2: Scaffold for the bone marrow model A. Comparison of the ceramic scaffold and the in vivo bone marrow. B. Schematic picture of the MOC viewed from below. The bone marrow model was positioned in the culture compartment opposing the micro pump. The black arrow indicates the direction of the medium flow. C. Flow around the ceramic within the MOC from flow simulations. Streamlines are color-coded with velocity magnitude.

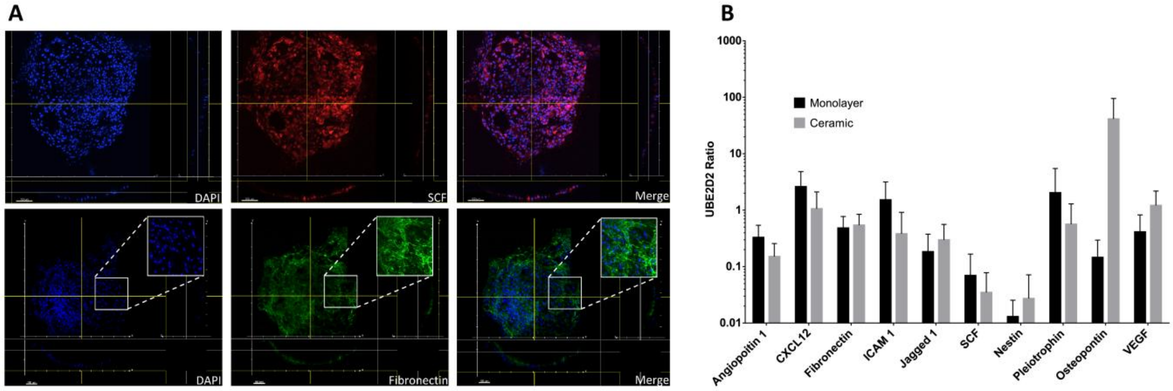

Fig. 3: MSCs build up a suitable environment for HSPC culture within 7 days. A. Immunofluorescent stainings of MSCs that were cultured for 7 days within a ceramic. MSCs express stem cell factor (SCF) (red) on their surface and secrete fibronectin (green). The white rectangles depict magnifications of the selected areas.Scale bars are 100µm. (n = 3 biological replicas). B. Expression of various bone marrow niche relevant genes in MSCs cultured in a monolayer or on the ceramic in DMEM + 10% FCS + 1% P/S for 1 week. The error bars represent the standard deviation. (n≥5 biological replica)

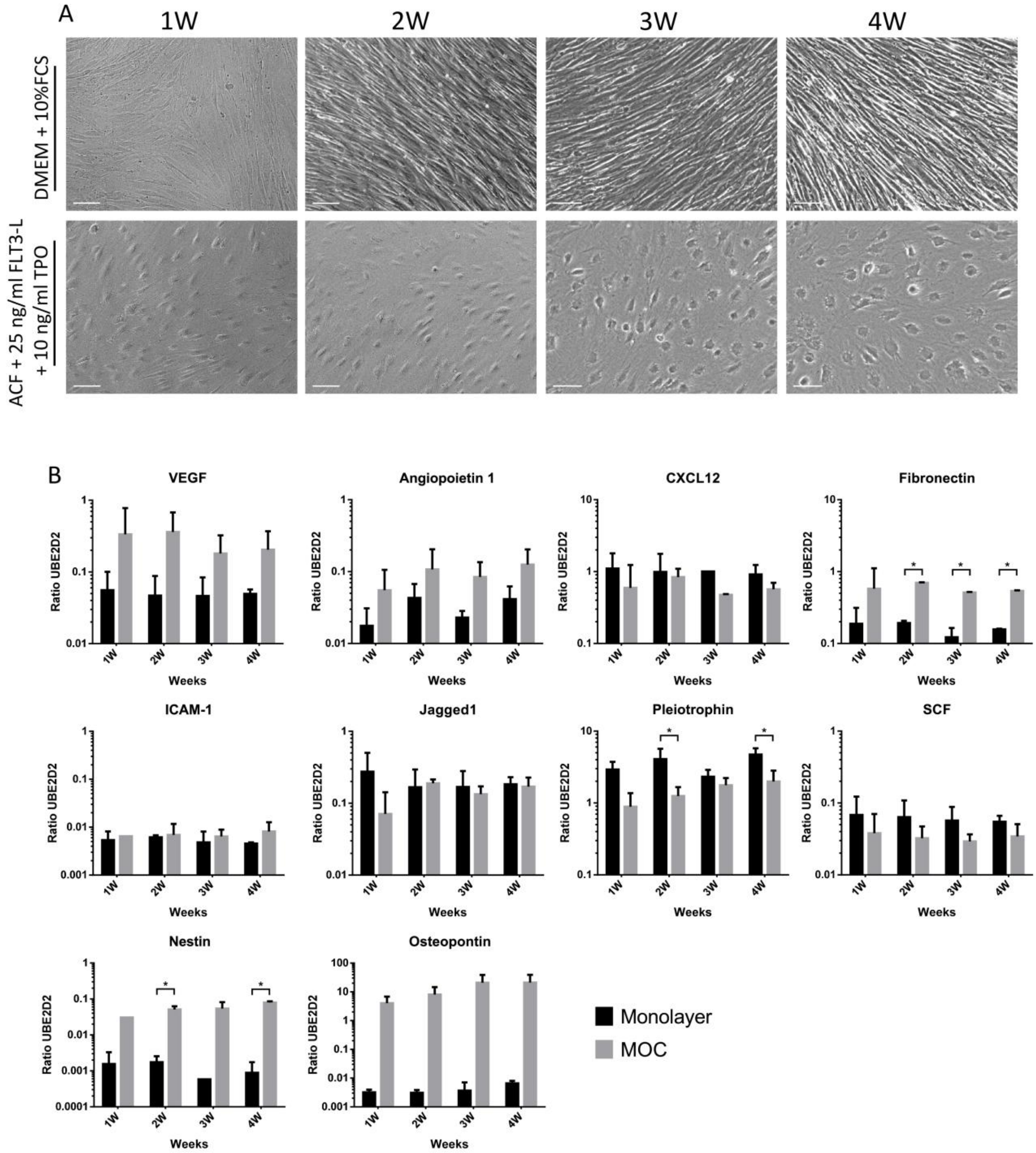

Fig. 4: MSCs express various bone marrow niche related genes in the ceramic A. Comparison of MSCs cultured in a 2D monolayer cultured in DMEM + 10% FCS + 1% P/S or ACF + 25 ng/ml FLT3-L + 10 ng/ml TPO +1% P/S over the course of 1, 2, 3 and 4 weeks. MSCs in the latter medium exhibited a poor phenotype while one donor completely died. B. Expression of various bone marrow niche relevant genes in MSCs cultured for 1, 2, 3 or 4 weeks on the ceramic or in a 2D monolayer. ACF + 25 ng/ml FLT3-L + 10 ng/ml TPO +1% P/S was used as medium. The error bars represent the standard deviation. (n = 3 biological replica).

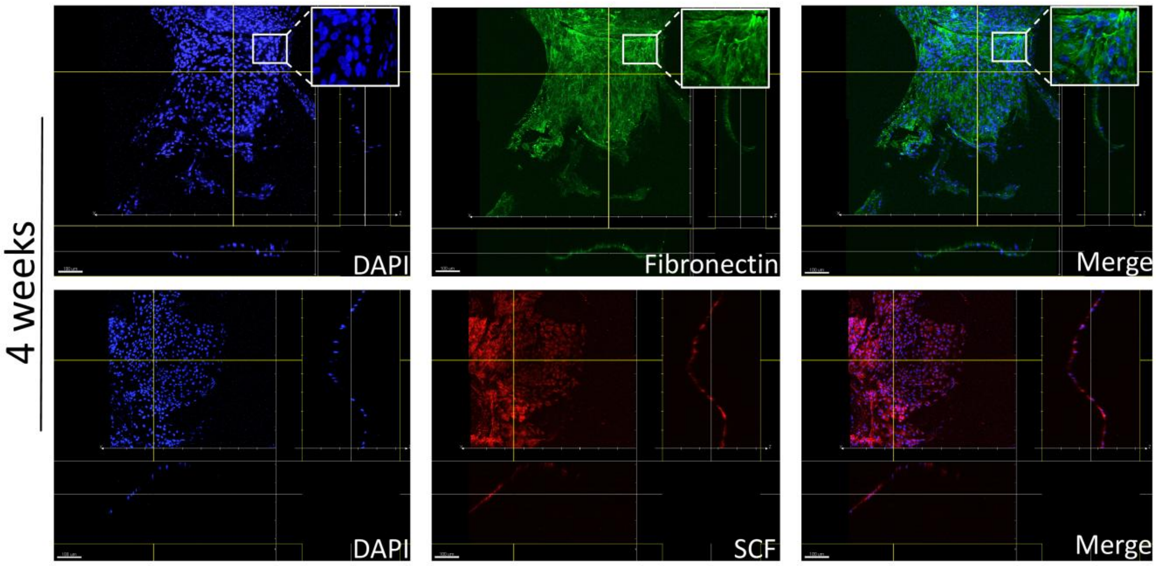

Fig.5:After 28 days of culture in serum-free HSC medium the ECM niche components are still intact Immunofluorescent stainings for fibronectin (green) and stem cell factor (SCF) (red) of MSCs that were cultured in serum-free HSC culture medium for four weeks. The white rectangles depict magnifications of the selected areas. The HSPCs were extracted before the stainings were carried out. Scale bars are 100µm. (n = 3 biological replicas).

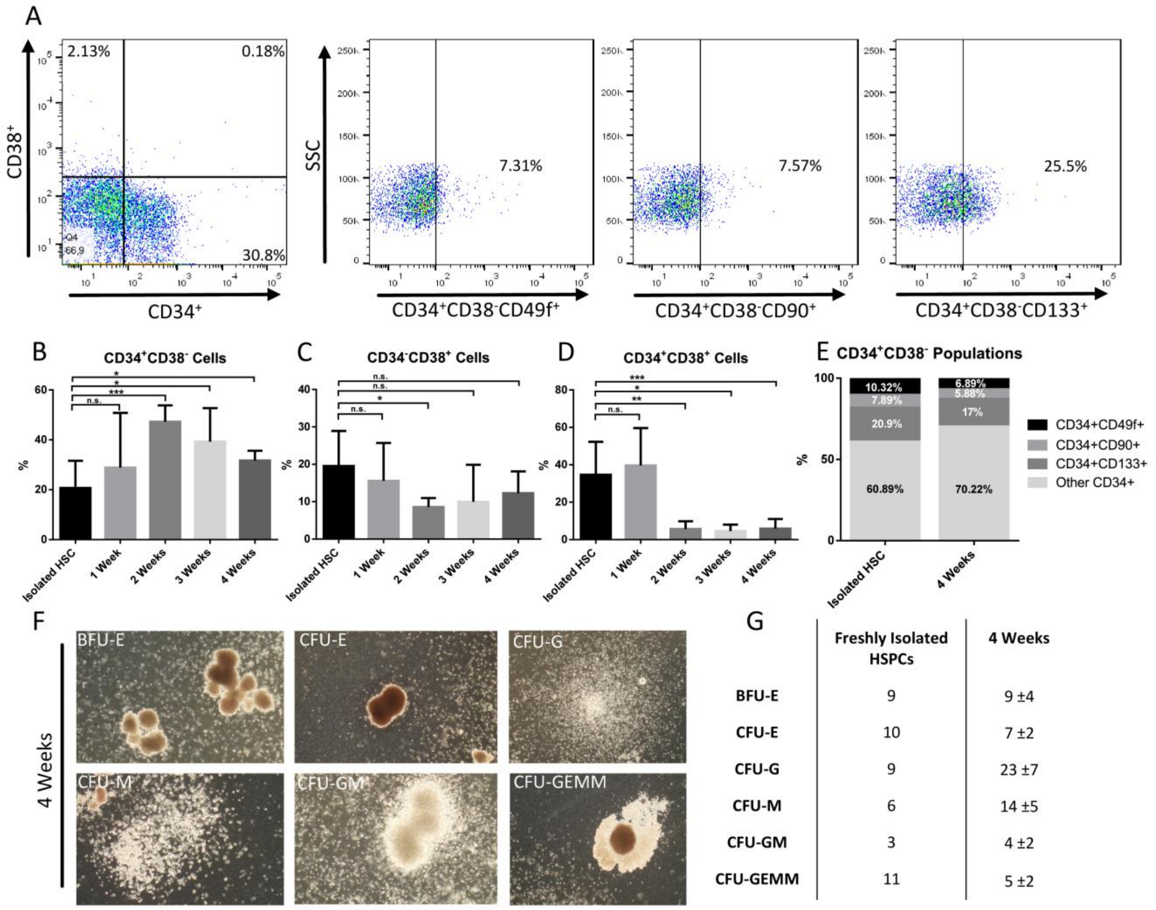

Fig.6: HSPCs remain in their native state after four weeks culture A. Representative FACS plots of HSPCs extracted from the ceramic after four weeks of culture. A significant proportion of the cells is still CD34+CD38-. Additionally, a proportion of CD34+CD49f+, CD34+CD90+ and CD34+CD133+ is measurable (n = 8 biological replicas). B. Percentages of CD34+CD38- cells. After four weeks of culture the amount of CD34+CD38- cells is still significantly higher in comparison to the starting population directly after isolation. C. Percentages of CD34-CD38+ cells. The population declines after week 2. D. Percentages of CD34+CD38+ cells. The amount of CD34+CD38+ cells decreases significantly after week 1 and remains at a low level. The error bars represent the standard deviation. E. Proportion of CD34+CD49f+, CD34+CD90+ and CD34+CD133+ is similar to the respective population directly after isolation. F. After 4 weeks of culture, isolated HSPCs are still able to differentiate into burst-forming-unit erythrocyte (BFU-E), colony-forming-unit erythrocyte (CFU-E), granulocyte (CFU-G), macrophage (CFU-M), granulocyte, macrophage (CFU-GM) and granulocyte,

erythrocyte, macrophage, megakaryocyte (CFU-GEMM) colonies. HSPCs were in culture for 1, 2, 3 and 4 weeks before being extracted from the ceramic Here, representative results from HSPCs cultured for 4 weeks are shown. G. Table showing the mean number of counted colonies of the CFU-GEMM assay performed with cells extracted after four weeks of culture (n = 7 biological replicas) or with freshly isolated HSPCs from umbilical cord blood.