Simulation of the bone marrow microenvironment

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Abstract

Hematopoietic stem and progenitor cells (HSPCs) are of immense significance, not only due to their use in traditional allogenic transplantation therapy, but also as a paradigm for adult stem cells capable of self-renewal as well as multi-potent differentiation. It is well established that the physiological microenvironment or 'niche' in which these cells reside is vital for their maintenance. The molecular and cellular mechanisms governing HSPC fate decisions however are yet to elucidated, largely due to the lack of a suitable in-vitro model.

Here, we present and characterize a novel 3D co-culture system comprising bone marrow mesenchymal stem/stromal cells (MSCs) and cord blood derived HSPCs, within a porous hydroxyapatite-coated ceramic scaffold, as a model for the main cellular interactions within the bone marrow HSPC niche.

Characterization of the 3D culture system revealed that MSCs spontaneously produce a bone marrow-like environment, when cultured in the ceramic scaffolds. Apart from physical resemblance to bone marrow, extracellular matrix molecules typically found in the bone marrow HSPC niche including fibronectin and collagen I were found to be produced. The MSCs also exhibit spontaneous osteogenic differentiation within 1 week of culture in the ceramic.

HSPC maintenance, phenotype, viability and functionality in this system were compared with traditional HSPC expansion and maintenance strategies. We were able to achieve stable long-term (8-week) maintenance of primitive HSPCs (CD34+ CD38-) only in the 3D system. This is the longest time period of *in vitro* HSPC maintenance described to date. These cells were found to be slow proliferating, viable and capable of GEMM colony formation, which is characteristic of long-term repopulating HSPCs.

Furthermore, the microenvironment within the ceramic bears close structural resemblance to that of bone marrow, and contains ECM and signaling molecules known to play a role in HSPC homeostasis. The HSPCs were shown to interact with these molecules as well as with the MSCs in the ceramic.

This co-culture system, therefore, not only presents a new means of HSPC maintenance, but also a medium to study the cellular and molecular interactions involved in niche homeostasis.

Zusammenfassung

Hämatopoetische Stammzellen (HSC) sind nicht nur wegen der allogenen Transplatationstherapie von großer Bedeutung, sondern zeichnen sich auch als Paradigma für adulte multipotente Stammzellen mit Fähigkeit zur Selbsterneuerung aus. Die Zellen persistieren dabei in einer Stammzellnische, welche ein physiologisches Milieu zum Erhalt des Stammzellphenotyps bereitstellt. Die molekularen und zellulären Mechanismen, welche über das Schicksal der HSCs innerhalb der Nische entscheiden, sind aber aufgrund des Fehlens eines geeigneten *in vitro* Modells noch weitgehend unbekannt.

Diese Arbeit beschreibt die Entwicklung und Charakterisierung eines neuartigen 3D Kulturmodells bestehend aus einer porösen Hydroxyapatit beschichteten Keramik, welche mit aus dem Knochenmark isolierten mesenchymalen Stammzellen und aus dem Nabelschnurblut aufgereinigten Hämatopoetischen Stammzellen besiedelt wird. Damit bietet diese 3D Kulturmodell das Potential zur Beschreibung zellulärer Interaktionen innerhalb der Hämatopoetischen Stammzellnische.

Aufgrund der Kultivierung in den Keramiken generierten die Mesenchymalen Stammzellen eine knochenmarkähnliche Umgebung. Neben einer starken Ähnlichkeit zu humanen Knochenmarksstrukturen, zeichnet sich diese durch die Expression typischer Vertreter extrazellulärer Matrixproteine des Knochenmarks wie beispielsweise Fibronectin oder Collagen Typ I aber auch Marker der osteogenen Differenzierung aus.

Ein Vergleich des Keramik-3D-Kulturmodells mit herkömmlichen HSPC Kultivierungsstrategien unter den Aspekten des Erhalts des Stammzellphenotyps, der Vitalität und Funktionalität zeigte, dass nur das Keramik-Kultur-System für die Langzeitkultivierung (8 Wochen) der HSPC bei gleichzeitiger Erhaltung des Stammzellphenotyps (CD34+ CD38-) geeignet war. Das ist die bisher längste Kultivierung Hämatopoetischer Stammzellen in vitro. Die Zellen zeigten ein langsames Proliferationsverhalten als auch ein für HSCs typisches Differenzierungspotential.

Untersuchungen der Mikroumgebung innerhalb der Keramik im Bezug auf Struktur, extrazelluläre Matrix und die Expression Homeostase relevanter Signalmoleküle zeigte eine starke Ähnlichkeit zu den Gegebenheiten des Knochenmarks. Dabei interagierten die Hämatopoetischen Stammzellen sowohl mit der neu generierten Matrix als auch mit den MSCs. Das System stellt somit nicht nur ein Langzeitkultivierungsmodell für Hämatopoetische Stammzellen dar, sondern ist

ebenfalls sehr gut für weitere Untersuchungen zellulärer und molekularer Interaktion innerhalb der Knochenmarksstammzellniche geeignet.

Abbreviations

2-PM	two-photon microscope
Ab	antibody
AGM	Aorta-gonad-mesonephros
ALP	alkaline phosphatase
Angptl	Angiopoietin-like protein
APC	Allophycocyanin
BM	bone marrow
BMP	bone Morphogenetic Protein
BMPR1A	BMP receptor 1A
bp	base pair
BSA	bovine serum albumin
CAR cells	CXCL-12 abundant reticular cells
CASR	Calcium sensing receptor
CB	cord blood
CD	cluster of differentiation
cDNA	complementary DANN
CFSE	carboxyfluorescein succinimidyl ester
CFU	colony forming unit
COL	Collagen
CXCL	chemokine (C-X-C motif) ligand
DMEM	Dulbeco´s modified Eagle medium
dNTP	deoxyribonucleotide triphosphate
ECM	extracellular matrix
EPO	Erythropoietin
FACS	fluorescence activated cell sorting

FBS	fetal bovine serum
Fig.	figure
FITC	Fluorescein isothiocyanate
FLT	Fms-like tyrosine kinase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GEMM	granulocyte, erythrocyte, monocyte, megakaryocyte
HKR_{GAPDH}	House Keeper Ratio (with corresponding housekeeping gene as foot note)
HSC	hematopoietic stem cell
HSPC	hematopoietic stem/progenitor cell
ICAM	intercellular adhesion molecule
IL	Interleukin
INT	Integrin
JAG	Jagged
LT-HSPC	long-term repopulating HSPC
MACS	Magnetic activated cell sorting
ML	monolayer
MNC	mononuclear cell
mRNA	messenger RNA
MSC	mesenchymal stem/stromal cell
N-CAD	N-cadherin
OP	Osteopontin
PBS	phosphate buffered saline
PE	Phycoerythrin
PS	Penicillin-Streptomycin
PTHR	parathyroid hormone receptor
PTHRP	parathyroid hormone related peptide

qPCR	quantitative PCR
SCF	stem cell factor
SEM	scanning electron microscope
TF	transcription factor
TPO	Thrombopoietin
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
WNT	wingless-type MMTV integration site

1. Introduction

1.1 Bone

Bone is a specialized, mesoderm-derived, highly vascularized and mineralized connective tissue. It is characterized physically by its hardness and resilience to mechanical stress and by its capacity for growth and remodeling. Bone remodeling is a dynamic process, which occurs throughout the lifetime of an organism. Microscopically, bone tissue is a dense multi-phase composite, composed of relatively few cells and a vast matrix made up of organic and inorganic components.

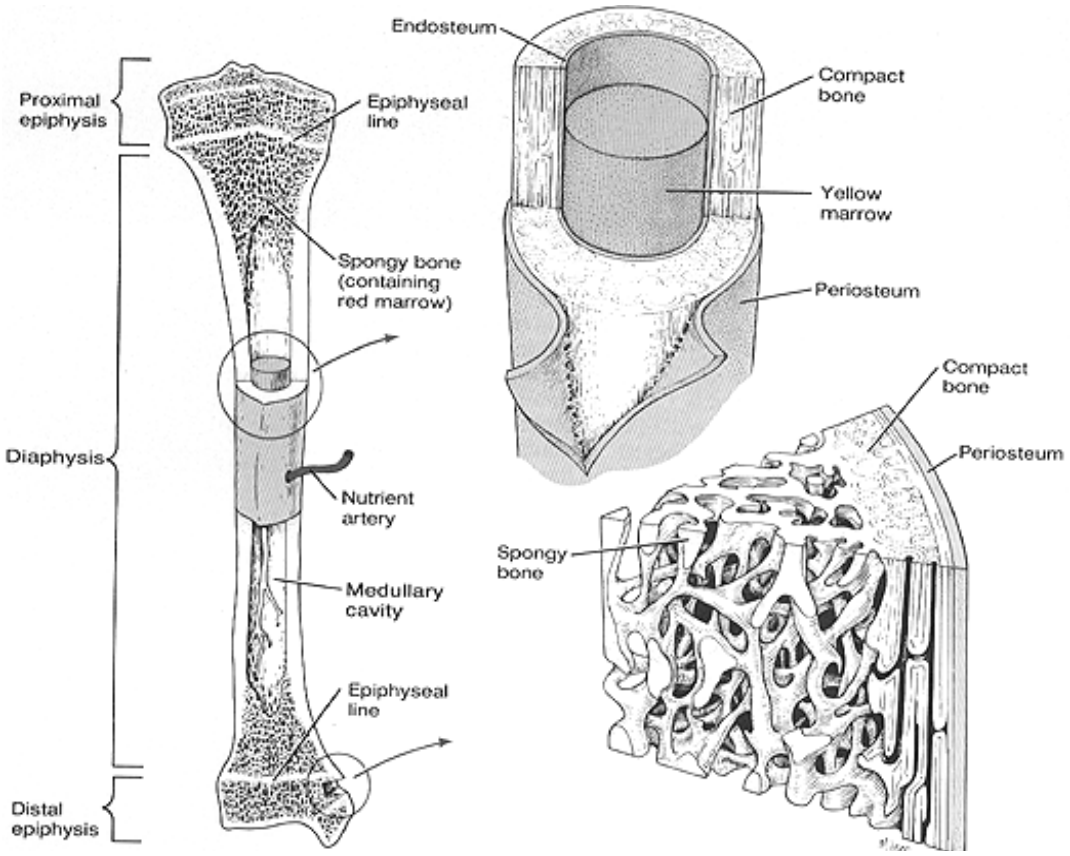

Figure 1.1. Schematic representation of bone macrostructure showing the parts of a typical bone and the location of spongy and cancellous bone (reproduced from Basic Medical Anatomy, by Alexander Spence (Benjamin/Cummings 1990).

The functions of bone, apart from providing structural support and protection of internal organs, include the maintenance of electrolyte and mineral homeostasis by

selective absorption or release of Ca^{2+} and other ions (Mundy, 1990), and as the site of hematopoiesis – the generation of all the blood cell types (Lord and Hendry, 1972).
Structurally, there are two distinct types of bone tissue: the dense ‘compact’ or cortical bone and the ‘spongy’ or cancellous bone (fig. 1.1).

Cortical or compact bone is mainly protective in function and is located on the periphery, such as on the shafts of long bones. The thickness of cortical bone increases based on the mechanical stress experienced by it. Cortical bone encloses the cancellous bone and the bone marrow.

Cancellousor spongy bone is interiorly located, such as in the femoral head and inside vertebrae. It is porous in structure and provides the surface area for the metabolic functions of bone. Bone marrow is found within the spaces of cancellous boneand extends into the bone cavity.

The boneis composed of three main components: the bone cells, bone matrix and bone marrow.

1.1.1 Bone cells

The main cellular components of the bone and bone marrow are the osteoblasts, osteocytes and osteoclasts. These are highly specialized cells responsible for bone tissue production, maintenance and resorption respectively. The relative number of these cells present at any given time is highly regulated. Each of these cell types originatesfrom specialized pluripotent stem or progenitor cells present in the adult bone marrow. Based on the requirement, distinct biochemical signals trigger the division and differentiation of the respective progenitors into the required cell type (Owen, 1978).

1.1.1.1 Osteoblasts

Osteoblasts are a heterogeneous population of cells that are responsible for the synthesis and maintenance of bone tissue. They are formed by the osteoblastic differentiation of multipotentmesenchymal stem cells (MSCs), found primarily in the bone marrow (Owen, 1988; Caplan, 1991), in response to activation of specific

signaling pathways. Osteoblasts are found in various degrees of differentiation and any given time, in healthy bone.

Osteoblasts play a crucial role in the process of bone formation, by the secretion of bone matrix components such as collagen I, fibronectin, osteopontin and osteocalcin, regulation of subsequent mineralization of the matrix and the formation of osteocytes. They also have a central role in bone remodeling by their direct interaction with osteoclasts and their role in activation of the same. Osteoblasts are considered indispensible to hematopoiesis in the bone marrow as they are thought to affect hematopoietic stem cell (HSC) homing and quiescence by direct interaction as well as secreted factors (Taichman et al., 1996; Calvi et al., 2003)

1.1.1.2 Osteocytes

Osteocytes comprise over 90% of bone cells. These are terminally differentiated cells derived from mesenchymal stem cells, through osteoblastic differentiation.

Osteocytes are involved in bone remodeling, mainly by regulation of osteoblast and osteoclast function. They also function as endocrine cells, producing soluble factors which target cells on the bone surface, muscle and other tissue (Baylinkand Wergedal, 1971; Bonewald, 2002). The death of osteocytes results in the formation of pores or spaces in the bone matrix, causing bone fragility. Osteocytes are known to induce osteoclast homing as well as bring about osteoclast formation and activation (Tanaka *et al*, 1995; Wang*et al*, 2005). They also stimulate osteoblast and mesenchymal stem cell differentiation (Heino*et al*, 2002; 2004), making them invaluable in the maintenance of bone homeostasis.

1.1.1.3 Osteoclasts

Osteoclasts are multinucleate cells found in relatively small quantities in the bone. They arise from hematopoietic stem cells (HSCs), which differentiate along the macrophagic lineage and fuse together, forming a large polykaryotic cells (Teitelbaum, 2007).

Osteoclasts have the unique ability to degrade mineralized matrix and are of particular importance in bone resorption (Everts et al., 1999). They are considered

instrumental in the degradation of injured bone during remodeling and work in concert with the osteoblasts, which then form new bone. Osteoclast recruitment and activation is thought to be effected by osteocytes (Zhao et al., 2005).

Despite some skepticism (Miyamoto et al., 2011), Osteoclasts are considered essential for the maintenance of the bone marrow HSC niche, since they are indispensible to bone cavity formation (Yoshida et al., 1990, Kong et al., 1999). They also affect the mobilization and homing of HSCs to the niche, directly, by signaling molecules, and through their influence on the differentiation of osteoblasts (Winkler et al., 2010; Lymperi et al., 2011).

1.1.2 Bone matrix

The bone matrix makes up the largest proportion of bone tissue, and provides structure and mechanical support to the bone. In addition to it's mechanical role, the bone matrix functions as a scaffold for the bone cells and marrow and also as a site for mineral storage.

Bone matrix is composed of an organic phase, consisting mainly of collagen fibers and a variety of other proteins, and an inorganic mineral phase.

1.1.2.1 Inorganic mineral phase

The main mineral component of the matrix is a carbonate- substituted calcium phosphate ceramic-like substance known as bone-apatite. Bone apatite exhibits characteristic internal crystal disorder, trace elements such as sulphur and carbon, and a hydroxyl deficiency. These factors impart important properties to the matrix, making it insoluble enough for stability, but sufficiently reactive to allow the minerals to beconstantly resorbed and reformed as required.

Hydroxyapatite (HA) is a close analog of bone-apatite, and is frequently used in bone grafting (Damien and Parsons, 1991). It is a hydrated calcium phosphate ceramic, andhas a similar crystallographic structure to natural bone mineral and chemical formula of $Ca_{10}(PO4)_6(OH)_2$.

1.1.2.2 Collagen fibers

Approximately 80% of the organic phase of bone matrix is made up of collagen, consisting of arrays of long, rigid tropocollagen molecules. These are composed of three left-handed helices of peptides, known as **α**-chains, which are bound together in a right-handed triple helix. Collagen I (Col1A) is the most abundant collagen molecule in the bone. It is composed of tropocollagen molecules containing two identical and one dissimilar **α**-chain (**α**1(I)2 **α**2).

Col1A has a pivotal role in the maintenance of bone density and integrity. It also directly affects apoptosis, differentiation and proliferation of the bone and bone marrow cells, by structural and molecular pathways (Young, 2003). Within the hematopoietic compartment of the bone marrow, collagen I isknown to mediate HSC homing, by binding to surface receptors and trapping secreted factors.

1.1.2.3 Non-collagenous proteins (NCPs)

The most abundant non- collagenous structural protein in bone is Fibronectin- a glycoprotein found at high levels at sites of osteogenesis. It contains a short amino acid sequence (Arg-Gly-Asp or RGD), which is critical for binding to integrin receptors, and therefore thought to have a role in osteoblast differentiation and proliferation (Gorski, 1998).

Other key NCPs include osteocalcin (OC), bone sialoprotein (BSP), osteopontin (OP) and osteonectin (ON).Bone cells produce these proteins. Their relative composition within the bone matrix is self-regulating through a feedback loop mechanism.They are all multi-functional, and are involved in regulating bone mineralizationand remodeling (Gorski, 1998).

Growth factors including fibroblast growth factors (FGFs), insulin-like growth factors (IGFs), platelet-derived growth factors (PDGF), transforming growth factor-beta (TGF**β**) superfamily, and bone morphogenic proteins (BMPs) are also found within the matrix. These regulate cell proliferation and differentiation and orchestrateendochondral bone formation- the process of bone formation wherein cartilage forms firstand is subsequently replaced by bone.

1.2 Bone marrow

'Bone marrow' is the highly vascularized tissue contained in the central cavity of long and axial bones and in the intra-trabecular spaces of spongy bones (illustrated in fig. 1.1). It is the principal site of adult blood cell formation or hematopoiesis.

Based on appearance, bone marrow is divided into two types: red and yellow marrow. Red marrow is the highly vascularized site of hematopoiesis, and gets its red appearance due to the presence of blood. Yellow marrow is associated with age. It lacks hematopoietic function and appears yellow due to the presence of a large number of adipocytes. At birth, all the bones contain red marrow. As age increases, the hematopoietic red marrow recedes towards the axial skeleton, and only yellow marrow remains in the peripheral bones.

Bone marrow is a well-organized and complex tissue, composed of several different cell types, which are maintained as well defined structures within the bone.

1.2.1 Bone marrow structure

Bone marrow consists of mainly of hematopoietic tissue islands and adipose cells surrounded by vascular sinuses. These are distributed within the meshwork of spongy bone matrix, as described earlier.

Bone marrow structure is dependent on the organization of the bone vasculature. In long bones, one or more feeding canals, each containing an artery and one or more veins pass through the cortical bone (Travlos, 2006). In flat bones, the marrow is traversed by numerous blood vessels of various sizes entering the marrow via large and small canals. The feeding arteries run parallel to the longitudinal axis in the central part of the marrow. They form branches, which run perpendicularly towards the bone cortex. This organization results in the delineation of specialized vascular structures – the bone marrow sinuses – the entry site for the mature hematopoietic elements, into the circulation (fig. 1.2).

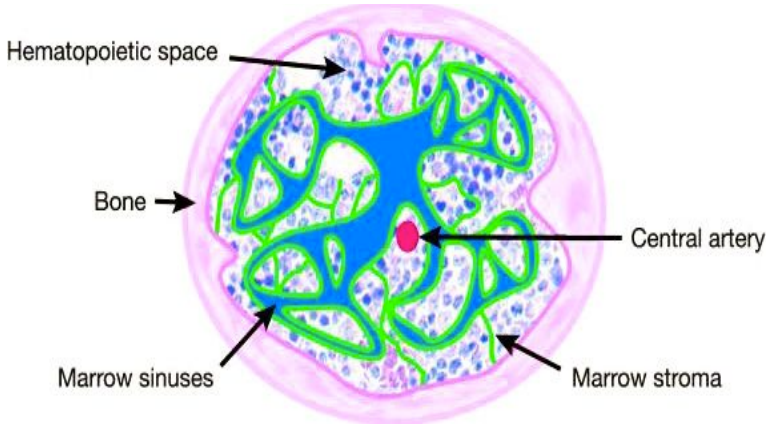

Figure 1.2. Schematic representation of bone marrow showing structure, location and organization of the various components(reproduced from Dunsmore and Shapiro, 2004).

The hematopoietic 'cords', wherein the maturation of the different blood elements takes place, occupy the space between the blood vessels. These are highly cellular areas. The hematopoietic activity (red marrow) is highest at the periphery of the marrow cavity, in proximity of the endosteal surface. This area is rich in hematopoietic stem and progenitor cells as well as other stromal cells types. The more central areas are rich in adipocytes (yellow marrow).

1.2.2 Cellular composition

There are two distinct cellular compartments, which are observed within the bone marrow: the stromal compartment and the hematopoietic compartment.

1.2.2.1 Stromal compartment

The stromal compartment, with its complex structure and variety of cells provides physical and physiological support for the hematopoietic compartment. The cell types that compose the stromal tissue include: macrophages, reticular cells and bone lining cells including osteoblasts, osteoclasts andmesenchymal progenitors or mesenchymal stromal cells (MSC).

The macrophages are located in proximity to the sinuses, towards the center of the hematopoietic islands. They are also responsible for the generation of the osteoclastic components.

The reticular cells are fibroblastic cells, found in close association with reticular fibers in the extra-cellular matrix. A subpopulation of reticular cells, termed as adventitial reticular cells, is located close to the sinuses, forming an adventitial layer on the wall of the blood vessel, similar to pericytes. These cells produce thin cytoplasmic processes from within the hematopoietic cords, which make contact with processes of other reticular cells, thus forming a three-dimensional scaffold for the hematopoietic compartment. The non-adventitial reticular cells have a regulatory function.

The bone-lining cells are a population of flat cells that covers the bone endosteal surface. Reticular cells, pre-osteoblasts, osteoblasts and osteoclasts can be found in these areas. All these cell types are characterized by the expression of alkaline phosphatase (ALP), which is considered a marker for the osteoblastic lineage. Hematopoietic stem cells are often found in close association with these cells (Tavassoli and Yoffey, 1983).

The mesenchymal stromal/stem cells (MSC) whose anatomical function and location remains controversial are also thought to be present in this area. MSCs are typically described as multi-potent, non-hematopoietic cells capable of osteogenic, chondrogenic and adipogenic differentiation. They were first isolated from bone marrow (Friedenstein*et al*., 1974; 1987), but have since been isolated from several adult and embryonic sources including adipose tissue (Zuk*et al*., 2001), Umbilical cord (Romanov *et al*., 2003), dental pulp (Pierdomenico*et al*.,2005), among others.

MSCs are widely cultured *in vitro*, and appear as adherent, spindle shaped cells. They are characterized by their adherence to plastic, capacity for osteogenic, chondrogenic and adipogenic differentiation, expression of a panel of surface markers, namely CD 105, CD 106, CD 90 and CD73 and non-expression of CD34 and CD 45 (Dominici*et al*., 2006).They are known to change phenotype in 3D culture and even undergo spontaneous differentiation in 3D, depending on the surroundings and physical properties of the scaffold (Kabiri*et al*., 2012; Neuss *et al*., 2008).

The ‘stem cell’ nature and the origin of MSCs are highly disputed. Since their physiological location is undetermined, it is not clear whether their plasticity, and

phenotype is in fact an *in vitro* artifact. They are, therefore referred to as stromal cells. Despite this uncertainty MSCs are considered a very promising candidate for use in tissue engineering, as stromal support cells and as vehicles for gene therapy.

1.2.2.2 Hematopoietic compartment

The hematopoietic compartment, as the name indicates, comprises the hematopoietic cells. These cells are responsible for the lifelong replenishment of all the blood cell types i.e. hematopoiesis. The hematopoietic cells are found within the stromal matrix produced by the stromal cells, often in association with a stromal cell type. All hematopoietic cells arise from a population of stem cells known as hematopoietic stem cells. Within the bone marrow, a mixed population of hematopoietic stem and progenitor cells (HSPCs) is observed. The eventual fate of these cells is related to their location within the bone marrow.

Maturation of the different blood lineages takes place in distinct stromal areas. Erythropoiesis takes place inerythroblastic islands located around a central macrophage. Megakaryopoiesis takes place under the sinus endothelium. Granulopoiesistakes place in foci always associated with a reticular cell. Primitive hematopoietic stem and progenitor cells are found concentrated at the endosteum, while differentiated progenitors are found in the perivascular area. This indicates an important role of the environment in cell fate, during the process of hematopoiesis.

1.2.3 Hematopoiesis

As mentioned earlier, the main function of adult bone marrow is hematopoiesis. Hematopoiesis refers to the systematic and controlled production of the different blood cell types, in response to molecular and environmental cues (Weissman, 2000).

A population of hematopoietic stem cells is maintained within the bone marrow throughout the life of an organism. These cells are able to undergo unequal division, allowing them to self-renew as well as differentiate into the required blood cell type. These processes are orchestrated by complex molecular mechanisms, for which the cellular and physiological environment of the cells is critical. All the aforementioned stromal cells are thought to have a role in hematopoietic stem cell maintenance or

differentiation. Their exact role however is not clear. Several molecular mechanisms have also been implicated in HSPC regulation; however, no definitive mechanism has been elucidated.

Bone marrow provides a specialized physiological environment suited to hematopoietic stem cells, assuring their maintenance and therefore a continuous production of all types of mature blood cells. This environment is referred to as the hematopoietic stem cell niche.

1.3 Hematopoietic stem cells (HSCs)

Hematopoietic stem cellsare adult stem cells, which are capable of self-renewal and differentiationinto specialized functional blood cells of all types (Till and McCulloch, 1968, Spangrude*et al*., 1988).These are located exclusively in the bone marrow, in adult humans. HSCs are multi-potent and have been shown to differentiate into several cell types like adipocytes (Sera *et al*., 2009), cardiomyocytes (Pozzobon*et al*., 2010),endothelial cells (Elkhafif*et al*., 2011) and fibroblasts (Ebihara*et al*., 2006) among others (Chotinantakuland Leeanansaksiri, 2012).They are of immense significance, not only due to their use in traditional allogenic transplantation therapy, but also as a paradigm for adult stem cells.

1.3.1 Origin and development

Although it has been established that HSCs migrate into the bone marrow during embryonic bone formation, and are maintained there throughout post-natal life, their origin is not completely understood.

Embryonic hematopoiesis in mammals starts after gastrulation, whena subset of specialized mesodermal precursor cells commit tobecoming blood cells. These precursors migrate to theyolk sac, and the AGM (Aorta-gonad-mesonephros) where they initiate embryonic red blood cell production. The first definitive HSCs, however,are thought to originate from a different subset ofmesodermal cells and develop in a different location, possibly the placenta and fetal liver (Jaffredo et al., 2005), where theycomplete a maturation process. The initial HSCpoolthen expands to establish anadequate supply of HSCs (Lessard et al.,2004), which eventually migrate to the

bone marrow (Boisset and Robin, 2012) and remain there throughout postnatal life (fig. 1.3).

The origin of the HSCs is largely thought to be from a 'hemangioblast' a common precursor forhematopoietic and endothelial cells (Sabin, 1920), which forms an intermediate population of cells known as hemogenic endothelium (Lancrin etal., 2009; Eilken et al., 2009). The factors governing the transition of these cells to HSCs, their subsequent expansion and homing are however yet to be elucidated.

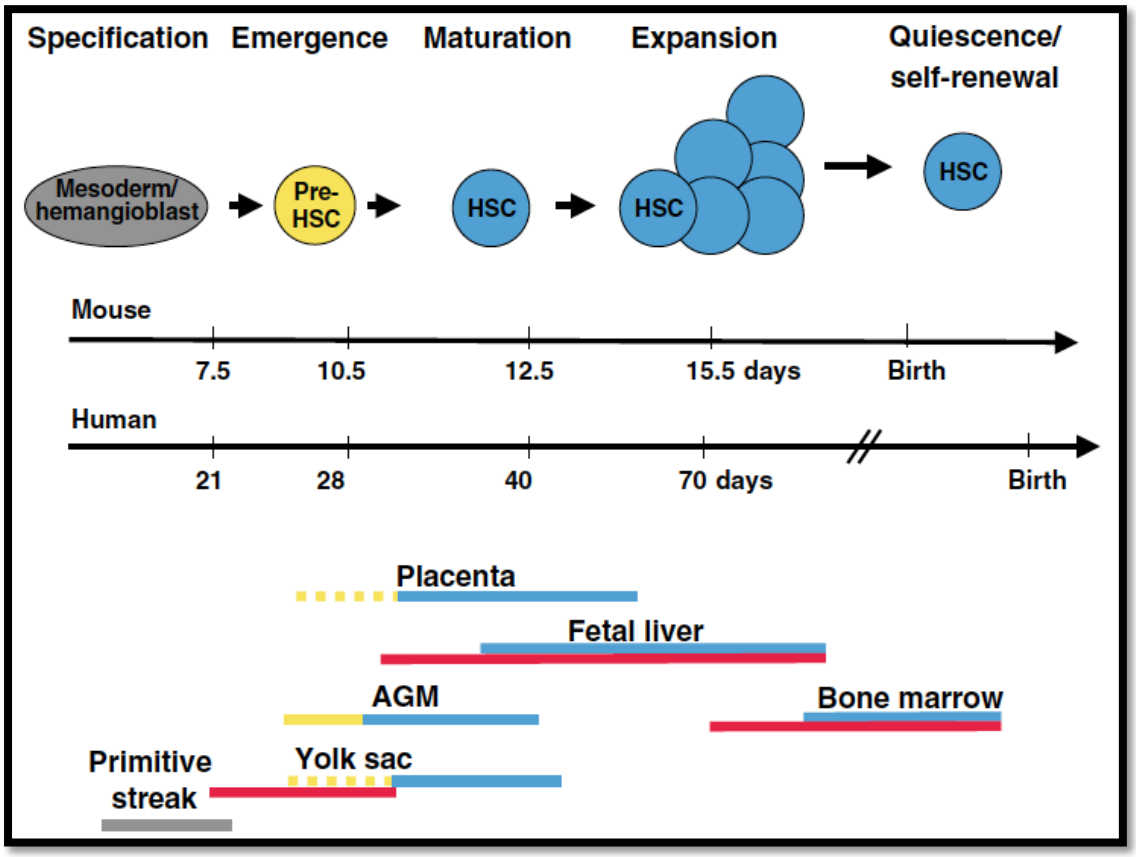

Figure 1.3. Schematic representation of the stages of HSC development in mouse and human showing different stages of hematopoietic cells, the point at which they appear and their anatomical location (Reproduced from Mikkola and Orkin, 2006).

Currently, it is widely accepted that the adult hematopoietic niche is derived from a self-renewing pool of cells in the bone marrow, which is established during fetal development. In recent times, however, it has been shown that adult cells derived from the muscle (Gussoni*et al*., 1999; Jackson *et al*., 1999), brain (Bjornson *et al*., 1999)and hair follicle are capable of hematopoietic activity (Lako*et al*., 2002) *in vivo*.

This has given rise to speculation as to whether HSCs are also generated in the adult bone marrow, by trans-differentiation of stromal cells. No evidence to support this theory has however, been presented.

1.3.2 Hematopoietic stem cell hierarchy

During postnatallife, a steady state is established in which HSC pool size is maintainedby the regulation of HSC self-renewal and differentiation. This occurs in specialized microenvironments or niches within the bone marrow. During homeostasis, most adult HSCs are non proliferative or quiescent and divide only rarely to maintain an appropriatequantity of differentiated blood cells and to renew the HSC pool(Cheshier et al., 1999). HSC pool maintenance and lineage differentiation are mediated either by asymmetric division, wherein specific cell fate determinants are redistributedunequally to the two daughter cells; or via environmental asymmetry, where one daughter cell leaves the niche and is then exposed to an environment that promotes lineagedifferentiation. As a result of these dynamic processes, there exists a homeostatic, balanced and mixed population of hematopoietic stem cells, multi-potent progenitor cells and cells at different stages of differentiation, at any given time, in the bone marrow (fig. 1.4).

Thus, there are several hematopoietic stem cells in the niche, such as the quiescent long term repopulating HSCs (LT-HSCs), the highly proliferative short term HSCs (ST-HSCs), multi-potent progenitor cells and differentiated cells within the bone marrow.

As depicted in fig 1.4,**in humans, the primitive hematopoietic stem cells as well as the proliferative progenitors are characterized by the Lin- CD34+ CD 38- phenotype.**A mixed population of these cells is typically used in all human hematopoietic studies, and is referred to as hematopoietic stem and progenitor cells (HSPCs). The terms HSPC and HSC are therefore usually used interchangeably in human hematopoietic stem cell research.

The homeostatic maintenance of all these cell types is largely dependent on their microenvironment or niche.

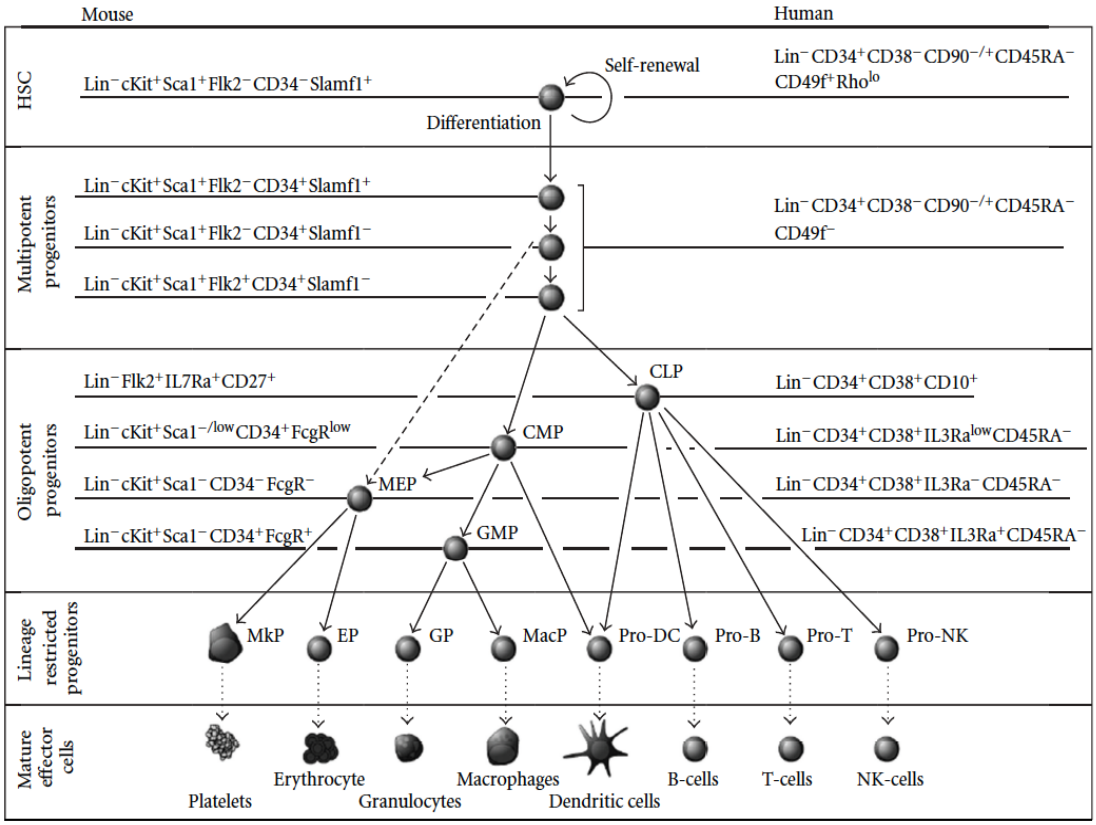

Figure 1.4. Schematic representation of the HSC hierarchy in mouse and humanshowing the different stages of hematopoietic differentiation and corresponding marker expression profiles. (Reproduced fromChotinantakul and Leeanansaksiri, 2012).

1.4 The hematopoietic stem cell niche

A stem-cell niche can be defined as a physiological microenvironment in which stem cells are housed and homeostaticallymaintained by allowing self-renewal in the absence of differentiation. During homeostasis,a proportion of stem cells are expected to divideat least occasionally (particularly in highly regenerativetissues such as the hematopoietic system), to maintaina constant flow of short-lived progenitors to generate enough differentiated cells to replace those that are constantlylost during normal turnover(Wilson and Trumpp, 2006).

The term ‘niche’ wasfirst coined by Schofield in 1978. It was proposed that HSCs arein intimate contact with bone, and that cell-to-cell contact is responsible for the apparently unlimited proliferativecapacity and inhibition of maturation of HSCs (Schofield, 1978).HSCs have been shown to reside close to the bone surface(Lord *et al.*, 1975). Further, scanning electron microscopy and histology of opened rat bone

have revealed that cells with hematopoietic stem and progenitor cell (HSPC) phenotypelocalize close to the endosteal liningof bone-marrow cavities (Gong, 1978), providing morphological evidencefor the presence of HSC niches in close association withthe endosteum.Since then, several subsequent studies in mice have established the endosteal surface as an area of HSPC engraftment.

More recent studies, utilizing immune-histology and intravital bone marrow imaging, have revealed that a large percentage of HSPCs (over 50%) are associated with the perivascular space, rather than the endosteum. Cell labeling and proliferation kinetic experiments have shown that the HSCs associated with the perivascular spaces are much more proliferative that those in the endosteal regions (Kiel *et al*., 2005; Sipkins*et al*., 2005).

These findings have resulted in the concept of two spatially and functionally distinctHSPC niches in the bone marrow: the vascular niche and the endosteal niche (fig.1.5). Due to the proximity and possible communication between these two niches, it has been suggested that they are, in fact, two functionally distinct parts of a single microenvironment or niche (Kiel and Morrison, 2008).

1.4.1 The perivascular niche

Due to the large numbers of perivascular HSPCs, the vascular niche is widely considered to be the area of HSPC proliferation and self-renewal (Wilson and Trumpp, 2006). HSPCs from this niche are considered to be ‘active’ or highly proliferative and predisposed to differentiation (Kopp *et al*., 2005), as they are conveniently located for subsequent mobilization into the blood stream.

Bone-marrow endothelial cells have been proposed to play a role in HSPC regulation within the perivascular niche. Primary CD31+ micro-vascular endothelial cells have been shown to stimulate HSC reconstitution and restore hematopoiesis in irradiated mice (Salter *et al*., 2009; Li *et al*., 2010).Sinusoidal endothelial cells are also known to be essential for engraftment of hematopoietic stem and progenitor cells (HSPCs) and restoration of hematopoiesis after myelo-ablation (Hooper *et al*, 2009). Therefore, the perivascular niche containing endothelial cells is considered to be a major HSC pool effecting proliferation and differentiation of HSPCs.

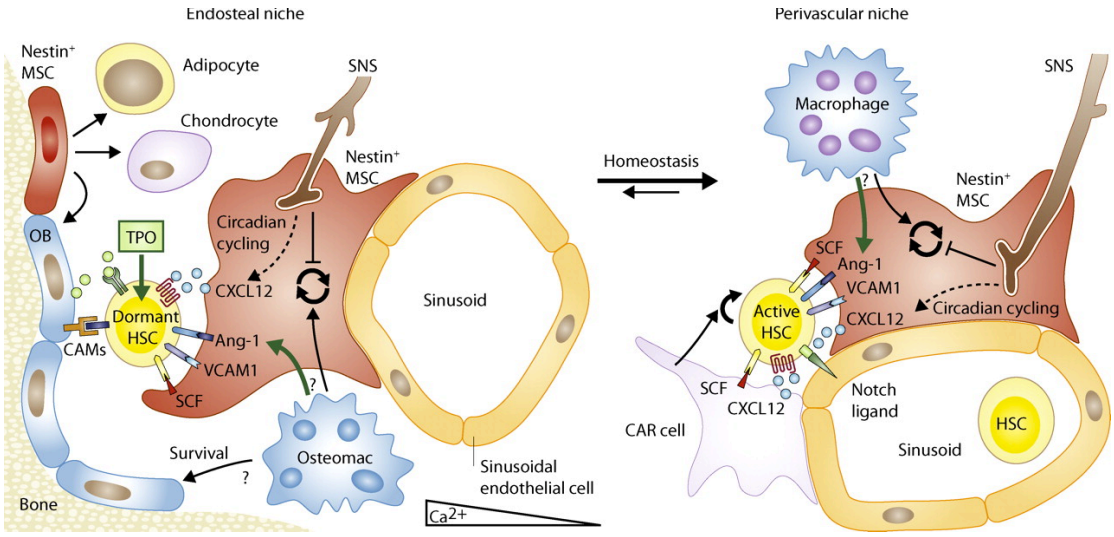

Figure 1.5. Schematic representation of the dormant and active HSCs within the bone marrow HSC niches showing the location and interactions between the endosteal and vascular niche. The different cell types and signaling pathways involved in the maintenance of each niche are also depicted (reproduced from Ehninger and Trumpp, 2011).

Osteoblast depletion in mice, however, was found to result in the drastic depletion of functional bone marrow HSPC and initiation of extra-medullary hematopoiesis (Visnjic*et al*., 2004). This indicates that the perivascular bonemarrowHSC niche alone is not sufficient tomaintain long-term hematopoiesis, suggesting that in the bone marrow the perivascular niche might bea secondary niche, requiring an influx of HSCs fromthe primary endosteal niche. It is therefore postulated that theperivascular and endosteal niches strongly cooperate (fig. 1.5) tocontrol HSC quiescence and self-renewing activity(and therefore HSC number), as well as the productionof early progenitors to maintain homeostasis orre-establish it after injury (Wilson and Trumpp, 2006).

1.4.2 The endosteal niche

The physical localization of HSCs close to the bone surfacewas first shown in 1975 (Lord *et al*., 1975). More recently, morphological evidencefor the presence of HSC niches in close association withthe endosteumhas been uncovered, as cells with HSCactivity and/or phenotypewere shown to localize close to the endosteal liningof bone-marrow cavities in trabecular regions of long bones. Further, more

differentiated hematopoietic cells were shown to localize around the central axis of the marrow (Zhang *et al*., 2003; Taichman, 2005; Kiel *et al*., 2005; Calvi*et al*., 2006).

While a large portion of the cells identified in these earlier studieswere subsequently classified as progenitors, rather than true stem cells,(Gong, 1978) which form colonies in the spleen of irradiatedanimals(colony-forming unit-spleen (CFU-S)), further investigations also revealed a similar spatialdistribution of undifferentiated cells near the endosteal region,over a time course of 15 hours after transplantation (Nilsson *et al*., 2001). Furthermore, BRDU retention studies have revealed that the undifferentiated HSCs on the endosteal surface are very slow dividing, undergoing one division in 30- 60 days (Bradford *et al*., 1997; Cheshier*et al*., 1999).

These results, when viewed together, provide irrefutable evidence of the presence of non-proliferative, 'dormant' HSCs as well as multi-potent progenitors in the endosteal region of the bone marrow. This suggests that the endosteal microenvironment or niche is the site for long- term maintenance of the quiescent HSCs. The niche maintenance is orchestrated by several molecular mechanisms, involving several different cell types.

1.4.2.1 Cells of the endosteal niche

Cell- cell contact has been considered essential in the homing and phenotype maintenance of HSCs within the endosteal niche. Several different cell types (fig. 1.5) have been implicated in niche function (Wilson and Trumpp, 2006). The most prominent cellular components of the endosteal niche can broadly be classified as follows:

Bone cellsincluding osteoblasts, osteocytes and osteoclasts (described on page 3) have been identified as mediators of the niche. Osteoblastic cells in particular are widely accepted as a key interaction partner of the HSCs in the niche (Taichman*et al*., 1996). A small subset of spindle-shaped osteoblasts, which line the endosteal surface, and express the marker N-Cadherin, have been shown to physically interact with quiescent HSCs in the niche (Zhang *et al*., 2003; Arai *et al*., 2004). These cells are referred to as SNO (spindle-shaped N-cadherin expressing osteoblast) cells, and are thought to be the specialized niche cells that interact with the HSCs. Further, the

depletion of cells of the osteoblastic lineage was shown to induce a marked reduction in B cells and erythroid progenitors (Visnjic*et al*., 2004) These results suggested that a mixed population of bone-liningosteoblasts, at different stages of differentiation, including SNO cells function as a niche forHSCs and hematopoietic progenitors.

Subsequent studies, however demonstrated that HSCs are not exclusively dependent on osteoblasts for their maintenance (Kiel *et al*, 2007; 2009), implying that while osteoblasts play an important role in the endosteal niche, they are not exclusively responsible for niche maintenance.

Osteoclasts, chondrocytesand adipocytes are also thought to have a role in the maintenance of the bone marrow HSC niche, due to their role in bone cavity formation (Yoshida et al., 1990, Kong et al., 1999;Zhu *et al*., 2007), and their effect on mobilization and homing of HSCs to the niche, directly, by signaling molecules, and through their influence on the differentiation of osteoblasts (Winkler et al., 2010; Lymperi et al., 2011).

Stromal cells including mesenchymal ‘stem’ cells and reticular cells have also been shown to have a leading role in niche maintenance.

Mesenchymal stromal or ‘stem’ cells (MSCs) are automatically considered to have a role in the niche, due to the fact that they are the progenitor cell which form osteoblasts, chondrocytes and adipocytes, all of which contribute to the bone marrow microenvironment. In vitro studies have shown that MSCs markedly improve HSC survival (Walenda*et al*., 2009). Recently, a populationof MSCs, expressing the neural marker nestin was identified *in vivo*. These cells self-renewed, displayed multi-lineagedifferentiation and were spatially associatedwith HSCs in vivo (Mendez-Ferrer*et al*., 2010). The elimination of nestin-positive MSCs from the bone marrowresulted in HSC loss. In addition, transplantedHSCs rapidly homed to nestin-expressing MSCs,and the loss of these cells from the microenvironmentdecreased stem cell homing, thus confirming the role of MSCs in the maintenance of quiescent HSCs.

In recent times, a population of reticular cells expressing CXCL-12, termed CXCL-12 abundant reticular (CAR) cells have been implicated in HSC homeostasis (Sugiyama *et al*., 2006; Omatsu*et al*., 2010). On ablation of CAR cells in vivo, HSC numbers were halved. The cells were smaller, morequiescent and their differentiation skewed (Tzeng*et al*., 2011), suggesting that these cells effect HSC proliferation and mobilization.

Cells of hematopoietic origin such as megakaryocytes and endostealmacrophages termed osteomacsare also thought to be components of the HSC niche, acting as positive regulators for MSCs and osteoblasts to retain HSCs within the bone marrow (Yoshihara *et al*., 2007; Chow *et al*., 2011). Neural cells, particularly non-myelinatedSchwann cells have also been shown to induce HSC quiescence (Yamazaki *et al*., 2011).

The maintenance of the endosteal niche is therefore clearly a complex process involving the inter-dependent functioning of several cell types, which act through several molecular pathways.

1.4.2.2 Molecular mechanisms of endosteal niche regulation

As mentioned earlier, the maintenance of the endosteal niche is a very complex process, and new molecular mechanisms are constantly being implicated in its regulation (fig. 1.6). Some of the key molecules and their mode of action are as follows:

Parathyroid hormone (PTH) /parathyroid hormone-related protein (PTHrP) receptormediated signaling has been found to dramatically increase HSC numbers and is therefore thought to mediate HSC proliferation (Calvi*et al*., 2003).

Bone morphogenetic proteins (BMPs) are knownto be important in regulating HSC specification during embryonic development and regulating the proliferation of adult HSCs. They are thought to act on the osteoblasts of the niche, via the BMP receptor 1A (BMPR1a). The ablation of BMPR1a in osteoblasts result in HSC proliferation, suggesting that BMP mediated signaling promotes quiescence (Larsson and Karlsson, 2005).

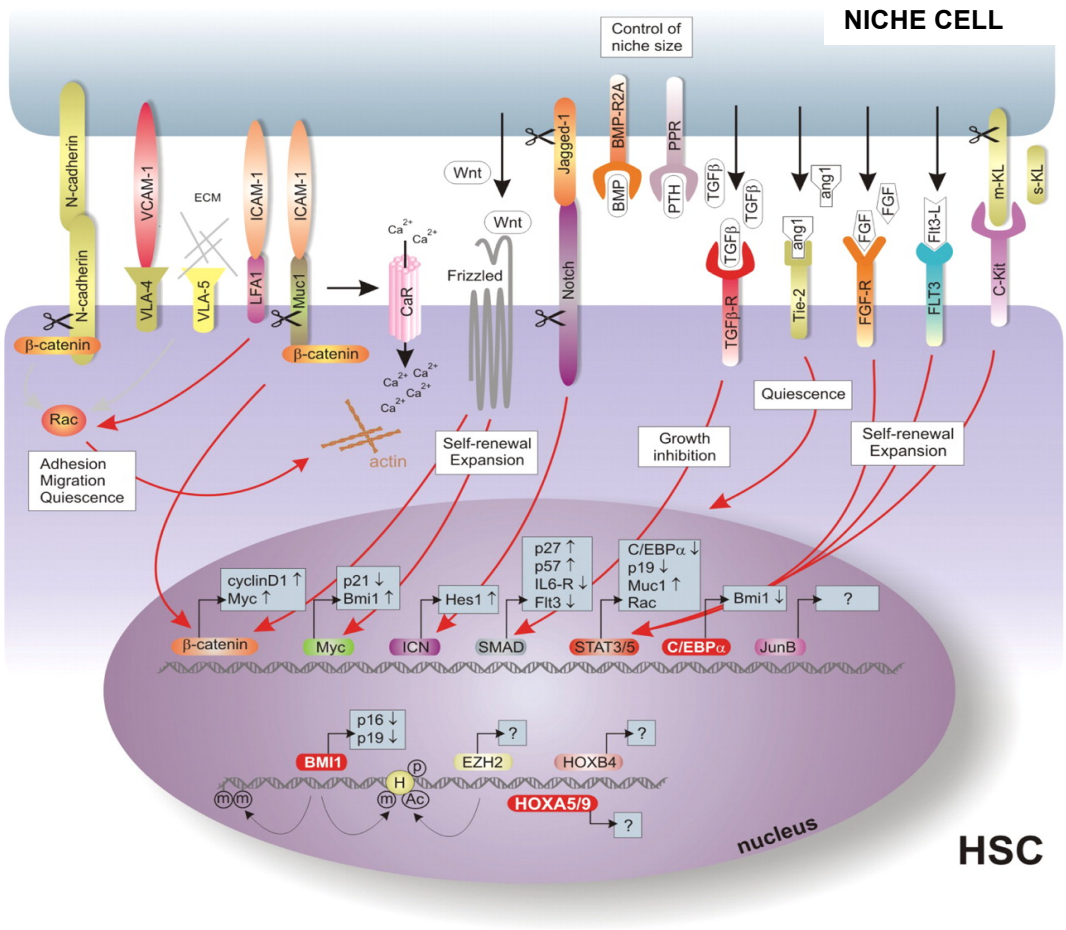

Figure 1.6. Schematic representation of selectedmolecular mechanisms involved in HSC niche maintenanceshowing the molecular interactions between the HSC and stromal cells of the niche. The different signaling pathwaysand their putative role in HSC maintenance are also depicted (reproduced from Rizo*et al*., 2006).

Osteopontin (Opn)is a glycoprotein produced by cells of the osteoblast and monocytelineages in the bone marrow. It binds to integrin 4a and CD44 present on HSCs. Ablation of Opn inmice resulted in HSC proliferation, suggesting thatOpn acts asa negative regulator of HSC proliferation and therefore promotes quiescence (Stier*et al*., 2005).

Trans-membrane Stem cell factor (SCF)is important for lodgment and detainment of HSCs in theniche (Driessen*et al*., 2003). Function of the SCF receptor c-kit hasbeen shown to be important for maintenance of quiescent HSCs in theniche (Thoren*et al*., 2008).

Angiopoietin 1 (Ang-1)interacts with its receptor **Tie2** on HSCs and has a role inmaintaining HSC quiescence in the HSC niche (Arai *et al*., 2004).

Jagged1 (Jag-1)is a surface receptor, which, by activation of Notch1 is known to be crucial for the increaseof HSCs in the PTH/PTHrP model (Stier*et al*., 2002). However the deletion of Jagged1 instromal cells did not affect HSC maintenance (Mancini *et al*., 2005). Transplantation of Notch1-negative HSCs also had no effect reconstitution. The role of Jagged1 in the HSCniche, therefore, is uncertain.

Chemokine (C-X-C motif) ligand 12 CXCL12is a stromal cell-derived cytokine. It binds CXCR4 on HSCs, and is important for retention of HSCs in the niche (Ara*et al*., 2003). Blocking of interaction of CXCL12 andCXCR4 results in mobilization of HSCs from the BM to the periphery(Broxmeyer*et al*., 2005).

Thrombopoietin (TPO)is a growth factor, which, by interacting with it's receptor MPL, on the HSC surface, maintains aquiescent HSC population in the HSC niche (Yoshihara *et al*., 2007).

Ca2+ ions, and the cell-surface calcium-sensing receptor (CaSR)plays a role in theengraftment of HSCs in the niche (Adams *et al*., 2006).

Vascular cell adhesion molecule 1 (VCAM1) and Intercellular adhesion molecule 1 (ICAM1) are adhesion molecules, which play a role in theHSC interaction with the niche cells, and therefore quiescence (Jung *et al*., 2007, Mercier*et al*., 2011).

N-cadherin: The expression and function of N-cadherin in niche maintenance is controversial because of conflicting reports regarding the necessity of N-cadherin for HSC function (Askmyr*et al*., 2009).

The variety of factors that might affect HSC phenotype maintenance, proliferation and differentiation reflect a very complex system, which is yet to be completely understood. The development of an *in vitro*model of these interactions would, therefore be of much use in this context.

1.5 *In vitro* HSC culture

The ex-vivo culture of hematopoietic stem cells, particularly long-term repopulating HSCs is of interest on three levels. First, transplantation of LT-HSCs is the only known treatment for congenital blood disorders, several types of anemia, myelo-proliferative disorders and hematological malignancies such as leukemias, lymphomas and myelomas. Currently, bone marrowand cord blood are used as sources of HSCs, but the number of HSCs obtained from these sources is rarely sufficient to treat adults. A strategy to successfully expand HSCs, while simultaneously retaining the primitive phenotype, is therefore of great value.

Secondly, the mechanisms involved in HSC cell fate determination, quiescence, differentiation and niche maintenance and modulation are not completely understood, particularly in humans. This is largely due to the lack of a suitable model. While mouse models and transplanted immune deficient mice and even humanized mice are used to study human HSCs (Rongvaux*et al*., 2011), the distinct differences between mouse and human hematopoietic cells, as illustrated in fig. 1.4 prevent accurate modeling. An *in vitro* culture system, which accurately models the interactions of the HSPCs within their niche, even in part, would be of great use in the study of niche maintenance. Finally, an *in vitro*system mimicking the HSC niche would serve as an ideal platform for testing drugs targeting the bone marrow, prior to animal experiments.

Several strategies to culture HSCs have been attempted. Some aim to expand primitive HSCs, while others aim to mimic the niche. None of these systems however, have been completely successful. The most prominent methods currently explored include the use of cytokine-supplemented media and the use of stromal support cells in 2D and 3D.

1.5.1 Expansion in cytokine supplemented media

During the last decades, many hematopoietic growthfactors and their receptors were identified and tested for efficacy in amplification and maintenance ofHSC *in vitro*. Some of the factors tested individually or in combination are: Interleukins (IL)-3 (Rennick*et al*., 1985), IL-6 (Ema*et al*., 2000), IL-11 (Lemieux *et al*.,1997),

Flt3-ligand (Flt3L), stem cell factor (SCF) (Miller and Eaves, 1997), thrombopoietin (TPO), fibroblast growth factor (FGF)-1 andAngiopoietin (Ang)-1 (reviewed: Takizawa *et al*.,2011).

Although the in vitro experimentalconditions and subsequent in vivo engraftment are highly variable, the net increase of HSC during short–term liquid cultures range from about 2–8 fold for mouse cellsand 2–4 fold for human cells. One of the highest HSC amplifications achieved to date is a 30-fold net increaseof functionally defined mouse HSC in serum free mediumsupplemented with Angiopoietin-like proteins (Angptls),secreted proteins with sequence homology to Angiopoietin (Zhang *et al*., 2006).However, a substantial HSCexpansion was only observed when Angptls were used incombination with other hematopoietic cytokines. Also, the resultant expanded HSC population was found to contain a large number or differentiated cells.

From these studies, it is clear that while cytokine supplemented media provides an efficient and serum free method of expanding HSPCs *ex vivo*, it is not sufficient to maintain the primitive phenotype of the HSPCs. Also, such culture systems are of little use in modeling and studying HSC niche interactions. Culture systems with stromal support cells are therefore a viable option to overcome both these drawbacks.

1.5.2 Culture with stromal support

As described earlier, cellular contact with partner cells is considered essential for HSPC maintenance in the bone marrow niche. It follows that the use of a stromal support cell population would promote HSPC culture *in vitro*.

To date, several studies have been carried out wherein monolayers of osteoblastic cell lines, bone marrow MSCs (Da Silva *et al*., 2005; Robinson *et al*., 2006; Madkaikar*et al*., 2007), umbilical cord-derived MSCs (Wang *et al*., 2004; Jang *et al*.,2006; Huang *et al*., 2007) and placental stromal cells(Zhang *et al*., 2004) have been used as stromal support to culture HSCs.

These studies revealed that the presence of stromal support cells, particularly MSCs distinctly improves HSC survival and proliferation and stabilizes the primitive phenotype (Walenda*et al*., 2009). However, such 2D culture systems bear nostructural resemblance to the physiological niche.

1.5.3 3D culture

Several groups have carried out 3D culture of HSPCs, in an attempt to recapitulatethe HSC niche *in vitro*. Recent work has demonstratedincreased maintenance of immature human and mouse hematopoieticcells when cultured in 3D scaffolds composed of polyurethane foam with stromal supportcells (Jozaki*et al*., 2010), cancellous bone with osteoblasts differentiated from MSC as support cells (Tan *et al*., 2010), poly(D, L -lactide-co-glycolide) or polyurethane withcollagen type-1 (Mortera-Blanco *et al*., 2011) and porous polyvinyl formalresin with stromal support cells (Miyoshi *et al*., 2011).

In the past year, maintenance and expansion of primitive human HSPCs has been demonstrated in 3D gel matrices composed of collagen I, and fibrin respectively (Leisten*et al*., 2012; Ferreira *et al*., 2012), in co-culture with MSCs. In these works, it has been suggested that the 3D scaffolds act as a stimulus and encourage the MSCs to mimic the bone marrow microenvironment.

What these studies lack however is a scaffolding system, which resembles bone marrow. In summary, these studies demonstrate the need for a combination of 3D scaffolding, appropriate ECM and partner cells for the successful maintenance of HSPCs *in vitro*.

2. Aims

As described in the previous chapter, a combination of 3D structure and cellular interaction is essential to mimic the physiological bone marrow niche, and achieve improved HSPC maintenance. To date, no work has been reported wherein the physical properties of bone marrow, the extracellular matrix (ECM) and stromal support cells have been brought together in a long- term culture system for HSPCs.

In this study, we present a 3D co-culture system, comprising MSCs and HSPCs, in zirconium oxide based ceramic scaffolds engineered to mimic bone marrow microstructure. The hydroxyapatite coated porous yet rigid scaffolds closely simulate the structural and chemical properties of bone marrow. It has been shown that MSCs seeded in such ceramic scaffolds have a tendency towards spontaneous osteogenic differentiation (Dietrichs *et al*., 2009). We hypothesized that co-culture of MSCs and HSPCs, in such a scaffold, would be conducive to the formation of a niche-like environment, due to the varying degrees of spontaneous osteogenic differentiation of MSCs, ECM production and production of other niche molecules. With such a system, we expected to mimic the cellular and molecular interactions of the bone marrow niche, wherein the HSPCs are maintained in a slow-proliferating, quiescent state due to their interaction with the MSCs as well as with the molecular microenvironment.

The first aim of this work was the establishment and characterization of a niche like microenvironment in the ceramic. Bone marrow derived MSCs were cultured in the ceramics for a period of 7 days, in order to allow attachment, ECM production and spontaneous differentiation. This 3D system was then characterized in terms of osteogenic differentiation of the MSCs, production of bone marrow ECM components including collagen I and fibronectin, production of niche- relevant molecules such a Jag-1, Ang, etc.

Next, we attempted to adapt the static 3D system to a perfused bioreactor setting, to achieve a dynamic culture system wherein the endosteal circulatory system would also be represented.

Finally, we introduced umbilical cord derived HSPCs into the static MSC-seeded ceramics, in order to mimic the interactions within the endosteal niche. We then studied their long-term maintenance, retention of primitive phenotype, proliferation kinetics and functionality. We also monitored the interactions of the HSPCs with the MSCs and ECM.

Such a system would find applications not only as an *in vitro* model to study specific niche interactions, but also as a platform for substance testing and possibly as a basis for a pre-transplantation expansion strategy for HSPCs.

3. Materials and Methods

3.1 Materials

3.1.1 Cell sources

Human mesenchymal stromal cells were obtained from femoral heads removed during bone replacement surgery, from the Immanuel Krankenhaus Berlin. These were transported and stored in Ringer's solution, until cell extraction.

Cord blood was used as a source of hematopoietic stem and progenitor cells. Cord blood was obtained from the Vivantes Humboldt Klinikum, Berlin. The blood was harvested and stored in PBS-BSA-EDTA, until cell extraction.

3.1.2 Cell culture media and supplements

The various cell culture media used in the different parts of this work, their components and compositions are listed in the following table.

Table 3.1. Cell culture media and supplements

Media	Composition	Manufacturer
DMEM (Dulbecco's modified Eagle's medium)		
DMEM High Glucose (4.5g/l)		PAA, Austria
Fetal bovine serum (FBS)	10%	PAA, Austria
Penicillin/ Streptomycin	1 unit/100 µg/ml	PAA, Austria
CFU-GEMM media		
MACS® HSC-CFU Media with EPO		Miltenyi Biotec, Germany

Table 3.1 contd.

StemSpan™ defined HSC expansion media		
StemSpan™ ACF		Stemcell technologies, France
IL-6	100ng/ml	Peprotech, UK
SCF	100ng/ml	Peprotech, UK
TPO	100ng/ml	Peprotech, UK
FLT-3L	100ng/ml	Peprotech, UK

3.1.3 Buffers and reagents

The following buffers and reagents were used in the course of this work, for the different methods.

Table 3.2. Buffers and miscellaneous reagents

Name	Composition	Manufacturer
Cell culture		
PBS (Phosphate buffered Saline) pH 7.4	140 mM NaCl 2,7mM KCl	Sigma,Germany
BSA (Bovine serum albumin)		PAA, Austria
EDTA		Sigma,Germany
Penicillin/ Streptomycin	1 unit/100 µg/ml	PAA, Austria
PBE (PBS-BSA-EDTA)	PBS	
	0.25% BSA	(see above)
	1mM EDTA	
Lymphocyte Separation Medium LSM 1077		PAA, Austria

Table 3.2 contd.

Trypsin-EDTA	10x	PAA, Austria
CFU-GEMM media		
MACS® HSC-CFU Media with EPO		Miltenyi Biotec, Germany
Flow cytometry		
Running buffer	PBS 0,5 % BSA 0,01% NaN_3	Sigma,Germany
Staining		
Oil Red O 0.5%	0.5g Oil Red O 100ml Propylene glycol	Sigma,Germany
Alcian Blue solution (pH 2.5)	3% Alcian Blue Acetic acid	Sigma,Germany
Alizarin Red	2% Alizarin Red S Distilled water	Sigma,Germany
Miscellaneous		
Acetone		Sigma,Germany
4% PFA (pH 7.4)	PBS 0.137 M NaCl, Paraformaldeyde 0.05 M NaH_2PO_4	PAA, Austria Sigma,Germany
Sponceram® HA ceramics		Zellwerk GmbH, Germany
Propidium iodide (PI)		AppliChem ,Germany
$CaCl_2$		Sigma,Germany

3.1.4 Antibodies

The following antibodies were used for FACS analysis and MACS sorting of cells in the course of this work.

Table 3.3. FACS Antibodies

Antibody against	Conjugate	Manufacturer
FACS Antibodies		
CD34	APC	Miltenyi Biotec, Germany
CD38	PE	Miltenyi Biotec, Germany
Annexin-V	Pacific-blue	BioLegend, USA
CD105	APC	Serotec, USA
CD106	PE	Pharmingen,USA
CD90	PE	BD Biosciences, Germany
CD73	PE	BD Biosciences, Germany
CD44	FITC	Pharmingen, Germany
CD13	FITC	Pharmingen, Germany
CD45	FITC	BD Biosciences, Germany
CD38	APC	Miltenyi Biotec, Germany
CD31	FITC	Miltenyi Biotec, Germany
CD105	Biotin	eBioscience®, USA
Streptavidin	PE-Cy7	BDBiosciences
MACS Antibodies		
CD34		Miltenyi Biotec, Germany

Table 3.4 lists the antibodies used for immunofluorescence staining. All the primary antibodies listed below are against human antigens.

Table 3.4. Antibodies for immunofluorescence

Antibody against	Species of origin	Manufacturer
Primary Antibodies		
Collagen I	Mouse	Sigma, USA
C-kit	Mouse	Santa Cruz Inc, USA
Fibronectin	Mouse	Millipore, USA
Integrin 4a	Mouse	Abcam, UK
N-cadherin	Mouse	Santa Cruz Inc, USA
Ki-67	Rabbit	Abcam, UK
Secondary Antibodies (with conjugates)		
anti-mouse/ Alexa 350		Invitrogen, USA
anti-mouse/ Alexa 594		Molecular Probes, USA
anti-rabbit/ Alexa 350		Santa Cruz Inc, USA

After immunofluorescence staining, samples were usually counterstained with Hoeschst 33342 (Invitrogen, USA)

3.1.5 Cell tracking and proliferation

The following reagents and kits, listed in table 3.5 were used in experiments wherein cells were tracked, and their proliferation rate monitored, after specific periods of time in culture.

Table 3.5. Cell tracking kits/reagents

Name	Application	Manufacturer
Qtracker® 525	Tracking	Invitrogen, USA
CellTracker™ Red CMTPX	Tracking	Invitrogen, USA
Carboxyfluorescein diacetate succinimidyl ester (CFSE)	Proliferation	Invitrogen, USA

3.1.6 Kits

The following staining and molecular biology kits (table 3.6) were utilized during the course of this work.

Table 3.6. Reagent kits

Name	Manufacturer
Microscopy	
ApopTag Fluorescein In situ Apoptosis detection Kit	Chemicon International
Molecular biology	
NucleoSpin® RNA II RNA isolation kit	Macherey-Nagel, Germany
Gel Extraction kit	Macherey-Nagel, Germany
SensiFAST™ Sybr No-ROX kit	Bioline, Germany
TaqMan® Reverse Transcription cDNA kit	Applied Biosystems, USA

3.1.7 Primers

Table 3.7 lists the primers used for quantitative or real-time PCR analysis. All primer-pairs were purchased from TIBMOLBIOL, Germany.

Table 3.7. qPCR primers

Name	Direction	Sequence
Jagged 1	Forward	5'- ATGGGAACCCGATCAAGGAA
	Reverse	5'- TCCGCAGGCACCAGTAGAAG
ICAM 1	Forward	5'- CCGACTGGACGAGAGGGATT
	Reverse	5'- TCGGCCCGACAGAGGTAGGT
BMPR1A	Forward	5'- TCACAGGAGGGATCGTGGAA
	Reverse	5'- AGTCTGGAGGCTGGATTGTGG
Osteopontin	Forward	5'- CACTGATTTTCCCACGGACCT
	Reverse	5'- CCATTCAACTCCTCGCTTTCC
N-Cadherin	Forward	5´- CATCCTGCTTATCCTTGTGCTG
	Reverse	5´- TCCTGGTCTTCTTCTCCTCCA
CXCL12	Forward	5'- CCAACCTGTGCCCTTCAGATTG
	Reverse	5'- CATATGCTATGGCGGAGTGTC
Osteocalcin	Forward	5'- CTGACCTCACAGATGCCAAG
	Reverse	5'- GTAGCGCCGGAGTCTGTTC
GAPDH	Forward	5'- TGTTGCCATCAATGACCCCTT
	Reverse	5'- CTCCACGACGTACTCAGCG

3.1.8 Instruments and software

Table 3.8 lists the various instruments used in this work. The software used in data analysis are shown in table 3.9

Table 3.8. Instruments

Instrument	Name	Manufacturer
Fluorescence microscope	BZ 9000	Keyence, Germany
2-photon microscope	Trimscope II	LaVision BioTec GmbH, Germany
Flow cytometer	MACSQuant Analyzer	Miltenyi Biotec, Germany
Multiplex Quantitative PCR System	Stratagene MX 3005P™	Agilent Technologies, USA
Bioreactor	Z®RP bioreactor	Zellwerk GmbH, Germany
Spectophotometer	NanoDrop ND-2000c	PEQLAB, Germany
Gel visualization	Fusion-FX7-Superbright	PEQLAB, Germany

Table 3.9. Software

Name	Application	Vendor
FlowJo version 7.6.5	Flow cytometry data analysis	Tree Star inc., USA
GraphPad Prism® version 5.0	Statistical analysis, graphs	GraphPad Software Inc., USA
Imaris version 7.5	Rendering 2-Photon images	Bitplane Scientific Software, Switzerland

3.2 Methods

3.2.1 Cell isolation and expansion

3.2.1.1 Mesenchymal stromal/stem cells (MSCs)

Human MSCs were isolated from femoral head marrow, obtained after joint replacement surgery, with written consent as per the guidelines of the Ethics board of the *Charité* - Universitätsmedizin Berlin. The cells from the bone spongiosa were vigorously flushed out, by forcefully pipetting PBS directly into the bone. Mononuclear cells were isolated from the resulting cell suspension, using standard Ficol® density gradient centrifugation. These cells were then placed in culture, in DMEM with 10% FCS. MSCs were selected based on the ability to adhere to plastic. The cells were then expanded in DMEM 10%FBS and Penicillin-Streptomycin. MSCs between passage 4 and 7 were used for the subsequent co-culture experiments.

3.2.1.2 Hematopoietic stem/progenitor cells (HSPCs)

Human HSPCs were isolated from umbilical cord blood, with written consent as per the guidelines of the Ethics board of the *Charité* – Universitätsmedizin Berlin. Cord blood was collected in PBS-BSA-EDTA solution, and the mononuclear cells isolated by density gradient centrifugation. The HSPCs were then separated by immunomagnetic separation, using the MACS CD34+ isolation kit (Miltenyi Biotec, Germany), and following manufacturers' instructions. The freshly isolated cells were then introduced into the different culture systems at a density of 2x 10^4 cells / culture.

3.2.2 MSC differentiation

In order to confirm the multi-lineage differentiation potential of the isolated, expanded MSCs, the following differentiation protocols were used:

3.2.2.1 Osteogenic differentiation of MSCs

Osteogenic differentiation was induced in monolayer cultures as described in Pittenger et al., 1999. MSCs were seeded in 6 well plates (100.000 cells per well)

and cultured in DMEM + 10% FCS until they reached confluence. Osteogenic differentiation media contained the following constituents:

- DMEM + 10 % FCS
- 10mM ß-glycerophosphate
- 10nM Dexamethasone
- 0,1mM L-ascorbic acid 2-phosphate

Medium was replenished every 4 days and after 21 days of osteogenic stimulation, the osteogenic differentiation was visualized by von Kossa and Alizarin red staining for secreted Ca2+ based mineralized matrix as marker for osteoblastic differentiation.

3.2.2.2 Adipogenic differentiation of MSCs

Adipogenic differentiation was induced in monolayer MSC cultures using well-established medium supplements (Pittenger et al., 1999). The cells were seeded in 6 well plates (100.000 cells per well) and cultured in DMEM + 10% FCS until they were confluent. They were then cultured in adipogenic differentiation media, which contained the following constituents:

- DMEM + 10% FCS
- 10 µg/ml Insulin
- 0,2 mM Indomethacin
- 1 µM Dexamethasone
- 0,5 mM 3-isobutyl – 1methyl-xanthine

The MSCs were stimulated for 28 days, with medium being replaced at 4-day intervals. After 4 weeks, adipogenic differentiation was visualized by histochemical analysis using oil-red staining for characteristic lipid vesicles.

3.2.2.3 Chondrogenic differentiation of MSCs

For chondrogenic differentiation, Cultured cells in monolayer, were trypsinized and transferred into 15 ml Falcon tubes at a concentration of $2x10^4$ cells / 2 ml to stimulate the formation of a micro-mass by centrifugation (4 min at 800xg). After 48 hours of incubation at 37°C, the cell pellets were detached from the bottom of the tube by gentle movement. Finally, differentiation was induced by incubation with

chondrogenesis medium, which has the following composition:

- DMEM 10% FCS
- 50µg/ml ascorbic acid
- 0.1µM Dexamethasone
- 100µg/ml Sodium Pyruvate
- 1x ITS
- 100 ng/ml TGFβ3.

Stimulation was carried out for 3 weeks and the differentiated micro-masses were frozen down into cryomolds and cryosections of 7µm and were prepared. Chondrogenic differentiation was visualized by Alcian blue staining of these sections.

3.2.3 3D co-culture

3.2.3.1 Ceramics

Zirconium oxide based, hydroxyapatite coated, Sponceram HA® ceramic discs of 1mm thickness and 1cm diameter were purchased from Zellwerk GmbH, Germany. The discs were autoclaved prior to use.

3.2.3.2 Cell culture systems

The ceramic discs were seeded with MSCs at a density of approximately 10^6cells/ disc, 7 days prior to seeding HSCs. The ceramic discs were submerged in DMEM-high glucose (PAA Laboratories, Austria) containing 10%FBS (PAA Laboratories, Austria) and Penicillin-Streptomycin, in ultra low attachment 24 well plates (Corning inc., USA). Plates were maintained at 37°C, 5% CO_2. Media was replaced every 48 hours. Simultaneously, 6 well plates were also seeded with MSCs, so that they achieved confluency in 24 hours. One set of these plates was cultured with the same medium as the ceramics, while another was treated with osteo-inductive medium as described previously.

After 7 days of culture, a sample of MSCs from each culture condition were lysed and prepared for RNA isolation and subsequent molecular biology analysis. Simultaneously, MSC-seeded ceramics, after 1 week of culture were introduced into

two perfusion-bioreactor systems (represented in fig. 3.1).

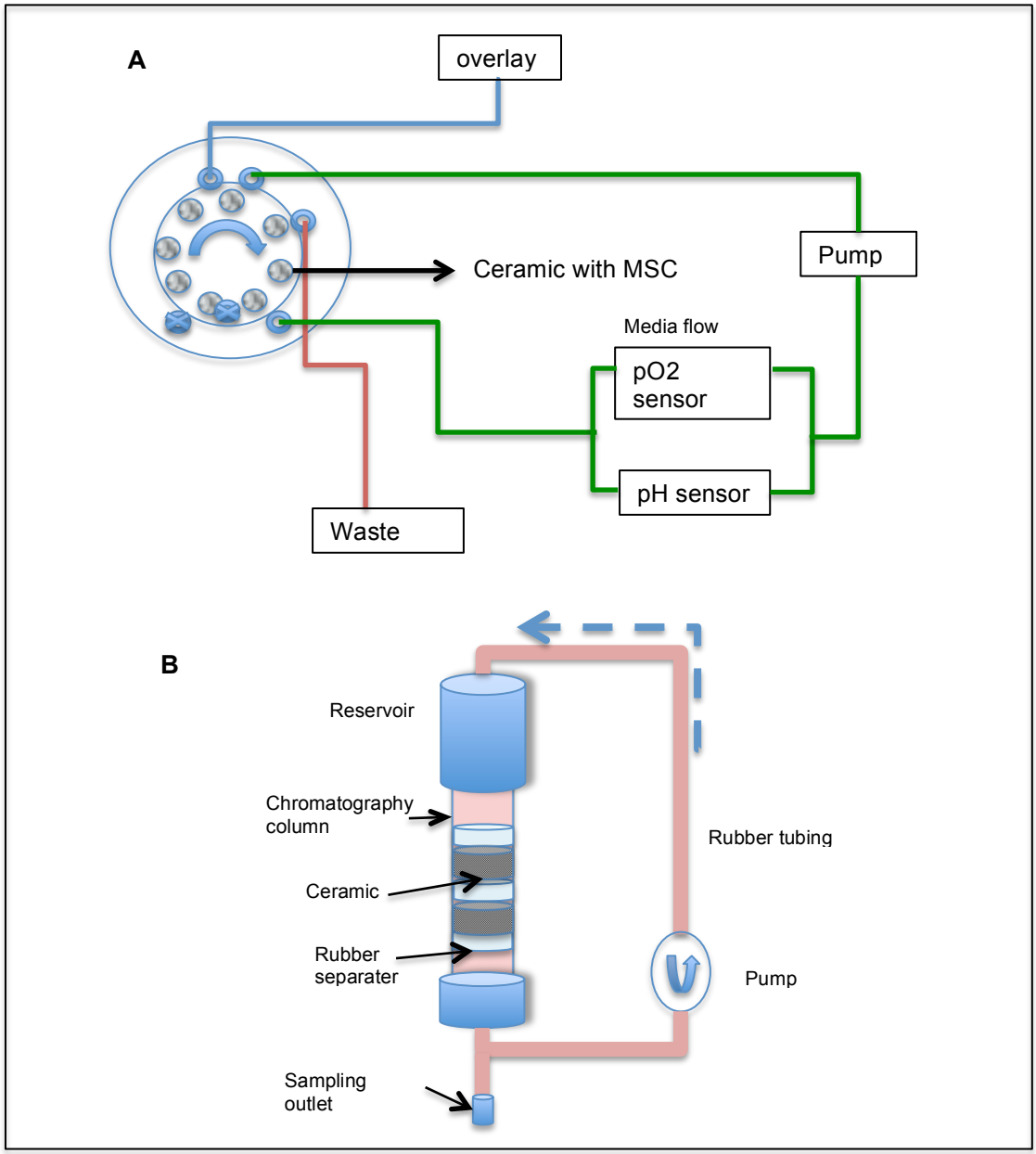

Figure 3.1. Bioreactor systems used for perfusion of MSC-seeded ceramics (A) Z®RP reactor system with rotational perfusion **(B) 'Tube' reactor system** with vertical flow-through perfusion.

As depicted in figure 3.1, two different perfusion strategies were used to investigate the effect of perfusion on the MSC-seeded ceramics. In the first method, the Z®RP bioreactor system (purchased from Zellwerk GmbH, Germany) was used (fig. 3.1A) while in the second; a modified chromatography column was used.

The MSC seeded ceramics were placed in a rotating plastic disc, in the case of the Z®RP system, and were placed in stacks of 2, separated by rubber tubing, in case of the tube reactor system. In the Z®RP system, the pH and pO_2 were constantly monitored by the pH and pO_2 electrodes. pH was maintained around 7 by the pumping in of CO_2. The ceramics were immersed in (50ml) media (DMEM+10% FCS), leaving a headspace of about 5ml, for aeration. The ceramic discs were constantly rotating through the media, at a rate of 2 rpm (rotations per minute). The media was circulated through the system at rates of 2, 5, 10 and 20 ml/min, in different experiments. Since the lateral rotation of the Z®RP system does not resemble the physiological flow of blood, we attempted to introduce unidirectional flow of media through the ceramics using the tube reactor system. In this system, a maximum of 2 ceramic discs were stacked within the tube, spaced with hollow rubber tubing. They were completely immersed in medium (25ml), with a headspace of about 5ml being left in the reservoir (see fig. 3.1B), to ensure aeration. As before, media was circulated through the system at rates of 2, 5, 10 and 20 ml/min, in different experiments. The pH and pO_2, however, could not be monitored real-time, and was manually measured 4 times a day.

Both systems were maintained in a Z®RP clean bench, at a constant temperature of 37°C. The cells were maintained in these systems for 1 week, with half the media volume being replaced every 3 days. After one week of culture, the effect of 3D perfusion culture on MSCs was analyzed by Hoechst staining, TUNEL-KI67. RNA was also isolated for further molecular biology studies. A corresponding control, using MSCs cultured for the same amount of time in static ceramics was also analyzed simultaneously.

3.2.3.3 Co-culture of HSPCs and MSCs

7 days after seeding of the MSCs, freshly isolated HSPCs were introduced into 4 culture conditions: 1. 3D co-culture with MSCs seeded in the static ceramic, 2. 2D co-culture with MSC monolayer seeded in 6 well plates, 3. 2D co-culture with osteo-induced MSC monolayer seeded in 6 well plates, and 4. Suspension culture in Stemspan media supplemented with 100ng/ml of IL-6, SCF, TPO and FLT-3L.

9 independent MSC and HSC samples were utilized for this study. The cells from each culture system were analyzed by flow cytometry and immunohistochemistry 1,2 and 4 weeks after start of culture. The 3D culture was analyzed at an additional time point of 8 weeks, to confirm long-term culture potential.

3.2.4 Gene expression analysis

Basic gene expression analysis of 3D (MSCs in static ceramic) and monolayer (MSCs, osteo-induced MSCs) culture cells and a comparison thereof was carried out on the mRNA level by semi-quantitative real time PCR.

3.2.4.1 RNA isolation and cDNA preparation

Isolation of RNA was performed using the NucleoSpin® RNA II kit (Macherey-Nagel, Germany) following the manufacturer's instructions. For monolayer cultures 350 µl RA1 buffer containing 1:1000 beta-mercaptoethanol were directly added onto the PBS-washed cell layer and detached using a cell scraper (Sarstedt, USA). Ceramic-cultures were washed with PBS and lysed directly on the ceramic with reconstituted RA1 buffer as above. For the elution step, 20 or 30 µl RNAse-free H20 were used. Isolated RNA was stored at -80 °C.

Reverse transcription of purified RNA was carried out using the TaqMan® Reverse Transcription Reagents cDNA kit (Applied Biosystems, USA) as follows:

200-400 ng RNA
2 µl TaqMan 10x buffer
0,5 µl oligo dTs
0,5 µl Random Hexamer
4,4 µl $MgCl_2$ (25 mM)
0,3 µl Reverse Transcriptase
0,4 µl RNase Inhibitor
4 µl dNTPs (25mM each)
in 20 µl H_20 (DMSO free).

Transcription was carried out in a thermo cycler (Peqlab, Germany) with an annealing step for 10 minutes at 25°C, an elongation step for 40 min at 48°C and an

inactivation step for 5 min at 95°C to inactivate the reverse transcriptase. Samples were stored at -20°C until further use.

3.2.4.2 Real time PCR analysis

Real time PCR was performed using 1 µl cDNA with 1µl primer mix and SensiFAST™ Sybr No-ROX kit (Bioline, Germany) in a volume of 20 µl. 96-well PCR plates (Biozym Scientific, Germany) were read with Stratagene MX 3005P™ Multiplex Quantitative PCR System (Agilent Technologies, USA).

First, samples were heated to 95°C for four minutes to achieve complete dissociation of the RNA-DNA double strain. Next, 45 cycles of DNA melting (95°C, 12 seconds), primer annealing (64°C, 15 seconds) and elongation (72°C, 12 seconds) of cDNA templates were performed. A dissociation curve was recorded by cooling the samples to 62°C and stepwise heating up to 95°C. The fluorescence intensity of SybrGreen was measured with every 0.5°C increment.

Melting curve analysis was performed to verify the amplification of the specific product without contamination by unspecific side products or primer dimers. The expression was normalized to housekeeping gene expression (GAPDH) and presented as housekeeping gene ratios in logarithmic charts. This data representation allows the possibility of comparing the expression strength of several genes. Expression rations were calculated according to the following formula:

Ratio: $E^{cp}_{HK} / E^{cp}_{gene}$

[cp: crossing point; E: amplification efficiency; gene, gene of interest; HK, house keeping gene]

As the deviation of the determined amplification efficiencies for particular target genes appeared to be smaller than 4%, the value was set to 1,95 for all calculations.

3.2.5 Flow cytometry and cell sorting

Magnetic-activated cell sorting (MACS) was used in the isolation of CD34+ cells for the co-culture experiments. Subsequently, the MSC phenotype and phenotype of the

HSPCs from all the culture systems was compared on the basis of surface marker expression, by flow cytometric analysis.

3.2.5.1 Magnetic-activated cell sorting (MACS)

The Miltenyi MACS (Magnetic-activated cell sorting) system was used for selection of CD34-expressing hematopoietic stem and progenitor cells from the mononucleate cells (MNCs) obtained from cord-blood. This system uses antibodies against surface markers (here, CD34) coupled to magnetizable micro-particles. Cells to be separated are incubated with the specific antibody, and the cell-antibody suspension is then applied to a column maintained in a magnetic field, which holds back all cells that were bound by the antibody. Upon removing the column from the magnetic field, the retained cells can be eluted.

In this work, cord-blood MNCs suspended at an approximate density of 10^8 cells/ml were incubated with 200µl of the CD34-MACS Micro beads, in a total volume of 800µl PBE, at 4°C for 30min. The cells were then washed with PBS and separated using MS Columns attached to the Midi MACS separator (Miltenyi, Germany) as described in the manufacturer's instructions. The positive fraction was then stained with CD34-APC antibodies (Miltenyi, Germany) and analyzed by flow cytometry, using the MACSQuant Analyzer (Miltenyi Biotec, Germany).

3.2.5.2 Flow cytometric analysis of surface markers

MSC surface marker expression as well as surface marker expression of the HSPCs from the co-culture was determined using flow cytometry.

In case of the MSCs, monolayer cells were harvested by trypsinization and centrifugation and the cell pellet was re-suspended in PBS/BSA (0.5%). Cells were incubated with fluorescent-labeled antibodies (for CD90, CD105, CD 106, CD73, CD44, CD13, CD34 and CD 45; see table 3.3) for 15 min at RT. Appropriate isotype controls were used. Staining assays were then washed with PBS/BSA and centrifuged to remove unbound antibodies. The cell pellets were suspended in 200µl PBE and analyzed on a MACSQuant® Analyzer (Miltenyi, Germany) flow cytometer. The data was analyzed using FlowJo software, version 7.6.5 (Tree Star Inc., USA).

When analyzing the surface marker expression of the cells from the various culture systems, cells were collected from all the adherent culture systems by incubating with 1x Trypsin-EDTA for 30 minutes, at 37°C in an incubator. The suspension cells were simply pipetted into collection tubes. The cells were then collected by centrifugation and washed with PBS. They were then stained with the following antibodies (see table 3.3), as described earlier: CD105-biotin followed by streptavidin-PE-Cy7, CD34- APC, CD 38- PE, Annexin V- Pacific Blue, Propidium Iodide. Flow cytometry analysis was carried out using a MACSQuant® Analyzer (Miltenyi, Germany) flow cytometer and the data was analyzed using FlowJo software, version 7.6.5 (Tree Star Inc., USA).

The distinction between HSPCs and MSCs was made by the analysis of CD105 expression. MSCs express CD105 while HSPCs do not. The HSPCs from the co-culture system were, therefore, selected from the FACS plots as the cells negative for CD105. Furthermore, these cells were counted using the flow cytometer, from samples of equal volume (from each culture condition). The count was then extrapolated to the entire volume of cell suspension obtained, for further quantification.

3.2.6 Immunohistochemistry and staining

Several aspects in this work were evaluated by immunofluorescence staining, fluorescence based cell tracking as well as other staining protocols, as described below.

3.2.6.1 Staining for detection of differentiation

Alcian blue staining: Alcian blue binds to negatively charged molecules and stains both sulfated and carboxylated sialomucins (glycoproteins). Thus, this dye is used to demonstrate the synthesis and secretion of proteoglycans during chondrogenic differentiation. Slides (with sections of chondogenic cell mass, described earlier) were thawed and preincubated in 3% acidic acid for 3 min before treatment with Alcian Blue (3% in 3% acetic acid) solution for 30 min at room temperature. The sections were then washed with water and dehydrated by an ascending ethanol row with terminal Xylene treatment, covered with Canada balsam and visualized by light

microscopy.

Oil-Red-O-staining: The Oil red O stain can identify neutral lipids and fatty acids within lipid filled vesicles of adipocytes. Staining with oil-soluble dyes is based on the greater solubility of the dye in the lipoid substances than in the usual hydro-alcoholic dye solvents. After 28 days of adipogenic induction, cells were fixed in 4% paraformaldehyde for 10 min, washed with PBS and subsequently incubated for 1hour with freshly filtrated Oil Red O staining solution (0,7 % in propylen glycerol. After rinsing the cells two times with aqua dest, the staining was evaluated by light microscopy.

Von Kossa staining: The principle of this staining method is the reduction od mineral (calcium) phosphates by silver nitrate, in the presence of strong light (UV-light) and replaced with silver deposits, visualized as metallic silver. Thus, von Kossa method is suitable to stain calcified matrix deposition characteristic of osteogenic differentiation. After 21 days of osteogenic differentiation, the monolayer cells were fixed with 4% paraformaldehyde. Silver nitrate solution (5%) was poured on the fixed cells and the culture plate was placed in UV light for 20 min. Finally, the cells were rinsed with water and evaluation was done by light microscopy.

Alizarin Red staining: The Alizarin Red stain is used to visualize the formation of calcified extracellular matrix associated with early osteogenesis. After 7 days of culture in the ceramic scaffolds with and without osteogenic media, cells were fixed on the ceramic, using 4% paraformaldehyde for 10 min, washed well with distilled water and subsequently incubated for 2 minutes with a freshly prepared solution of Alizarin Red S staining solution (2 % in distilled water, pH 4.1- 4.3; adjusted with 0.5% ammonium hydroxide). After washing off the excess dye and rinsing the cells two times with distilled water, the staining was evaluated by light microscopy.

3.2.6.2 Cell tracking

For long term tracking of HSPCs and MSCs by fluorescence microscopy, the cells were labeled using Qtracker® 525 Cell Labeling Kit and CellTracker™ Red CMTPX (Invitrogen, USA), respectively, as per manufacturers' instructions, prior to culturing.

To track cell division, the HSPCs were labeled with Carboxyfluorescein diacetate succinimidyl ester, (CFSE), at a concentration of 2.5μM, as per manufacturers' instructions, immediately after isolation. Cell division was tracked after 1, 2 and 4 weeks of culture, using flow cytometry.

3.2.6.3 TUNEL staining

DNA fragmentation localized in the nuclei and apoptotic bodies of apoptotic cells was detected using Fluorescein TUNEL assay. This assay detects the DNA breaks by enzymatic labeling the free 3'OH termini with modified nucleotides. The nucleotides contained in the reaction buffer are enzymatically added to the DNA by terminal deoxynucleotidyl transferase (TdT). TdT catalyzes a template independent addition of nucleotide triphosphate to the 3'OH ends of double or single stranded DNA. The incorporated nucleotides form an oligomer composed of digoxigenin nucleotide and unlabeled nucleotide in a random sequence. Signal of DNA fragments then labeled with the nucleotides is developed through binding of an antii-digoxigenin antibody conjugated with fluorescein. Ceramics for staining with Tunel kit were fixed with 10% formalin at room temperature during 10 min, washed 3 times for 5 min with PBS and fixed again with ETOH-acetic acid at -20°C for 10 min, washing steps were repeated and incubation with TdT enzyme for 30 min at 37°C was performed. Reaction was stopped with stop buffer solution and washed. Samples were co-stained at 4 °C with the proliferation marker Ki67 was allowed after blocking step. The following day, signal development for TdT enzyme reaction with Anti-Dig antibody and for Ki67 with Alexa 594-Anti rabbit antibody was done like all other immunofluorescence protocols.

3.2.6.4 Immunofluorescence staining

In order to visualize the ECM and signaling molecules within the ceramic scaffolds, the ceramics with cells were fixed in Acetone (Sigma, USA) at -20°C, 1 and 2 weeks after seeding the tracked HSPCs. The discs were then cut using a scalpel and stained in 96 well plates, in a total volume of 150μl as follows:

- 3x wash with PBS for 5 min
- Block with serum for 10 min at RT
- Incubate with primary antibodies (table 3.4) overnight at 4°C.

- 3x wash with PBS for 5 min
- Incubate with secondary antibody for 1 hour at RT
- Counterstain nuclei with Hoechst 3342

The samples were then visualized by fluorescence or 2-photon microscopy.

3.2.7 Microscopy

Fluorescence microscopy and two-photon microscopy were extensively used in this work, for tracking cells and analyzing the culture systems. Electron microscopy was also used extensively, for making comparisons with native bone marrow.

3.2.7.1 Fluorescence and 2-photon microscopy

The presence of the Qtracker ® green tracked HSPCs in the co-culture system after 1,2, 4 and 8 weeks was confirmed by visualizing them under a digital fluorescence microscope (BZ 9000, Keyence, Germany), after counterstaining the nuclei with DAPI (Sigma, US).

The ECM and signaling molecules were visualized as 3D stacks using a 2 photon microscope (Trimscope II, LaVision BioTec, Germany), and rendered using Imaris version 7.5 (Bitplane Scientific Software, Switzerland).

3.2.7.2 Scanning electron microscopy (SEM)

SEM was used to visualize the structural similarity between the cell-seeded ceramic culture system and bone marrow, and also the physical interaction on HSPCs and MSCs. Ceramic discs with MSCs and HSPCs, after 2 weeks of co-culture, and 1cm^2 pieces of bone marrow excised from femoral heads were fixed and dehydrated using acetone and prepared by critical point drying, as described in literature (Pearce, 2003). These samples were then coated with gold and silver, and visualized using a Hitachi S-520 SEM (Hitachi, Japan).

3.2.8 Statistical Analysis

Statistical analyses were required to accurately evaluate data from the FACS analysis and the qPCR experiments. 2-way ANOVA analysis, followed by Bonferroni corrections were applied to the data sets, using *GraphPad Prism*® software version 5.0 (GraphPad Software Inc., USA). P values greater than or equal to 0.05 were considered significant. Data is represented as means +/- standard deviation.

4. Results

The establishment of our novel 3D co-culture system required systematic characterization of each cellular component, monitoring of their behavior in the 3D environment, and finally, confirmation that their interactions conform to the *in vivo* 'niche' phenotype, and that they remain functional.

4.1 Characterization of MSCs

Mesenchymal stem/stromal cells (MSCs) isolated from bone marrow constitute an essential component of the 3D co-culture system. It was, therefore, necessary to confirm their phenotype, before they were seeded into the ceramic.

4.1.1 Expression of surface molecules

MSCs are traditionally characterized, not by a single surface marker, but by the expression of a combination or panel of markers. Flow cytometric analysis showed that the MSCs used throughout this study expressed the established MSC marker molecules- CD73 (SH3), CD13, CD90 (Thy1), CD44, CD105 (SH2) and CD106 (V-cam). They were also negative for the hematopoietic markers CD34 and CD45.

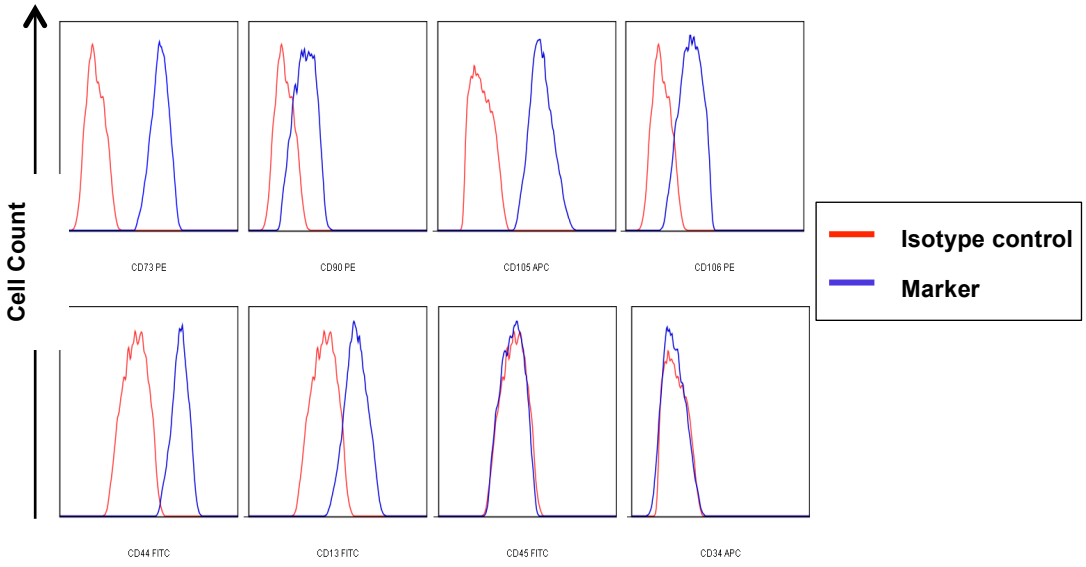

Figure 4.1. Expression of standard MSC marker panel MSCs express CD73, CD90, CD105, CD106, CD44 and CD13 but not CD45 and CD34 (n=8).

Figure 4.1 depicts representative surface marker staining of 8 independent cultures of MSCs, between passage numbers 3 and 7, used in the co-culture systems with the HSCs. Each staining was compared to an appropriate isotype control (CD 38-PE, CD38-APC and CD31-FITC). The MSCs used for the subsequent experiments showed surface marker expression consistent with published data.

4.1.2 Multi-lineage differentiation potential

The second criterion by which MSCs are characterized is their ability to differentiate into the osteogenic, adipogenic and chondrogenic lineages. The MSCs used in this study were therefore induced to undergo differentiation into these three cell types.

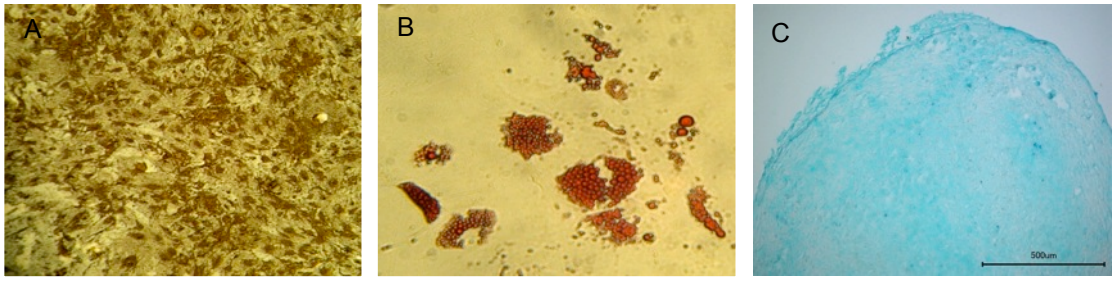

Figure 4.2. Multi-lineage differentiation of MSC (A) Von Kossa staining indicating osteogenic differentiation **(B) Oil Red staining** indicating adipogenic differentiation **(C) Alcian blue staining** indicating chondrogenesis**.**

The 8 independent MSCs cultures tested were found to undergo multi-lineage differentiation when appropriately induced. Figure 4.2 depicts representative cultures of differentiated MSCs, exhibiting osteogenic, adipogenic and chondrogenic differentiation, as visualized by Von Kossa, Oil-red and Alcian blue staining respectively. Von Kossa staining reveals mineralization, particularly mineral phosphates formed during osteogenic differentiation. Oil red stains lipids and Alcian blue stains cartilage ECM molecules.

Thus, the MSCs used in this study were shown to express the appropriate surface markers and undergo differentiation as expected. The phenotype of the MSCs used for the subsequent experiments was therefore established to correspond to that described in literature.

4.2 Behavior of MSCs in 3D ceramic culture

It has been extensively documented that 3D culture conditions influence the differentiation and ECM production in bone marrow MSCs. Rigid scaffolds, such as the ceramics used in this study, have been shown to predispose MSCs to osteogenic differentiation. The effect of 3D culture in the ceramic scaffolds on the MSCs was, therefore determined prior to introduction of HSPCs, by testing the spontaneous osteogenic differentiation, physical appearance and expression of ECM components.

4.2.1 Spontaneous osteogenic differentiation

Osteogenic differentiation was compared in 7-day cultures of (a) MSCs seeded in the ceramic, cultured in osteogenic media and (b) MSCs seeded in the ceramics, cultured in DMEM with 10% FCS. Alizarin Red staining (fig. 4.3Ai), Von Kossa staining (fig. 4.3Aiii) and Q-PCR analysis of the early osteogenic marker, Osteopontin (fig. 4.3B), indicated that MSCs seeded in the ceramic do undergo spontaneous osteogenic differentiation in the ceramic. The late osteogenic marker-Osteocalcin was not expressed.

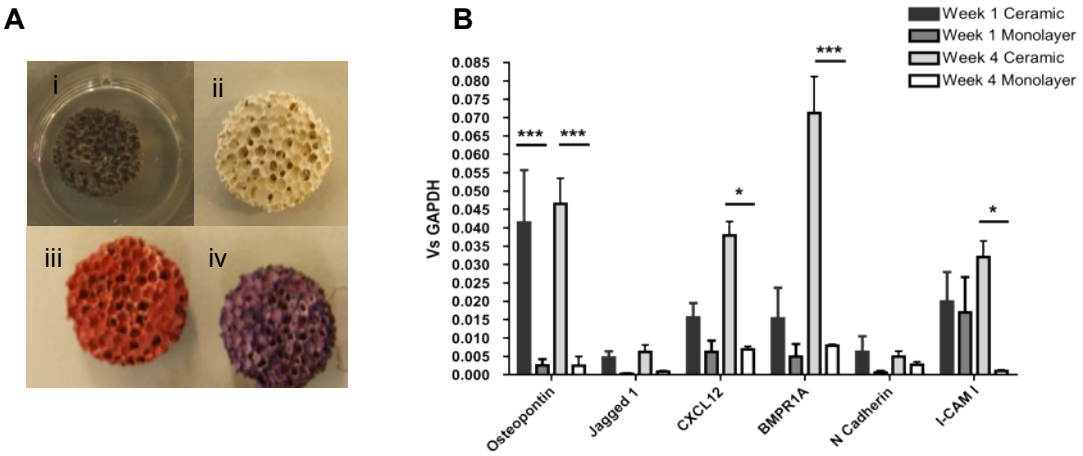

Figure 4.3. (A) Spontaneous osteogenic differentiation of MSCs after 1 week of 3D culture, visualized by positive Von Kossa (i) staining and Alizarin red (iii) staining, compared to unseeded ceramics (ii, iv). **(B) Real time PCR analysis of known niche molecules in the co-culture system, 1 and 4 weeks after seeding, compared to monolayer.** (n=3, Error bars: SD of mean, ***$p < 0.001$, *$p < 0.05$).

Figures 4.3A shows the staining of mineralized matrix in MSC-seeded and non-seeded ceramics, indicating that the MSCs are predisposed towards osteogenic differentiation, even without induction. Figure 4.3B shows that osteopontin is upregulated in the un-induced 3D culture, when compared to monolayer cultures. It has been previously shown that there is spontaneous osteogenic differentiation of MSCs in un-induced ceramic, but not as much as in osteo-induced 3D culture (Griensven *et al*., 2009), suggesting incomplete osteogenic differentiation in the un-induced ceramic.

4.2.2 Expression of niche markers

In addition to osteopontin, the expression of selected molecules implicated in bone marrow endosteal niche function was also determined, by qPCR. These are also represented in figure 4.3B

As represented in fig. 4.3B, the qPCR analysis of 3 independent samples of MSCs seeded in the ceramic and in monolayer and maintained for 7 and 28 days respectively indicate the differential expression of the putative niche maintenance molecules jagged-1, CXCL12, BMP receptor 1A(BMPR1A), N-cadherin and ICAM1.

Jagged-1 and N-cadherin were found to be marginally upregulated in the ceramic cultures as compared to the monolayer. The level of expression did not however vary with duration of culture.

CXCL12 and BMPR1A expression was significantly higher in the ceramic cultures than in the monolayer at both time points, and was found to increase with time in culture in the 3D culture system. The expression level in the monolayer however, remained low and did not vary with time.

ICAM1 was found to be expressed at similar levels in the 3D and monolayer cultures, 7 days after seeding. After 28 days however, the expression of ICAM1 by the MSCs was significantly reduced in the monolayer, while it was sustained in the ceramic culture.

4.2.3 Network formation

The MSC-seeded ceramics were visualized by light microscopy, 1 week after seeding. The MSCs were found to form filament-like structures, spanning the pores of the ceramic, as depicted in figure 4.4A.

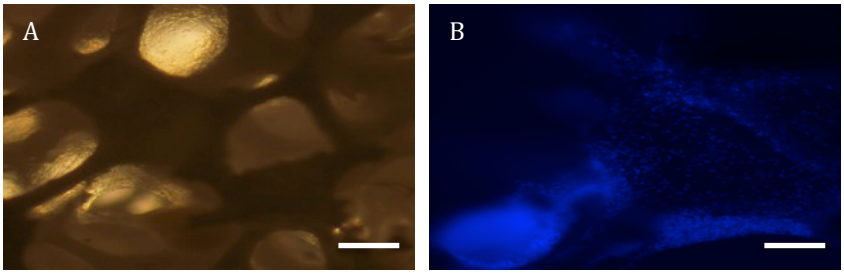

Figure 4.4. Formation of highly cellularized networks by MSCs in the ceramic as illustrated by (A) Light microscopy, (B) Hoechst 33342 staining 1 week after seeding. (Scale bars: 500 µm)

In order to determine the composition of these 'networks', we first determined the location of the MSCs within the ceramic by nuclear staining using Hoechst 33342 (fig. 4.4B). This showed that the networks were highly cellularized and contained densely packed MSCs. Having determined that the MSCs not only coat the surface of the ceramic but form microstructures which span the pores of the ceramic, we then further examined the composition of these structures in terms of ECM and cell adhesion molecules by immunohistochemistry.

4.2.4 ECM production

The presence of extracellular matrix proteins within the bone marrow stroma is well documented. Collagen I and fibronectin, in particular, are known to be highly expressed in the bone marrow and to have a role in HSPC niche maintenance. The expression of these molecules in the 3D culture system is therefore highly likely and would prove conducive to the effective engraftment of the HSPCs. Immunofluorescence staining followed by 2 photon microscopy (fig. 4.5), was used to determine whether the MSCs produced ECM components after 1 day and 1 week of culture in the ceramic scaffolds.

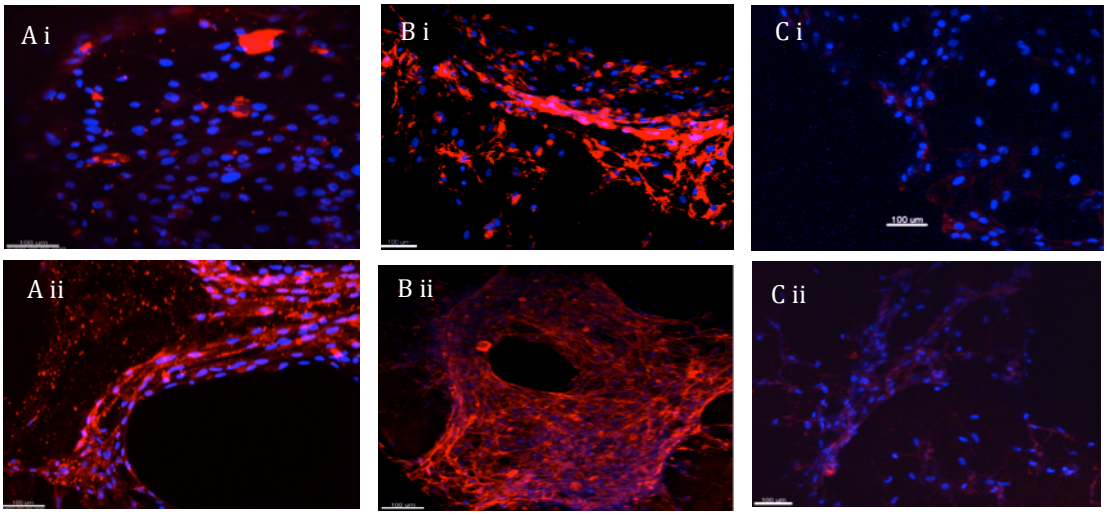

Figure 4.5. Production of ECM molecules by MSCs. (A) Collagen I, (B) Fibronectin, (C) Integrin4a (i) Day 1 and (ii) Day 7 after seeding**.** Nuclei are counterstained with Hoechst 33342. (Scale bars: 100µm)

Figure 4.5 shows 2-photon microscopy pictures of MSCs in the ceramic, 1 day (upper panel) and 7 days (lower panel) after seeding. The MSCs were found to produce web like networks composed of Collagen I (fig. 4.5A) and Fibronectin (fig. 4.5B), after 7 days of seeding. The MSCs are seen dispersed in these structures. Integrin 4a, which is important for cell contact, is also produced (fig 4.5C).

Collagen I and Fibronectin are known to be involved in maintenance of the HSC niche in the bone marrow, and are thought to mediate the securement of the HSPCs to the bone marrow. Integrin 4a is known to play a role in MSC-HSC interaction within the niche.

Having demonstrated the compositional similarity of the MSC-seeded ceramic to bone marrow, we then investigated the physical similarity of this system to human bone marrow. In order to elucidate the close resemblance of the 3D culture system to human bone marrow, scanning electron microscopy (fig. 4.6) was used to compare the structure of the MSC-seeded ceramic to that of human bone marrow matrix. Figure 4.6 clearly shows the structural similarity between the MSC-seeded ceramic (left panels) and bone spongiosa (right panels). Both showed the presence of filamentous networks spanning a porous structure, and the presence of bead-like proteins.

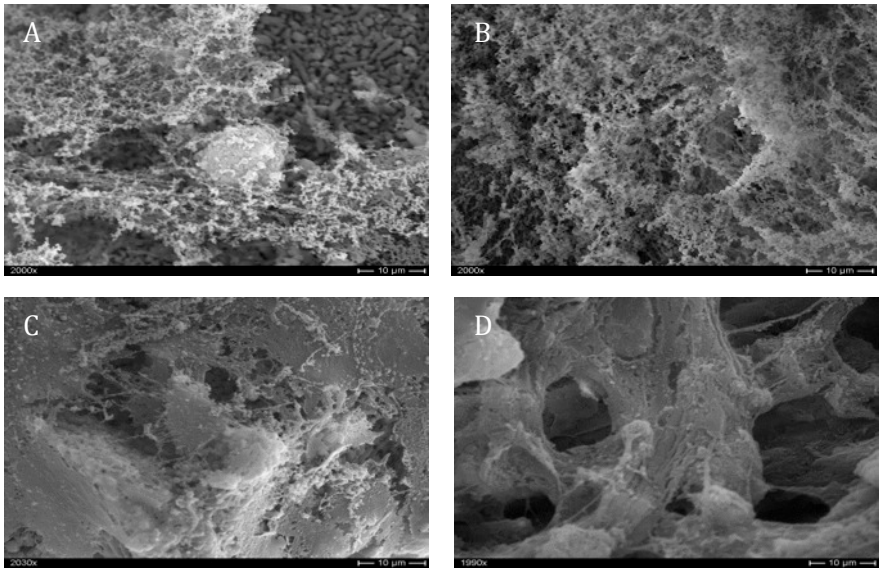

Figure 4.6. Structural similarity between (A, C) MSC-seeded ceramic and (B, D) bone marrow, visualized by SEM. Networks of matrix and cells are seen. The bead -like structures are ECM proteins. (Scale bars: 10μm)

These results indicate that the MSCs spontaneously produce a microenvironment, when cultured in the ceramic scaffold, which is conducive to HSC maintenance. 7 days was, therefore, determined to be a suitable time point to introduce hematopoietic stem and progenitor cells into the system. We then tested the effect of perfusion on the MSC-seeded ceramic.

4.3 Perfusion culture of MSCs in ceramic

In order to mimic vascularization, we attempted to introduce circulation of media within the MSC-seeded ceramics, first in a rotating-bed bioreactor system (Z®RP system- Zellwek Gmbh, Germany) and later in a vertical flow column, as described earlier. The MSC seeded ceramics were introduced into the perfusion system 7 days after seeding and removed and analyzed after 7 days, by bright field microscopy, nuclear staining and total RNA isolation.

In the Zellwerk reactor, a large proportion of MSCs were found to detach within 1 week. No proliferation was observed, and the network-like structures observed in the static ceramic were completely disrupted (fig. 4.7)

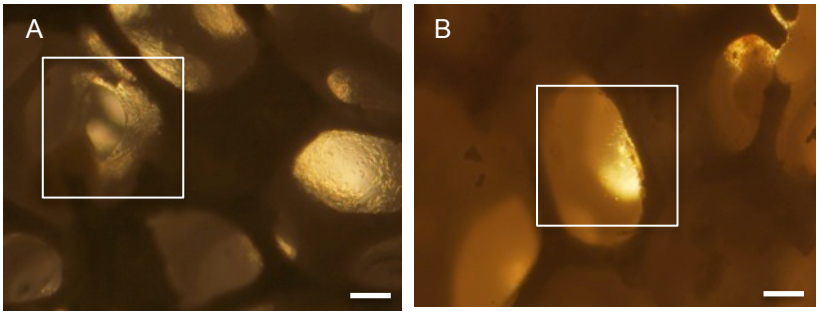

Figure 4.7. MSC culture in (A) static ceramic and (B) rotating-bed perfusion(Zellwerk) reactor, visualized by bright-field microscopy. Network-like structures are seen spanning the pores of the static but not perfused ceramic (n= 12; Scale bars: 100µm).

As depicted in figure 4.7, bright-field microscopy revealed that rotating-bed perfusion of the ceramic was severely disrupted MSC proliferation and organization. In addition to this, the system was found to be highly susceptible to bacterial and fungal contamination. Over 80% of the samples were discarded due to contamination. The cells in the surviving cultures yielded quantities of RNA, which were insufficient for further analysis.

In order to determine whether perfusion or the rotation induced shear stress was the cause for MSC disruption, we attempted to perfuse the system by a steady vertical flow, in a narrow cylindrical column. MSC seeded ceramics were introduced into these columns 7 days after seeding and perfused for 7 days. The cell number and viability was determined by nuclear staining, TUNEL staining and fluorescence microscopy (fig. 4.7).

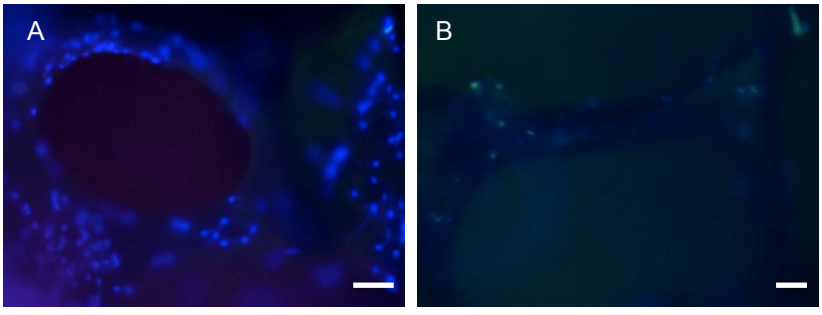

Figure 4.8. Apoptosis in MSC culture in (A) static ceramic and (B) vertical flow perfusion (tube) reactor, visualized by fluorescence microscopy. Apoptotic cells are stained green (TUNEL) and nuclei are counterstained (blue) with Hoechst 33342. (n= 6; Scale bars: 100µm).

The vertical flow perfusion system was superior to the Zellwerk system in terms of sterility- no contamination was observed. However, as depicted in figure 4.8, the number of MSCs was much higher in the static ceramics than in the perfused ceramics. TUNEL staining (fig. 4.8) followed by fluorescence microscopy of 6 independent MSC samples revealed that the cells in the perfused ceramic were mostly apoptotic.

Thus, the two perfusion culture systems were judged to be unsuitable for the cultivation of MSCs, particularly in the context of the generation of a marrow-like environment. We concluded that the static MSC-seeded ceramic was, therefore, the most suited system for HSPC co-culture.

4.4 Characterization of HSPCs

The hematopoietic stem and progenitor cells (HSPCs) are the main component of our co-culture system. It was therefore imperative to ensure that the cord blood derived HSPC population, initially seeded into the MSC-pre seeded ceramics, was as pure as possible.

4.4.1 Purity of seeding culture (MACS check)

Since human HSPCs are widely characterized by expression of CD34, the HSPCs used for subsequent experiments were isolated from umbilical cord mono-nucleate cells by magnetic-activated cell sorting (MACS) for CD34 expressing cells.

In order to confirm the ‘purity’ of the seeding culture, the efficiency of the sorting was confirmed by FACS analysis of CD34+ cells in the sorted cell fraction.

Flow cytometric analysis revealed that the sorted cells do indeed express CD34, confirming that the MACS sort was efficient and specific, and that little or no unspecific binding occurs during the sort.

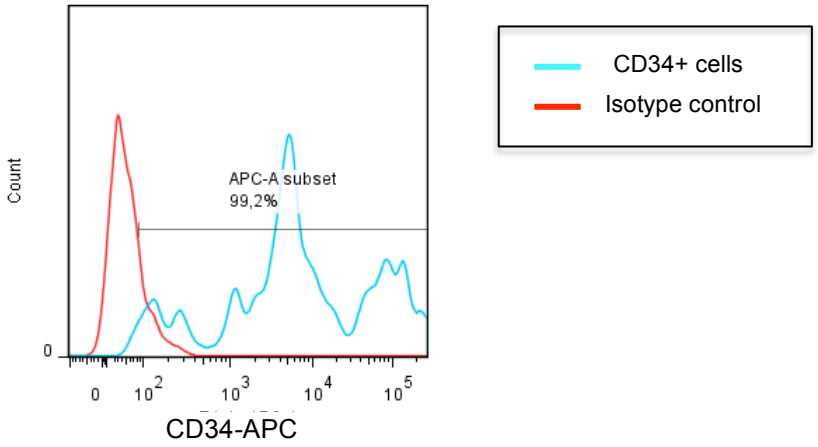

Figure 4.9. FACS based verification of HSPCs (CD34+) purity before seeding in 3D co-culture with MSCs. Over 99% of the cells selected for CD34 expression were found to be CD34 positive. (isotype control: CD106-APC; n=8)

Figure 4.9 is a representative flow cytometry histogram of 8 independent cord blood derived HSCs samples. It illustrates that the cell populations seeded in the MSC pre-seeded ceramics are over 99% CD34 positive, and can be considered pure HSPC populations thereby. The heterogeneity in CD34 expression is also evident in figure 4.9; this is due to the fact that all non-committed human hematopoietic stem and progenitor cells express CD34 (fig. 1.4), and the traditionally defined HSPC fraction does, in fact, consist of a mixed population of hematopoietic stem and progenitor cells.

In subsequent experiments, this heterogeneity was taken into account, and the most primitive HSPCs were defined based on CD34 expression and lack of CD38 expression (CD34+ CD38-).

4.4.2 Characterization as primitive HSPCs

Since CD34+ HSPCs are a mixed population, the seeding population was further characterized based on expression of CD38. This was determined by flow cytometric analysis of CD38 expression, in combination with CD34 expression. The samples were compared to unstained controls (fig.4.10)

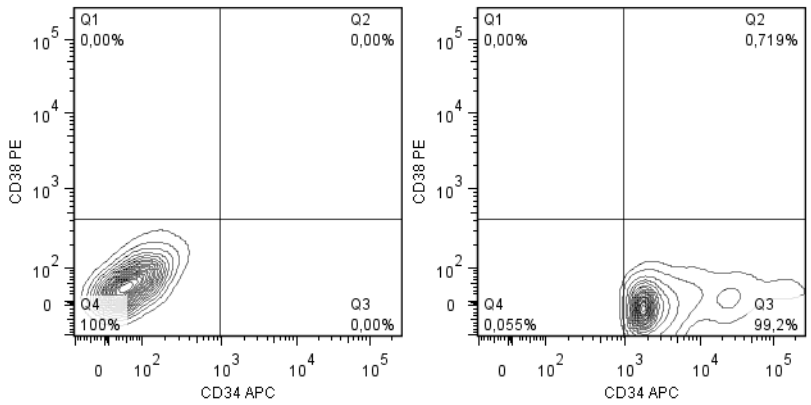

Figure 4.10. FACS based characterization of HSPC phenotype before seeding in 3D co-culture with MSCs. Over 99% of the cells selected for CD34 expression were found to be CD38 negative (right panel) when compared to the unstained control (left panel; n=8).

Figure 4.10 shows representative FACS plots of the 8 independent HSPCs samples prior to seeding in the co-culture systems. FACS analysis for the co-expression of CD34 and CD38 in these cells revealed that 99% of the HSPCs seeded in the ceramic were CD34+CD38-, which is established as the phenotype of the primitive, long term repopulating HSPCs in humans.

Thus, we confirmed that we had indeed selected the primitive, long term repopulating HSPCs from the cord blood for the subsequent experiments.

4.5 HSPC-MSC Co-culture

In the bone marrow, HSPCs are retained within their niche throughout the life of the individual. This retention is brought about, to a large extent, by their interaction with bone marrow stromal cells- MSCs and their differentiated progeny. We sought to achieve a similar effect, by mimicking the structural, cellular and extracellular environment of the bone marrow with our ceramic co-culture system. To this end, we monitored HSPC survival, phenotype maintenance, viability and functionality within our system. HSPC behavior in the 3D co-culture system was compared to traditional HSPC culture methods, such as suspension culture in defined serum free media, and co-culture with MSC monolayers- in osteo-inductive media, as well as regular cell culture media.

4.5.1 HSPC survival

The first consideration for validating the 3D co-culture system was whether the HSPCs seeded into the ceramic entered into it, and remained there for long periods of time. Cell tracker labeled HSPCs were used to determine whether the seeded HSPCs were retained in the co-culture system for up to 8 weeks, with biweekly media change.

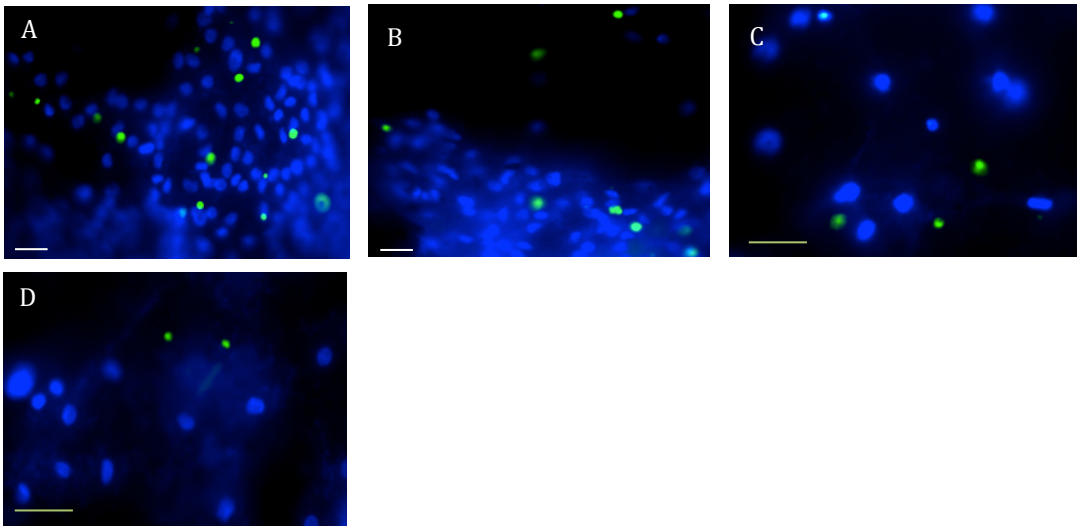

Figure 4.11. Retention of HSPCs in the ceramic. (A) Day 7 after seeding **(B)** Day 14 after seeding **(C)** Day 28 after seeding **(D)** Day 56 after seeding. HSPCs are labelled with cell tracker green and nuclei of MSCs are counterstained with Hoechst 33342 (nuclear staining is not detected in the HSPCs due to small size of the cells and cytoplasim concentration of cell tracker). (Scale bars: 100μm)

Figure 4.11 shows the presence of green-tracked HSPCs in the ceramic pores, in close proximity to the adherent MSCs, which were only stained with Hoechst 33342. Nuclear staining is not detected in the HSPCs due to their small size and the high cytoplasmic concentration of the cell tracker. Using fluorescence microscopy, we were able to detect green cells in the co-culture system at day 7, day 14, day 28 and day 56 (8 weeks) after seeding, indicating that the HSPCs are maintained stably within the ceramic for up to 8 weeks.

Having established the presence of green-tracked cells in the ceramic at these time points, we then proceeded to confirm the HSPC phenotype of these cells.

4.5.2 Separation of MSCs and HSPCs after co-culture

Separating the MSCs and the HSPCs after co-culture is imperative for the subsequent characterization of the HSPCs, and confirmation of their phenotype. FACS based separation by size and surface properties (FSC-SSC) proved ineffective due to the wide variation of these parameters in MSCs. Cell tracking of HSPCs with fluorescent probes also failed because of the high auto-fluorescence of MSCs. Finally, we were able to gate out the MSCs by staining for the surface marker CD105, which is highly expressed in MSCs, but weakly or not expressed in HSPCs.

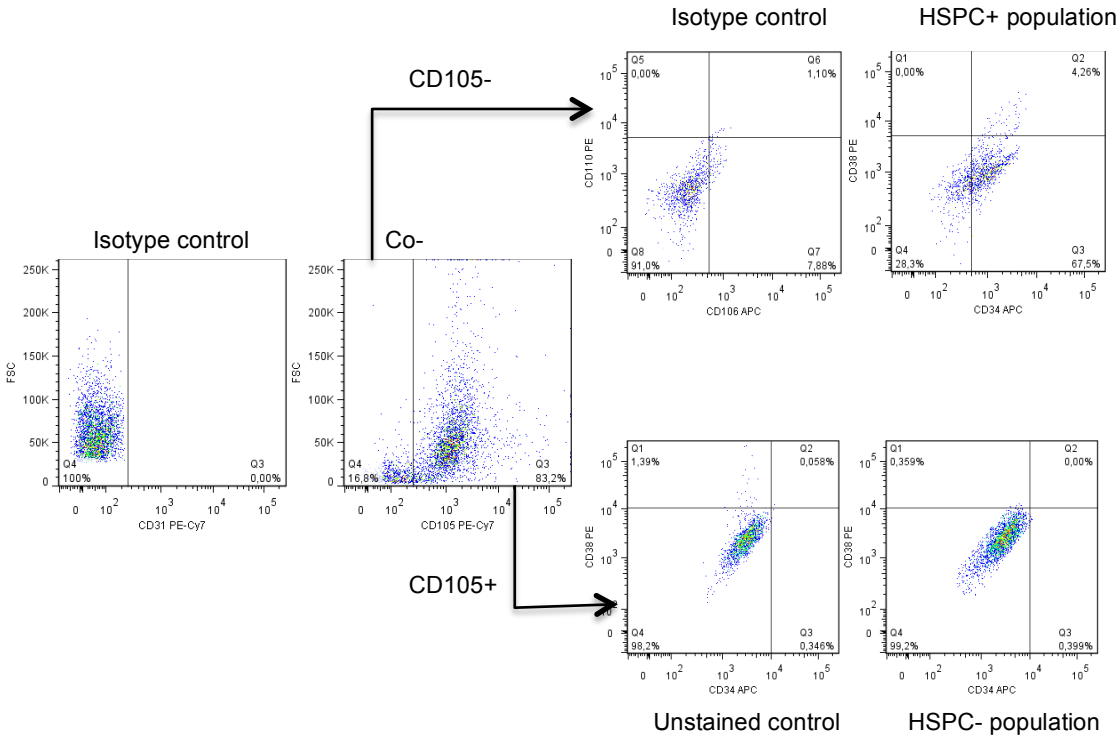

Figure 4.12. FACS based gating of MSCs (CD105+) cells from 3D co-culture with HSPCs. The CD105 positive and negative fractions were stained for CD34 and CD38, to confirm cell phenotype. All samples are gated against unstained controls.

As shown in figure 4.12, the MSCs could easily be gated out based on high CD105 expression. The CD105+ fraction was confirmed to be CD34- CD38- (non-hematopoietic), while the CD105- fraction contained all the CD34+ CD38-, CD34-CD38+ and CD34+CD38+ cells, all of which are considered to be of hematopoietic origin.

4.5.3 Engraftment efficiency of HSPCs

The efficiency of engraftment of the HSPCs in the ceramic and monolayer co-culture systems was investigated by determining the number of CD105- cells obtained from each system at 1,2,4 and 8 weeks after seeding of HSPCs.

Co-culture condition	Cell count ± SD Week 1	Cell count ± SD Week 2	Cell count ± SD Week 4	Cell count ± SD Week 8
Ceramic	2093 ± 433	2150 ± 432	2508 ± 563	2462 ± 567
Monolayer	1670 ± 209	1899 ± 447	1986 ± 452	NM
Osteo-induced Monolayer	1489 ± 181	1686 ± 212	1640 ± 248	NM

Table 4.1. Cell numbers of HSPCs (CD105- cells) obtained from the 3 co-culture systems at different time points. Initial seeding count: 2x 10^4 cells/culture (n=8; SD- Standard deviation from mean; NM- Not measured).

As represented in table 4.1, the mean cell count of the HSPCs obtained from the different co-culture systems, in 8 independent experiments was much less than the initial seeding density of 2 x 10^4 cells. The cell count obtained after one week of culture was in fact reduced to about 10% (about 2 x 10^3 cells) in the ceramic and monolayer co-culture about 7.5% (about 1.5 x 10^3 cells) in the monolayer co-culture with osteo-induced MSCs.

The cell count did not, however, significantly differ at the different time points of measurement, as also indicated in table 4.1. This suggests that although the initial engraftment and adaptation of the HSPCs to the different co-culture systems results in the loss of a large proportion of cells, the engraftment is stable and the cells, once they enter the respective systems are stably maintained, for up to 8 weeks, in the case of the ceramic co-culture system.

4.5.4 Maintenance of HSPC phenotype

In order to determine whether the HSPCs maintained their primitive CD34+ CD38- phenotype and explore the advantages of the 3D co-culture system over the HSPC culture strategies already in use, we compared the percentage of primitive HSPCs maintained in our ceramic co-culture system with that in suspension culture with defined serum free media and co-culture with osteo-induced and non induced MSC monolayers for up to 28 days (4 weeks) in culture, by FACS analysis of CD34 and CD38 expression (fig. 4.13). Each of these experiments was carried out with 8 independent MSC samples and 8 independent HSPC samples.

To further establish the longevity of our 3D system, we checked for HSPC phenotype maintenance in the ceramic co-culture system after 8 weeks of culture. As HSPC count in the other culture systems was found to steadily decrease to less that 5% (fig. 4.13B), this time point was not investigated in these systems.

Figure 4.13A shows representative FACS plots for CD34 and CD38 expression in 8 independent HSPC and MSC samples. The plots indicate that the percentages of CD34+, CD38- cells maintained in the ceramic co-culture system remain stable at around 50% up to 8 weeks (56 days) after seeding. In all the other culture conditions, however, the proportion of CD34+ CD38- cells steadily decrease, until there are less than 5% primitive HSPCs in culture (at 4 weeks).

Cumulative results of the flow cytometric analyses (fig 4.13B) confirm the above observations. They also show that the proportion of CD34+CD38- cells is highest in the ceramic co-culture system at week 1, 2 and 4 respectively, when compared to the other conventional culture strategies.

The percentage of differentiated progenitor cells, determined by the CD34+CD38+ phenotype was found to be lowest in the ceramic co-culture system, at all time points, and highest in the monolayer co-culture with MSCs (fig. 4.13C). The number of differentiated cells was found to decrease dramatically between week 2 and week 4, suggesting that the cells either differentiate further or die between these time points.

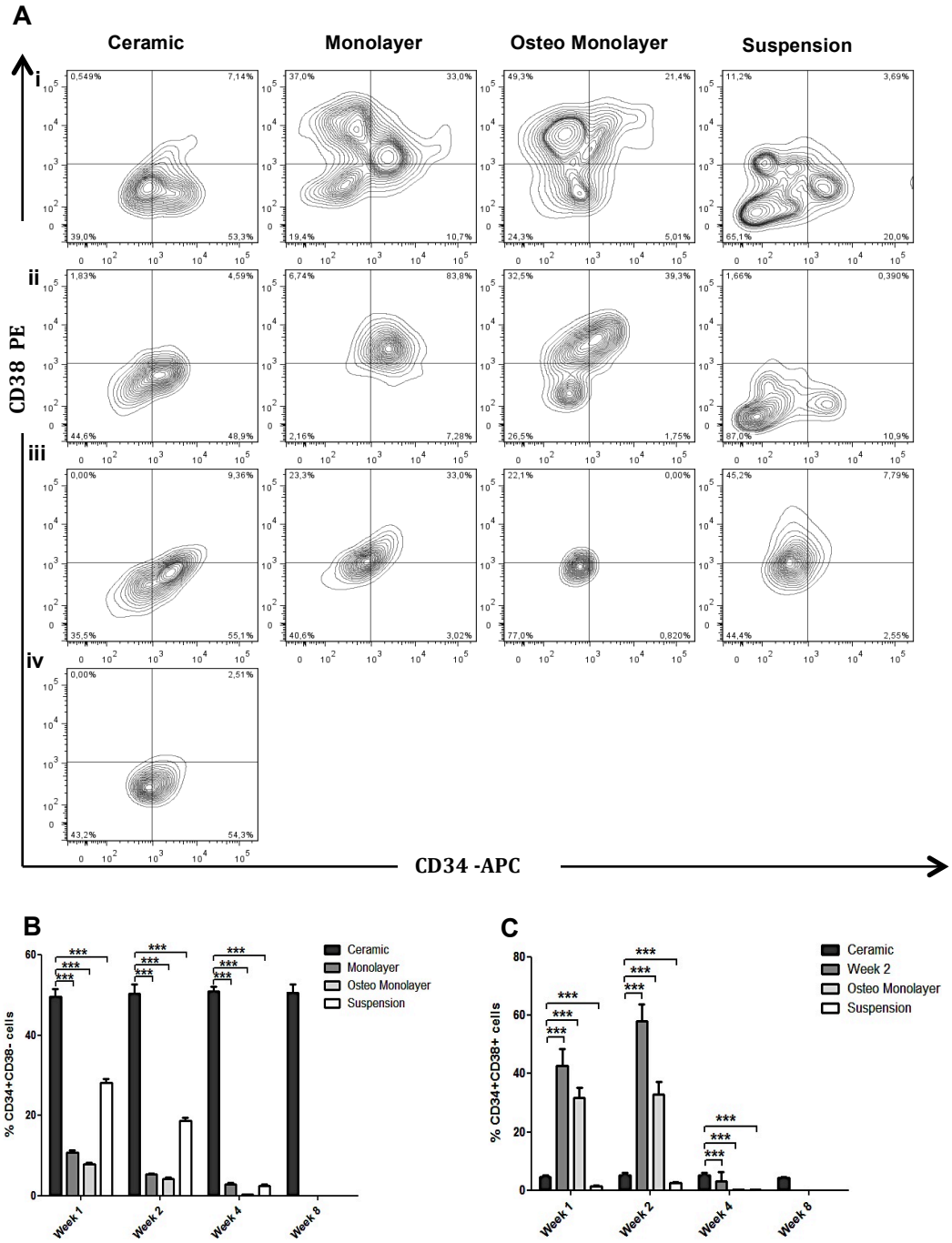

Figure 4.13. HSPC phenotype. (A) Representative FACS plots of cells (i) 1 week, (ii) 2 weeks, (iii) 4 weeks and (iv) 8 weeks after seeding **and (B, C) quantification** of primitive (CD34+CD38-) and differentiated (CD34+ CD38+) HSPCs in 3D and traditional co-culture systems at week 1, 2 and 4 after seeding of HSPCs . (n=8, error bars- SD of mean, **p < 0.01, ***p < 0.001).

This data indicates that our 3D co-culture system is capable of maintaining HSPCs in their primitive state in the long term, much more efficiently than the other conventional culture strategies.

4.5.5 HSPC viability

Having established that the primitive HSPC phenotype is maintained in the 3D culture system, long term, the question of whether the cells remain viable and non-apoptotic arose. We investigated this by checking the expression of annexin V- a widely used indicator of early apoptosis, and propidium iodide (PI), which stains late apoptotic and necrotic cells, in all the culture systems described previously.

As shown in the representative FACS plots and cumulative values (from 8 independent HSPC and MSC samples) in figure 4.14, over 98% of the HSPCs in all the culture systems tested do not express Annexin V on their surface, and more than 95% do not take up PI. Among the 4 culture conditions, the suspension culture and monolayer co-culture with osteo-induced MSCs exhibited the greatest extent of cell death (fig.4.14 B,C).

Annexin V expression was highest in the suspension cultures, but the proportion of cells expressing this marker did not change with time (fig. 4.14 B). In the ceramic co-culture system, the expression of annexin V was observed in very few cells at week 1 and 2, but not in week 4. In the monolayer co-culture with MSCs, the number of annexin V-expressing cells increased between weeks 2 and 4. As mentioned earlier, however, the total proportion of annexin V- expressing cells in all the cultures was less than 2% of the total cell count.

The number of PI-positive cells was observed to be highest at week 2, in the osteo-induced monolayer culture and at week 4 in the other culture systems. The proportion of these cells was however very low (< 3%).

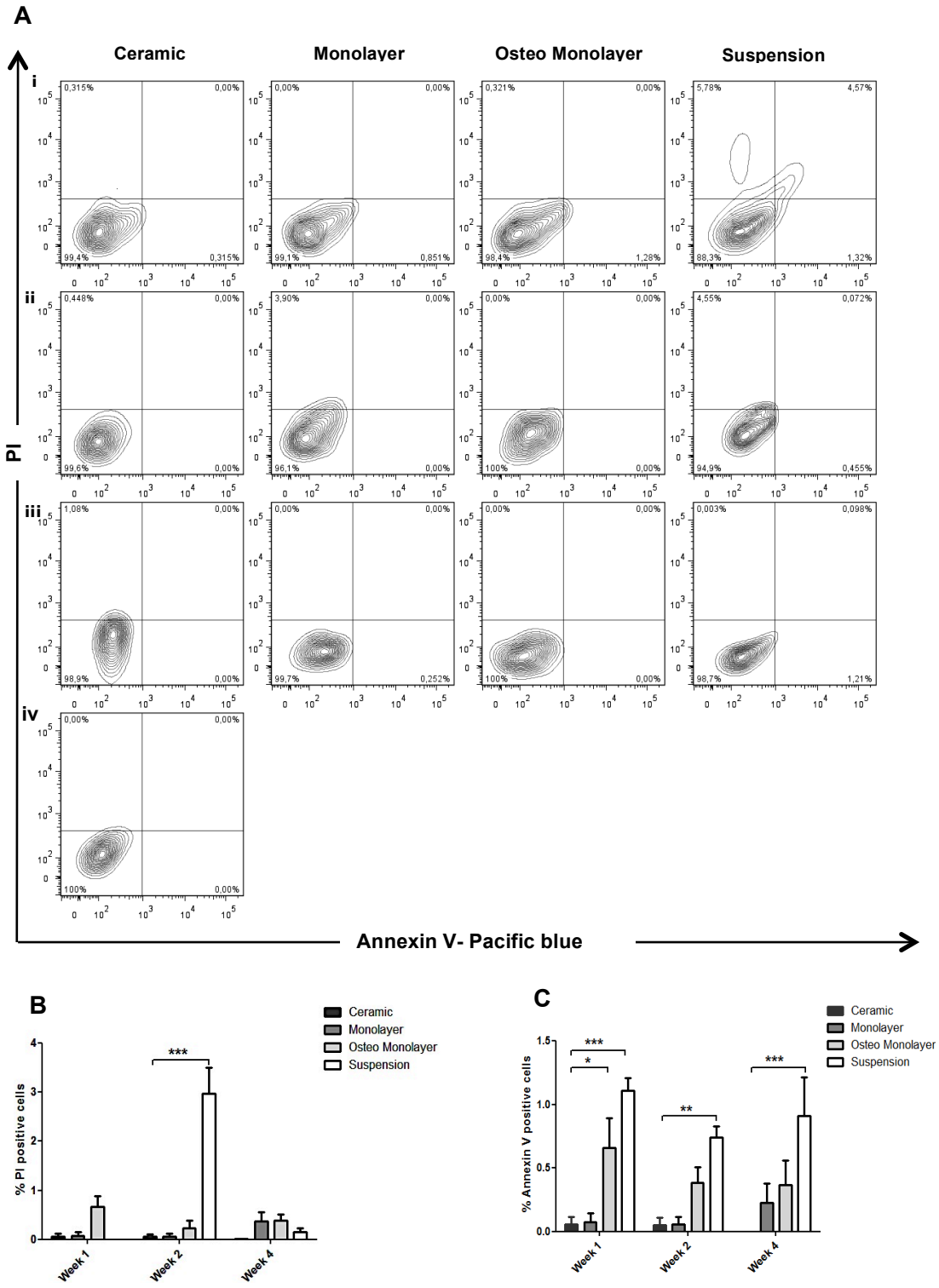

Figure 4.14. HSPC viability (A) Representative FACS plots and (B, C) quantification of necrotic (PI positive) and apoptotic (Annexin V positive) HSPCs in 3D and traditional co-culture systems. (n=8, error bars- SD of mean, *p < 0.05 **p < 0.01, ***p < 0.001).

Thus, we concluded that the HSPCs, especially in the ceramic co-culture system remain viable and non-apoptotic for up to 8 weeks, irrespective of the culture conditions.

4.5.6 MSC-HSPC interaction

After establishing that the HSPCs maintained in our co-culture system are indeed viable and primitive hematopoietic cells, we sought to illustrate that our 3D system simulates the interactions within the bone marrow HSPC niche. We addressed the question as to whether the HSPCs had any physical interaction with their partner cells- the MSCs, within the ceramic system. The co-localization of the HSPCs and MSCs shown in figure 4.11 is an initial indication that the two cells do, in fact interact, but does not give evidence of physical contact or any indication of the mechanism of interaction.

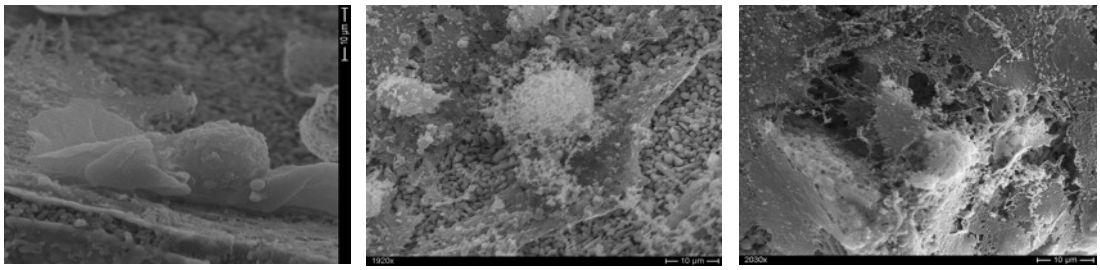

Fig 4.15. HSPC- MSC interaction SEM analysis of the ceramic co-culture system showed HSPCs (small, rounded cells) to be in close association with MSCs (large, flat cells) (Scale bars: 10μm).

Scanning electron microscopy revealed that the HSPCs and MSCs are in contact, within the ceramic, as depicted in figure 4.15. The small, rounded HSPCs, which have a diameter of about 10μm were always found to be in close contact with one of more flat, large MSCs, within the ceramic co-culture system, closely resembling the interaction thought to exist in the endosteal hematopoietic niche.

The possible modes of interaction of the HSCs within the ceramic co-culture system were then investigated. ECM molecules (Fibronectin and collagen I) and signaling molecules (C-kit, integrin 4a and N-cadherin) known to play a role in the bone marrow HSPC niche were detected in the ceramic system by immunofluorescence staining and 2-photon microscopy.

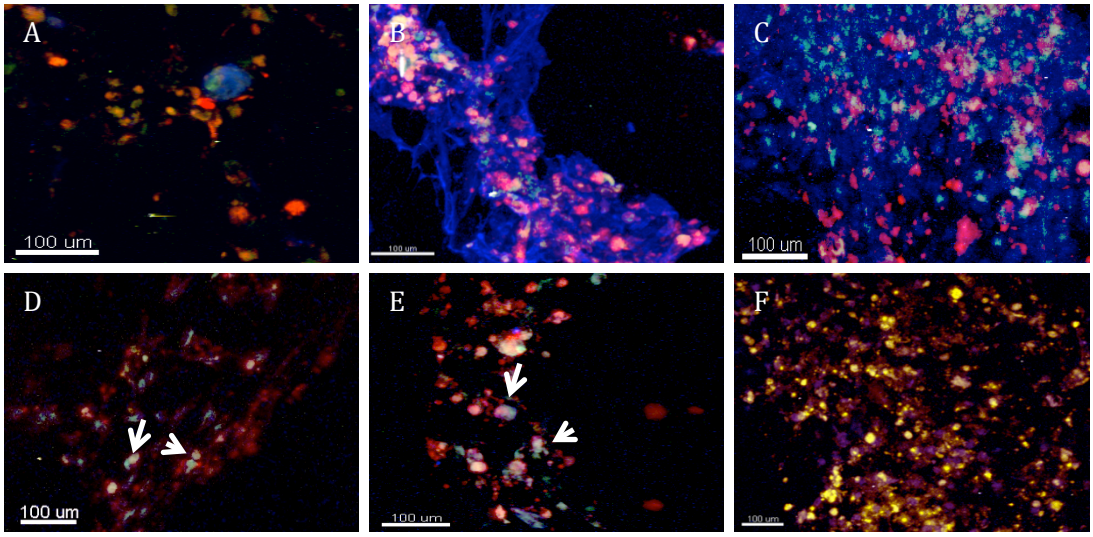

Figure 4.16. Molecular interactions in the 28 day 3D co-culture: Tracked MSCs (red) and HSPCs (green) with Alexa 350 (blue) stained: (A) unstained control (B) Fibronectin (C) Collagen I (D) C-Kit (E) Intergrin 4a (F) N Cadherin. Colocalization indicates interactions with the ECM molecules as well as signaling molecules. (n=3; Scale bars: 100μm)

Figure 4.16 shows 2-photon microscopy images of immunofluoscence staining for various ECM and signaling molecules known to be important in the endosteal HSPC niche, in the 4-week ceramic co-culture system. The fluorescent-labeled MSCs (red) and HSPCs (green) were found to be embedded in an ECM network composed mainly of Fibronectin and Collagen I (fig. 4.16, panels B, C).

Immunostaining of the signaling molecules C-kit, Integrin 4α and N-cadherin showed that these molecules are found in areas where HSPCs and MSCs are in contact (fig. 4.16, panels D, E, F), indicating that the HSPCs and MSCs in our 3D co-culture system interact via these molecules, and that this system successfully mimics the interactions within the endosteal hematopoietic niche.

4.5.7 HSPC proliferation

Next, the proliferation of HSPCs in the 3D co-culture system was compared with the conventional culture methods mentioned earlier, by flow cytometric analysis of CFSE labeled HPSCs at week 1, 2 and 4. Cells that had undergone 1 or fewer divisions (the first 2 peaks from right in the CFSE histogram) were considered as slow proliferating, while all other cells were considered to be fast proliferating (fig. 4.17).

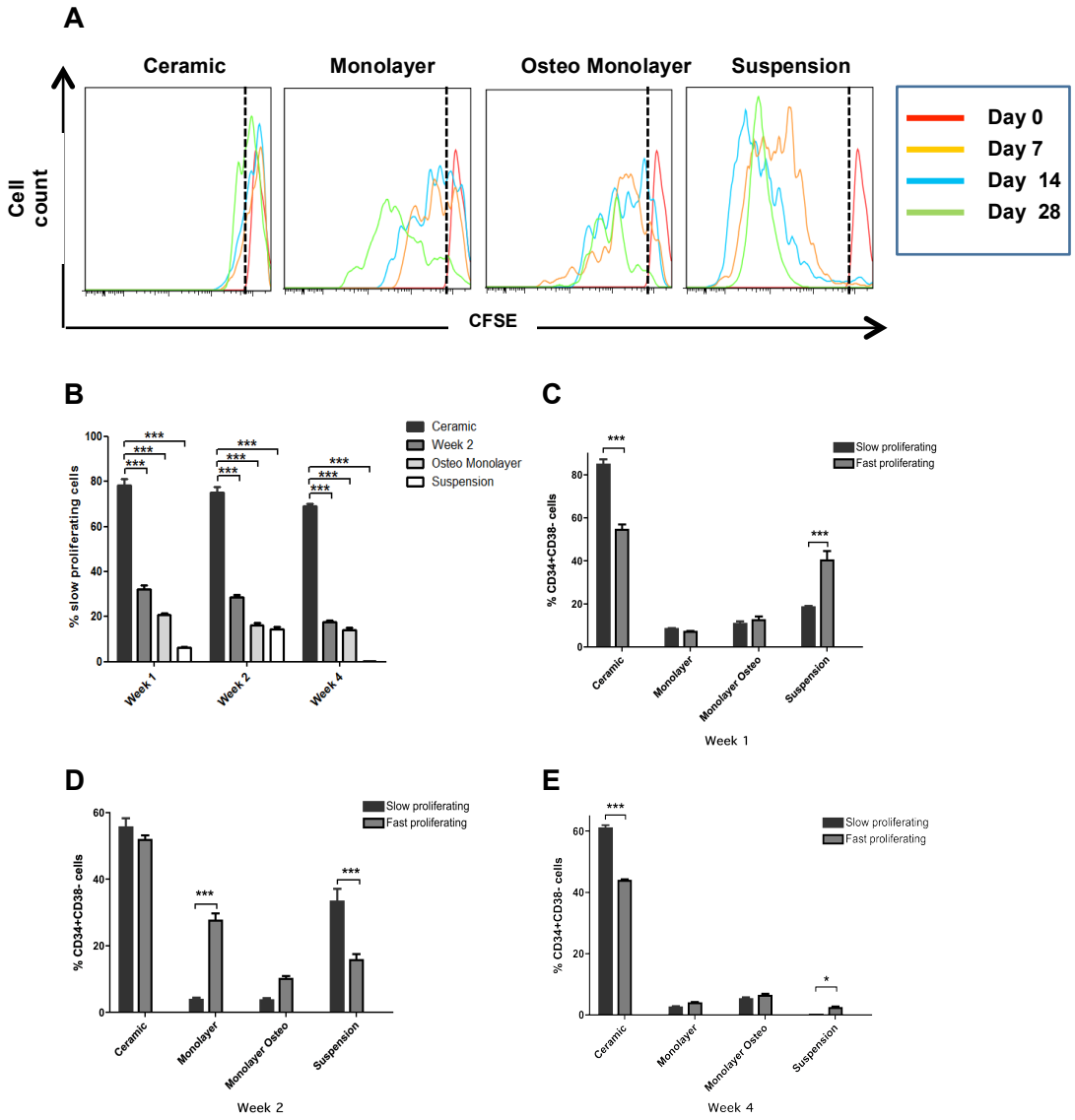

Figure 4.17. HSPC proliferation. (A) Representative histograms of CFSE-FACS analysis showing the differential proliferation of HSPCs in the different culture systems. Dotted lines delineate the slow (right) and fast (left) proliferating cell fractions. **(B) Quantification of slow proliferating cells in the four culture systems. (C, D, E) Quantification of primitive (CD34+CD38-) HSPCs in slow and fast proliferating cell fractions in each culture system, after 1,2 and 4 weeks of culture.** (n=6, error bars- SD of mean, *p < 0.05, ***p < 0.001).

Flow cytometry analysis and the subsequent generation of histograms (fig. 4.17A) indicated that the cells in the 3D co-culture system are the slowest proliferating of all culture systems.

A large proportion (> 70%) of the HSPCs in the 3D co-culture were found to be slow proliferating cells. This percentage was found to decrease marginally (<10%) between weeks 2 and 4(fig. 4.17B). The proportion of slow proliferating cells was found to be relatively lower in all the conventional cultures than the 3D co-culture system, and decreased to fewer than 10% by 4 weeks of culture (fig. 4.17B).

On investigating the phenotype of the slow and fast proliferating HSPCs after 1,2 and 4 weeks of culture, by flow cytometric analysis of CD34 and CD38, it was found that the HSPCs from the 3D co-culture system retained the largest proportion of cells with the primitive CD34+ CD38- phenotype (fig. 4.17 C, D, E) after 1,2 and 4 weeks of culture. Over 50% of slow and fast proliferating HSPCs from the 3D co-culture retained the primitive phenotype for up to 4 weeks. The proportion of CD34+CD38- cells in the fast proliferating cells was less than that in the slow proliferating cells, as expected. The percentage of primitive cells in the slow and fast proliferating fractions, from the traditional co-cultures, steadily decreases with time (fig. 4.17C, D, E).

Thus, we determined that the ceramic co-culture system maintains HSPCs as primitive and slowly proliferating cells.

4.5.8 Effect of cellular contact

The previous results establish that our 3D co-culture system is able to successfully maintain HSPCs at a stable percentage. We also showed that these HSPCs physically interact with the MSCs within the co-culture system. Next, we investigated the importance of this cellular contact for the maintenance of the HSC phenotype- whether physical contact is necessary, or secreted factors from the MSCs in 3D culture are sufficient.

To this end, we compared the HSPCs cultivated in (a) conditioned media from MSCs cultivated for 1 week on the ceramic scaffold, (b) a 1:1 mixture of this conditioned media and the defined serum free HSPC media used for suspension culture in all our earlier experiments, and (c) only the defined serum free media.

The HSPCs were analyzed by flow cytometry after 14 days of culture, as the proportion of CD34+ cells was found to decrease dramatically after this point.

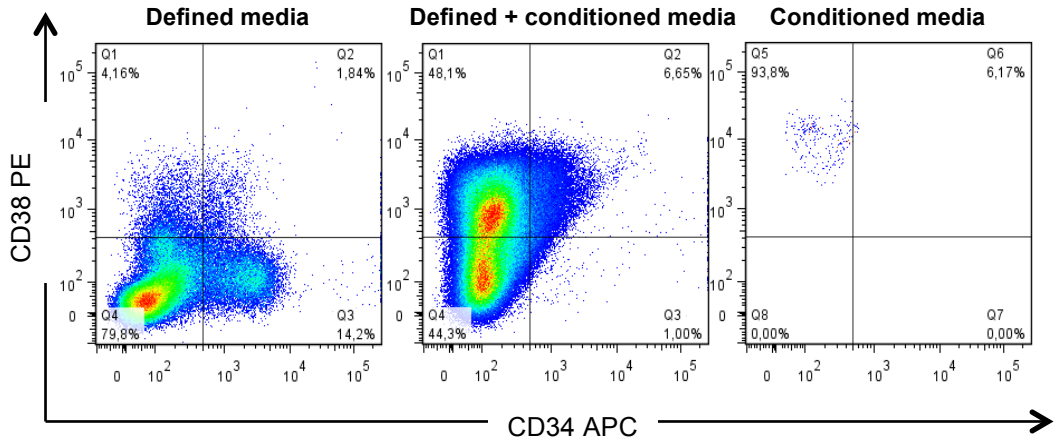

Figure 4.18. Flow cytometric analysis of HSPC phenotype in suspension culture for 2 weeks, in conventional defined media, a combination of defined and conditioned media, and only conditioned media. All samples are gated against unstained controls (n=3).

Figure 4.18 shows FACS plots representative of 3 independent HSPC samples cultures under the conditions mentioned earlier. As illustrated by this data, the presence of conditioned media from the MSCs in 3D culture does not favor the retention of the primitive CD34+ CD38- phenotype. On the contrary, it results in differentiation of the primitive HSPCs into a more mature CD34-CD38+ phenotype.

The final cell number represented in these plots also suggest that the defined media used is highly suited to proliferation of the HSPCs, since a significant (about 10-fold) increase in cell number was seen in the cultures with defined media. Defined media, was, however, not suited to the maintenance of the primitive phenotype.

We then further analyzed the cells for CD45, which is considered a marker of pan-leucocyte differentiation, in order to investigate whether the media composition and lack of stromal cell contact had any effect on HSPC differentiation.

From the data represented in figure 4.19, the presence of conditioned media mediates the retention of the CD38+ phenotype, which represents a degree of differentiation greater than that of the CD34+ phenotype, but within the range of multipotent progenitor cells.

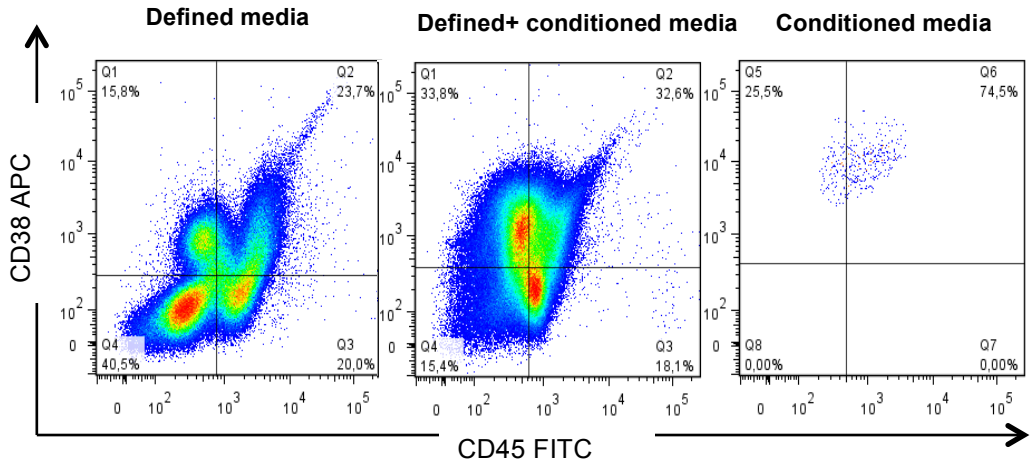

Figure 4.19. Flow cytometric analysis of HSPC differentiation in suspension culture for 2 weeks, in conventional defined media, a combination of defined and conditioned media, and only conditioned media. All samples are gated against unstained controls (n=3).

In the cultures containing both media types, a marked decrease in CD45+ phenotype was observed, in addition to the increase in CD38+ phenotype, indicating less differentiation. In only conditioned media, however, the HSPCs showed a marked tendency to differentiate into leucocytes, as illustrated by the increase in CD45+ cells. Cells cultured in only defined media comprised a comparatively large percentage of CD38-CD45+ cells, and CD38-CD45- cells, in comparison with the other culture conditions. These phenotypes indicate a larger degree of differentiation than the CD38+ phenotype.

From this data, we concluded that cellular contact between the MSCs and HSPCs within the 3D co-culture system is essential in the maintenance of the primitive CD34+ CD38- HSPC phenotype.

4.5.9 HSPC functionality

After confirming that the HSPCs maintained in the 3D co-culture system retain their primitive CD34+CD38-, slow proliferating phenotype and are viable, we tested whether these cells as capable of differentiating into the different blood cell lineages, using the CFU-GEMM assay.

CD34+ cells isolated from 3 independent samples of the 3D co-culture system, 1, 2 and 4 weeks after seeding HSPCs yielded mixed granulocyte, erythrocyte,

macrophage, megakaryocyte (CFU-GEMM), early erythrocyte (BFU- E) and mixed granulocyte megakaryocyte (CFU-GM) colonies (fig. 4.20).

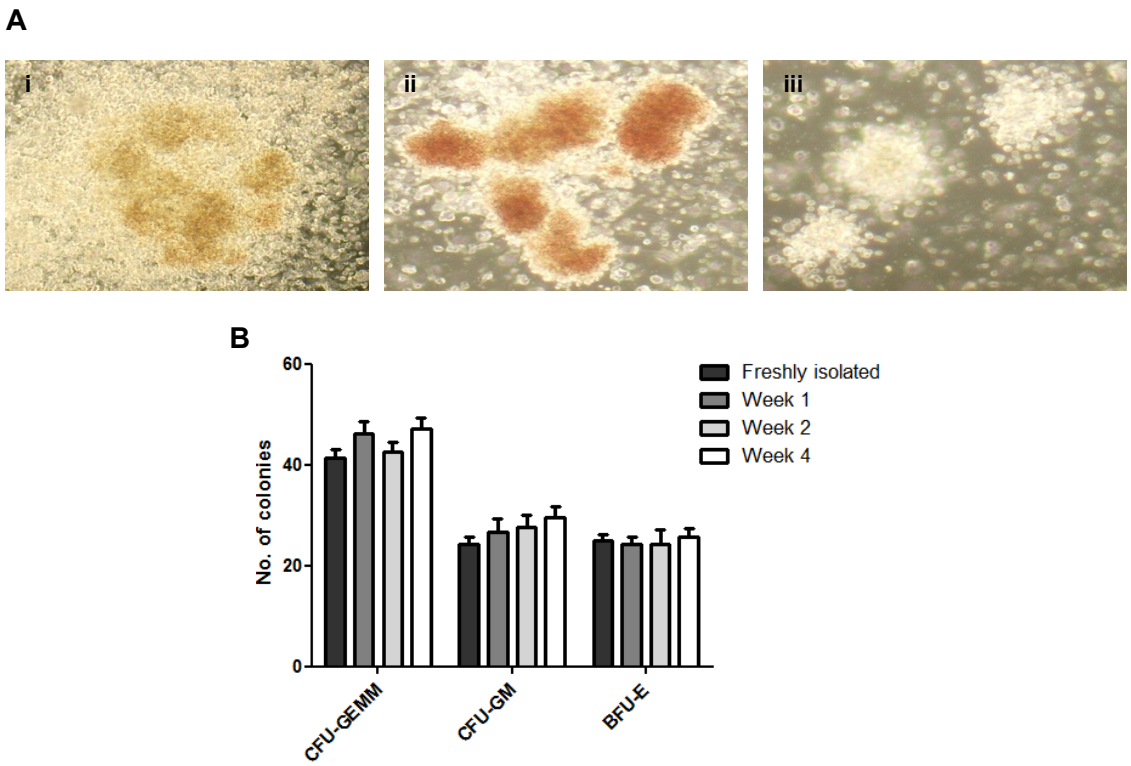

Figure 4.20. HSPC functionality: Multi-lineage differentiation potential of HSPCs from 3D co-culture system. (A) Representative (i) GEMM, (ii) BFU-E, (iii) GM colonies and (B) scoring of colonies after CFU-GEMM assays (n=3).

On counting and cumulative scoring of these colonies, no significant difference was found in colony number (for every 1000 CD34+ cells seeded) from the ceramic co-culture system after 1,2 and 4 weeks of culture and from the freshly isolated cord blood HSPCs.

Thus, we concluded that the CD34+ cells maintained in the 3D co-culture system are not only viable, but also retain their characteristic multi-lineage differentiation potential.

5. Discussion

The goal of this work was the establishment and characterization of a 3D co-culture system simulating the interactions of the bone marrow endosteal hematopoietic stem cell niche, in which human primary HSPCs are maintained as quiescent, slow-dividing, functional cells. To this end, we combined a well-defined, reproducible three-dimensional scaffold, which provides a suitable environment for HSPC maintenance by facilitating the correct assembly of the niche cellular components and physiological extra- cellular matrix deposition, and two primary niche cell types- the HSPCs and mesenchymal stromal cells. The generation and characterization of this system was a two-step process, with the 3D microenvironment being generated, using MSCs and the ceramic scaffold, and the HSPCs later being introduced, stably engrafted and maintained long-term within this system.

5.1 Generation of a bone marrow-like microenvironment

The first step in the development of our 3D co-culture system was the production of a three-dimensional microenvironment bearing close resemblance to that of bone marrow, into which the HSPCs could subsequently be engrafted. This was achieved by the spontaneous differentiation and ECM production of the MSCs, in response to the 3D microenvironment provided by the Sponceram® HA ceramics.

5.1.1 Efficacy of Sponceram® HA ceramic scaffolds

The behavior of mesenchymal stem/stromal cells, when cultured in 3D has been established to be very different from the traditional 2D culture (reviewed: Tortelli and Cancedda, 2009; Baker and Chen, 2012). It has been shown that MSCs, when cultured in 3D, with or without scaffolds, exhibit increased extracellular matrix (ECM) production (Dawson *et al.*, 2008), and altered proliferation (Hosseinkhani *et al*., 2005; Diederichs *et al.*, 2009; Kasten *et al.*, 2005; Frith *et al*., 2010) and differentiation capacity (Benoit *et al.*, 2008; Wang *et al.*, 2009). The use of scaffolds for MSC culture has been extensively documented, particularly in the field of bone and cartilage tissue engineering (Oliviera *et al*., 2006; Dawson *et al*., 2008). Scaffold composition, surface properties and rigidity have been shown to have a marked

influence on the direction of differentiation of MSCs. Stiffness or rigidity in scaffolds been found to predispose MSCs to osteogenic differentiation *in vitro* (Huebsch *et al.*, 2010). It has also been shown that porous scaffolds such as ceramics are best suited to osteogenic differentiation, with a gradient of pore sizes between 300-500μm being recommended as ideal for spontaneous dynamic bone and bone matrix formation (Karageorgiou and Kaplan, 2005). The use of hydroxyapatite (HA) as a scaffolding surface for osteogenesis has been extensively studied, due to the presence of bone-apatite- a mineral compound analogous to HA, within the bone matrix. HA coatings have been shown to improve MSC adherence and promote ECM production and osteogenic differentiation, *in vitro* (Storrie and Stupp, 2005; Dawson *et al.*, 2008; Tortelli and Cancedda, 2009).

The features required in the scaffolding system for this work were: Defined structure and composition, suitability for MSC adherence, ECM production and osteogenic differentiation. The Sponceram® HA ceramics fulfilled all these criteria, have a micro and macro-structure comparable to bone marrow, are coated with HA and are well defined and reproducible. Previous work has shown that these scaffolds, in static culture promote limited growth and partial spontaneous osteogenic differentiation of MSCs (Diederichs *et al.*, 2009). These were therefore chosen as an ideal scaffold for the simulation of the bone marrow microenvironment.

5.1.2 Spontaneous partial osteogenic differentiation of MSCs

It has been well documented that osteoblasts are among the main interaction partners of HSPCs in the bone marrow niche (Arai and Suda, 2007). In recent times, however, more and more cell types including neural cells, macrophages, stromal reticular cells and endothelial cells (reviewed: Shen and Nilsson, 2012) are emerging as potential niche regulators. Of these, mesenchymal stem/stromal cells are considered most important, not only as direct partners, but also as potential progenitors of the other partner cell types. *In vivo* data also suggests that partially committed stromal cells with a predisposition to osteogenic differentiation are the most likely cellular partners of quiescent HSCs (Balduino *et al.*, 2005; 2012).

It was therefore important for our 3D culture system, into which HSPCs were to be introduced, to contain a mixed population of MSCs, and partially differentiated or committed osteo-progenitor cells. To achieve this, we used the Sponceram® HA

scaffolds, which have been found to induce partial osteogenic differentiation of adipocyte-derived MSCs (Diederichs *et al.*, 2009). We determined the osteogenic differentiation of the MSCs seeded in the ceramic, one week after seeding, by detecting mineralization and the expression of the early osteogenic marker, osteopontin (OPN).

Von Kossa staining and Alizarin red staining of the ceramics revealed deposition of mineralized matrix in the ceramic, one week after seeding of MSC, indicating the formation of a bone marrow-like mineralized matrix. The mineralization, while indicative is not conclusive, and we therefore checked for the expression of OPN, at the transcriptional level. Real time PCR analysis of this culture system showed that expression of the early osteogenic marker- osteopontin was higher in the 3D culture than in monolayer, confirming the spontaneous osteogenic differentiation of the MSCs. The lack of expression of the late osteogenic marker, osteocalcin, however, indicates that the MSCs are not terminally differentiated. This is in accordance with our requirements.

Having established that the cellular composition in the ceramic scaffolds is comparable to certain aspects of the endosteal hematopoietic niche, we then investigated the structural aspects and the production of ECM components, by the MSCs within the ceramic.

5.1.3 Structure and ECM production

On immunohistochemical analysis of the ceramic seeded with MSCs for a week, we observed the extensive expression of the ECM molecules- collagen I and fibronectin. These molecules formed the main components of dense network-like structures that were observed spanning the pores throughout the ceramic.

Collagen I is the most abundant non-mineralized component of the bone matrix and known to mediate HSC homing, by binding to surface receptors and trapping secreted factors. Fibronectin is a glycoprotein found at high levels at sites of osteogenesis. It contains a short amino acid sequence (Arg-Gly-Asp or RGD), which is critical for binding to integrin receptors, and is therefore thought to have a role in the homing and maintenance of HSPCs, *in vivo* (Voermans *et al.*, 1999).

In vitro studies investigating the effect of collagen I, fibronectin and combinations of these (Gu *et al.*, 2003; Celebi *et al.*, 2011), on HSPC migration and binding, have shown that both collagen I and fibronectin, independently and in concert with other ECM components, bring about HSPC quiescence and dormancy (Kurth *et al.*, 2011). Fibronectin, in particular, has been shown to play a distinct role in determining fate of HSCs (Feng *et al*., 2006). Further studies have shown that fibronectin, by integrin 4a based cell adherence, is closely involved in cell function, protein expression and the status of cell cycle, thereby maintaining HSPCs as quiescent, non-proliferative cells, and promoting engraftment in transplants (Garcia *et al*., 1999). Collagen I has also been shown to mediate the stable engraftment and quiescent maintenance of HSPCs *in vitro* (Ostwald *et al*., 2006).

Our 3D culture system therefore exhibits the presence of ECM components characteristic of the bone marrow microenvironment. The bone marrow ECM (including collagen I and fibronectin) is known to modulated HSPC homing and maintenance (Klein, 1995), and is therefore essential to out co-culture system. We then compared the structure of this microenvironment to that of native human bone marrow.

Scanning electron microscopic analysis of the MSC-seeded ceramic and bone-spongiosa from the femoral head revealed a great degree of structural similarity between the two. Extensive interconnected networks comprising cells and ECM were observed in both MSC-seeded ceramic and in human bone marrow. Additionally, bead like structures comprising ECM proteins were also observed, confirming the presence of a microenvironment with a close physical and structural resemblance to bone marrow, within the ceramic scaffolds.

Having thus confirmed that the 3D culture of MSCs in the Sponceram® HA ceramics results in the formation of ECM, and is comparable to bone marrow stroma in terms of structure and cellular composition, we then investigated the molecular composition of the system, particularly with respect to molecules implicated in the maintenance of the HSPC niche.

5.1.4 Production of niche-specific molecules

Real time PCR analysis of this culture system, 7 days after seeding MSCs, showed that expression of molecules with known roles in the endosteal niche, namely Jagged-1, C-X-C chemokine receptor ligand type 12 (CXCL-12), BMP receptor 1A (BMPR1A), N-cadherin and Intercellular Adhesion Molecule-1 (ICAM-1) were up-regulated in the 3D cultures, in comparison to monolayer culture. As mentioned earlier, expression of OPN was also found to increase in 3D. All these molecules have putative roles in the maintenance of quiescent HSPCs in bone marrow.

Osteopontin is known to bind to integrin 4a and CD44, which are extensively expressed on the surface of HSCs. Ablation of this protein in mice resulted in HSC proliferation, migration and eventually differentiation (Stier *et al*., 2005), suggesting that it acts as a negative regulator of HSC proliferation and therefore promotes HSPC homing and dormant maintenance of long-term repopulating HSPCs.

Jagged-1 is thought to affect HSPCs through Notch- mediated signaling, which in turn is linked to HSPC proliferation and self-renewal by the parathyroid hormone pathway (Stier *et al*., 2002). The ablation of both Jagged-1 and Notch, however, has been shown to have no significant effect on HSPC function (Mancini *et al*., 2005), resulting in uncertainty about the role of these molecules in HSPC maintenance. N-cadherin is expressed on specialized bone-lining osteoblastic cells, and was initially thought to be the main player in the interaction of HSPC with the niche osteoblasts (Arai *et al*., 2004), its role has however, been disputed, and there is currently a lack of concrete evidence of it's importance in niche function (Kiel *et al*., 2007). In the current culture system, however, both Jagged-1 and N-cadherin are present, indicating the possibility of niche-like signaling via these pathways.

CXCL-12, BMPR1A and ICAM-1 have all been implicated in HSPC homing, and are all significantly up regulated in the 3D culture, in comparison with monolayer. CXCL12 interacts with its receptor- CXCR4, which is expressed on the surface of HSPCs, and is important for retention of HSCs in the niche (Ara *et al*., 2003). Blocking of interaction of CXCL12 and CXCR4 results in mobilization of HSCs from the BM to the peripheral blood (Broxmeyer *et al*., 2005). BMPR1a is expressed on osteoblastic cells in the bone marrow. The ablation of this molecule was found to

result in HSC proliferation, suggesting that BMPR1a mediates the BMP signaling in the niche and promotes quiescence (Larsson and Karlsson, 2005). ICAM-1 is a surface protein found on several stromal cell types. It binds to the lymphocyte function-associated antigen 1 (LFA1) on the HSPC surface and is thought to mediate the engraftment of the HSPCs within the bone marrow niche. The expression level of these molecules was also found to increase with time of culture in the ceramics, suggesting that the 3D culture system is highly supportive of HSPC engraftment and maintenance of quiescence.

Thus, within 1 week of seeding MSCs into the ceramic scaffolds, we observed that a microenvironment bearing structural and molecular similarity to the bone marrow niche was formed. This microenvironment fulfills all the requirements- structural, molecular and cellular, to support and maintain primitive HSPCs. In order to complete this putative artificial niche, we then introduced CD 34+ umbilical derived HSPCs into this system. These cells were then analyzed at regular intervals, up to 8 weeks of culture, to ensure long-term maintenance.

5.2 Engraftment and maintenance of HSPCs

Hematopoietic stem and progenitor cells are maintained in their niche as quiescent, dormant cells, in the G_0 Phase of cell division, in order to prevent stem cell depletion and maintain a constant pool of stem cells (Cheshier *et al*., 1999; Glauche *et al*., 2009). The primary aim of the co-culture system described in this work is the maintenance of HSPCs in this state, thereby effectively simulating the endosteal hematopoietic niche. The HSPCs introduced into the MSC-seeded ceramic were therefore monitored closely for HSPC marker expression, viability, function and proliferation. Using fluorescence microscopy, we were able to detect fluorescent labeled HSPCs in the 3D co-culture system up to 8 weeks after their introduction into the ceramic, indicating that the HSPCs not only enter the ceramic, but are also retained there, long term.

On counting the HSPCs obtained from the co-culture system, it was found that only 10% of the original seeding volume was retained in the ceramic. This could be due to the HSPC phenotype- since the starting population is a mixture of stem cells and more committed progenitors, which could have a differential rate of engraftment. The

number of HSPCs after engraftment, however, remained constant with time in culture, suggesting that although the rate of engraftment of the HSPCs into the system is not optimal, the subsequent retention and maintenance of HSPCs is stable and constant. We then proceeded to investigate the phenotype of these HSPCs, for the primitive CD34+ CD38- marker expression pattern, viability and proliferation rate. The CD34+CD38- phenotype is widely accepted as the primitive HSPC phenotype in humans (reviewed: Chotinantakul and Leeanansaksiri, 2012). However, this phenotype includes the long term repopulating HSCs and a population of proliferative and more differentiated progenitors. This discrepancy is due to a limited knowledge of differentiation-specific markers in the human HSPCs. In order to establish the efficiency of the 3D culture system, the maintenance of HSPCs in this system was compared with that of three conventional culture methods, namely suspension culture in defined, cytokine-supplemented media and co-culture with monolayers of osteo-induced and non-induced MSCs.

5.2.1 Phenotype of HSPCs in co-culture system

By flow cytometric analysis, it was demonstrated that a large proportion (over 50%) of the HSPCs in the ceramic co-culture system retained the primitive CD34+CD38- phenotype stably, from one week up to 8 weeks of culture. This is the longest known period for which HSPCs have been maintained stably in culture. Time points after 8 weeks were not investigated, but it is hypothesized that since the system is stable up to 8 weeks, it is likely to remain so. In contrast, HSPCs cultured in conventional monolayer co-cultures or as suspension cultures in defined, cytokine- supplemented media were found to consistently lose their primitive phenotype, with less than 5% of the cells retaining CD34 expression after 4 weeks of culture. It was also confirmed that the cells in all the culture systems were not apoptotic or necrotic, by measuring annexin-V expression and PI uptake. Thus, the HSPCs differentiated to a much larger extent in the conventional culture systems, but not in the 3D co-culture system.

Since primitive HSPCs in the endosteal niche are also characterized by their slow rate of proliferation (Cheshier *et al*., 1999), we investigated HSPC proliferation in each of the culture systems, using CFSE dilution studies. We demonstrated that the HSPCs in the 3D co-culture system underwent very few divisions (<2) in 4 weeks, in comparison to those from the conventional cultures, which divided several times. The

cells in the suspension culture, in particular were highly proliferative. We also found that a larger percentage of the slowly proliferating cells from the 3D co-culture retained the CD34+CD38- phenotype, compared to the fast proliferating cells, which is consistent with the primitive phenotype of long-term repopulating quiescent HSPCs that we aimed to maintain in our co-culture system. The above findings suggest that the 3D co-culture system described in this work is highly efficient in the maintenance of primitive HSPCs in the quiescent state, but the expansion of these cells requires additional factors, such as cytokine supplements.

Having thus established that our 3D co-culture system is capable of maintaining viable, slow-proliferating, CD34+CD38- HSPCs for extended periods of time, *in vitro*, we used CFU-GEMM assays to demonstrate that these cells are functional and capable of forming erythroid and myeloid colonies. The colony forming capacity of the HSPCs from the ceramic did not change with time in culture, indicating stable maintenance of functional HSPCs.

5.2.2 Interaction of HSPCs with the microenvironment

Having confirmed that the co-culture system does in fact mimic the endosteal niche, and that primitive HSPCs are maintained in this system, as in the physiological state *in vivo*, the possible mechanisms for this maintenance and the ways in which the HSPCs interact with their microenvironment within the co-culture system were investigated.

FACS based comparison of CD34, CD38 and the pan-leucocyte differentiation marker CD45 (Lightstone and Marvel, 1990) expression in 2-week suspension culture of HSPCs in defined, cytokine-supplemented media, conditioned media from the MSC-seeded ceramics and a 1:1 mixture of the two media types was used to determine the role of secreted molecules and cell-to-cell contact in the 3D co-culture system. These analyses revealed that the presence of MSC-conditioned media limits the expression of CD45, i.e. terminal differentiation of the HSPCs, but is insufficient to maintain the CD34+ CD38- phenotype, and promotes the passage of HSPCs into the CD38+ (committed progenitor) phenotype. Cytokine supplemented media, on the other hand, causes terminal differentiation, as evidenced by the distinct increase of CD45+ cells. The cell numbers in each of these culture conditions suggest that cytokine supplementation highly favors HSPC proliferation, but not maintenance.

This is consistent with several published studies (Bryder and Jacobsen, 2000; Bruno *et al*., 2001) On the basis of these findings, it was concluded that the maintenance of the primitive phenotype in the ceramic co-culture system is a result of physical interactions between the HSPCs and the other cellular and ECM component of the co-culture system.

Immunohistochemical analysis and 2-photon microscopy were then utilized to investigate the molecular interactions within the co-culture system. It was found that pre-labeled MSCs and HSPCs were located in close proximity within the fibronectin and collagen I composed network described earlier. These ECM molecules are not only important in HSPC maintenance *in vivo*, but have also been extensively investigated as mediators of HSPC adherence *in vitro* as described earlier. The niche mediating molecules stem cell growth factor receptor (C-kit), N-Cadherin and integrin 4a were also seen to co-localize with the HSPCs and MSCs, indicating that these two cell types might interact via these molecules. C-kit is a receptor for stem cell growth factor, on the HSPC surface and is found to promote HSC maintenance, by mediating self-renewal (Yonemura *et al*., 1997). The putative role of N-Cadherin in the niche, as described before, is controversial, and Integrin 4a is known to play a role in specific cellular interactions with fibronectin (Askmyr *et al*., 2009). The interactions of HSPCs in the 3D co-culture system, therefore, are modeled closely upon those seen in the bone marrow niche.

Further, SEM imaging revealed physical interaction between the adherent MSCs and HSPCs, clearly indicating niche-like cellular interactions within the 3D co-culture system. These interactions bore striking similarity to interactions visualized *in vivo*, between HSPCs and bone marrow stromal cells, in the mouse femur (Balduino *et al*., 2005; 2012). Thus, the 3D co-culture system described in this work efficiently mimics the most important aspects of the endosteal HSPC niche and can be considered an accurate simulation of the bone marrow endosteal niche.

5.3 Comparison with previously described systems

In the last two decades, extensive work has been carried out to expand primitive HSPCs *in vitro* (reviewed: Aggarwal *et al*., 2012). Studies have investigated the use of cytokine-supplemented media (reviewed: Takizawa *et al*., 2011), and the use of

2D and 3D culture systems, with or without stromal support (Feng *et al*., 2006; Da Silva *et al*., 2005; Robinson *et al*., 2006; Miyoshi *et al*., 2011). Currently, the general consensus is that 3D culture, especially with stromal support cells, promotes better adhesion and expansion than 2D or stromal cell-free systems and better mimics the *in vivo* bone marrow microenvironment. Several studies have therefore investigated the 3D culture of HSPCs, in an attempt to recapitulate the HSC niche *in vitro*. The current 3D systems being investigated fall largely into two categories; Scaffold-free and scaffold based systems. These systems attempt to combine 3D structure and bone marrow cells to simulate the bone marrow environment *in vitro*.

Scaffold-free 3D systems for the expansion or maintenance of HSPCs rely on the aggregation of stromal support cells- usually mesenchymal stem/ stromal cells into three-dimensional structures, wherein a niche like microenvironment is thought to be spontaneously produced. HSPCs are then introduced into these aggregates. Three studies have been reported where HSPC migration and retention was studied in MSC-based aggregates (Bug *et al*., 2002; de Barros *et al*., 2010; Cook *et al*., 2012). In all these studies, it was observed that HSPCs are actively recruited into MSC or osteoblastic cell aggregates, aggregated by different methods. The long-term survival, self-renewal and functionality of the HSPCs, however could not be adequately demonstrated in these studies. The main drawbacks of these systems are; the lack of reproducibility in the formation of the aggregates and the inability to control or monitor their cellular composition, the structural dissimilarity of the aggregate microenvironment to bone marrow and the limited ability to manipulate this microenvironment. In order to obtain a more reproducible and controllable 3D microenvironment, scaffolds composed of a variety of biomaterials have been tested as a platform to co-culture stromal cells and HSPCs and thereby mimic the HSPC niche. Biomaterials used for scaffolds range from polyurethane foams to ECM protein gels to decellularized bone matrix. Such scaffold-based experiments have shown that immature hematopoietic cells cultured in 3D polyurethane foam scaffolds seeded with stromal support cells are marginally expanded and stably maintained for up to 2 weeks (Jozaki *et al*., 2010). Other scaffolds such as poly (D, L -lactide-co-glycolide) coated with collagen-1 (Mortera-Blanco *et al*., 2011) and porous polyvinyl formal resin with stromal support cells (Miyoshi *et al*., 2011) have also yielded similar results. In the past year, three studies have investigated the use of ECM protein based gels, seeded with stromal support cells, as a platform for HSPC maintenance. Leisten *et al* (2012) reported that a 3D scaffold of collagen gel with MSCs expanded HSPC in two

distinct populations: highly proliferative and differentiating cells in suspension above the collagen gel and proliferative, but primitive HPCs within the collagen scaffold. This report states that this is the best parallel of the entire HSPC niche, wherein the collagen-embedded MSCs mimic the endosteal region, and the suspension above the gel, the perivascular region. This model, however, lacks the cellular diversity of the endosteal niche, fails to demonstrate a quiescent HSPC pool which is characteristic of the endosteal niche, does not contain bone-stroma ECM molecules, and lacks the basic structural features of bone marrow, including rigidity and mineralization. Similar studies using fibrin gels (Ferreira *et al*., 2012) and other hydrogels (Sharma *et al*., 2012) also showed increased HSC proliferation and the maintenance of HSC function as demonstrated by multi-lineage reconstitution in a competitive transplant setting. These systems too fail to demonstrate structural and molecular similarity to the physiological condition. A single study has been reported wherein decellularized cancellous bone seeded with osteoblasts differentiated from MSC as support cells (Tan *et al*., 2010) were found to maintain quiescent HSPCs for up to 5 weeks. The system was, however, not tested for niche-like molecular interactions, or ECM production. The 3D co-culture system described in this work improves upon these results and combines a bone-like mineralized scaffold, appropriate ECM and partner cells for the successful long-term maintenance of HSPCs *in vitro*.

5.4 Conclusion

As depicted in figure 5.1, the 3D co-culture system described in this work was found to effectively and stably sustain hematopoietic stem/ progenitor cells with a primitive CD34+ CD38- phenotype for up to, and possibly longer than, 8 weeks (56 days). In addition to the CD34+CD38- phenotype, these cells also exhibited little or no proliferation, but were viable and capable of multi-lineage differentiation, upon appropriate stimulation. These features correspond to the majority (over 75%) of the dormant long-term repopulating hematopoietic stem cells maintained as a self-renewing quiescent population in the bone marrow (Cheshier *et al*., 1999). Our first conclusion, therefore, is that the three dimensional co-culture of MSCs in the Sponceram® HA ceramics with HSPCs results in the efficient engraftment and subsequent maintenance of primitive HSPCs, as in the bone marrow microenvironment. This system thus efficiently mimics the quiescent dormant fraction of the hematopoietic stem cell niche.

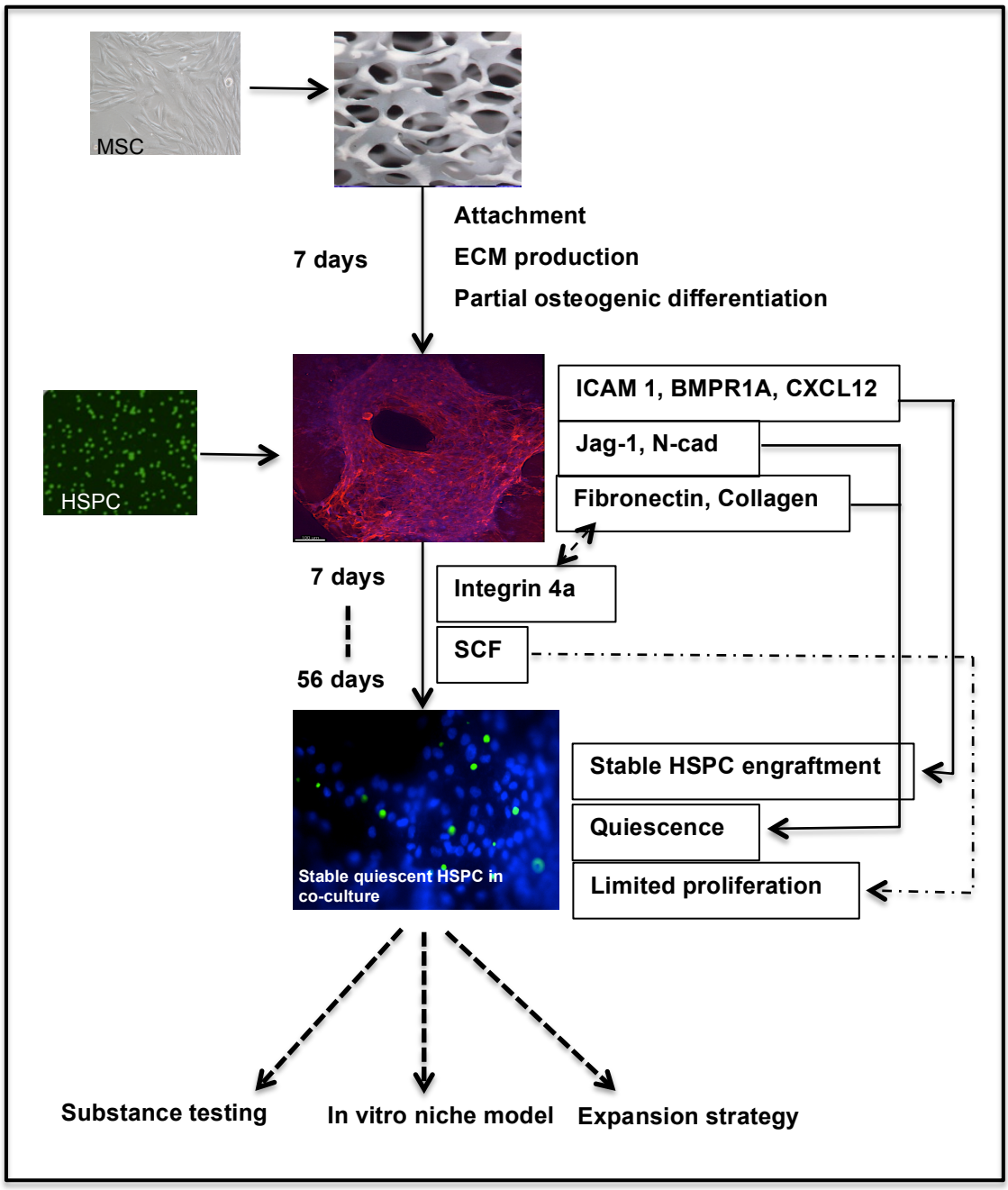

Figure 5.1. Schematic representation of the development the ceramic-scaffold 3D co-culture system and putative mechanisms by which HSPC maintenance is regulated in this system. HSPCs introduced into the system are maintained as primitive and quiescent, slow proliferating cells, as in the endosteal hematopoietic niche, indicating the successful simulation of the niche environment in the 3D system (BMPR1A, BMP receptor 1A; CXCL12, CXC chemokine ligand 12; HSPC, hematopoietic stem/progenitor cell; ICAM1, intercellular adhesion molecule 1).

Further, the possible mechanisms by which this maintenance is brought about were investigated, as discussed earlier. The increase in expression level Osteopontin, ICAM1, CXCL12 and BMPR1A in the 3D culture, in combination with their well documented role in HSPC engraftment to the niche (reviewed: Wilson and Trumpp, 2006), lead to the conclusion that the initial recruitment or migration of the HSPCs into the co-culture system, and their subsequent stable engraftment is primarily brought about by the activity of these molecules. The production and bone stoma-like organization of collagen I and fibronectin, in the co-culture system, whose role in the attachment of HSPCs in the bone marrow has been extensively investigated (reviewed: Lund *et al*., 2009), and the presence of HSPCs within this matrix, suggests that the HSPCs in the co-culture system have access to a bone-like ECM structure, which promotes their engraftment. Furthermore, the co-localization and physical contact of the HSPCs with the MSCs within the ceramic scaffold, as demonstrated by SEM imaging closely resembles the physiological niche (Balduino *et al*., 2005). Finally, the micro and macro-structure of the ceramic scaffolds and the presence of MSCs at different stages of differentiation are all reminiscent of the endosteal HSPC niche. All these observations highlight the presence of a distinctly bone marrow endosteal niche-like environment within the ceramic scaffold and strongly suggest that the maintenance of the HSPCs within this system is largely a result of niche-like interactions with the cellular, molecular and structural components of the co-culture system i.e. the differentiating MSCs, niche-specific signaling molecules and the spontaneously generated ECM.

The co-culture system described in this work, therefore, **accurately simulates the quiescent HSPC pool of the endosteal niche, not just in terms of stable maintenance of slow dividing, functional multi-potent HSPCs, but also with regard to the molecular cues, ECM, and physical aspects.** It surpasses other reported 3D systems in terms of duration of HSPC maintenance, reproducibility of the scaffolds and most importantly, the close resemblance to the physiological state. This system has great potential; as a starting point for the effective *in vitro* simulation of the complete adult hematopoietic system, as a platform to dissect the mechanics of endosteal niche maintenance, as a means to test substances targeting specific components the bone marrow prior to animal trials and last but not least, as the basis for the development of novel pre-transplantation expansion strategies for HSPCs.

6. Perspectives

The 3D co-culture system established in this work effectively combines the most vital aspects of the endosteal HSPC niche, namely; structure, cellular components and molecular interactions, and is able to successfully maintain a population of viable, quiescent hematopoietic stem and progenitor cells. It can therefore be considered an accurate simulation of one aspect of the bone marrow HSPC niche and be utilized for several potential applications. This system, due to it's well defined and relatively simple construction can also be manipulated in various ways, thereby significantly increasing it's range of potential applications.

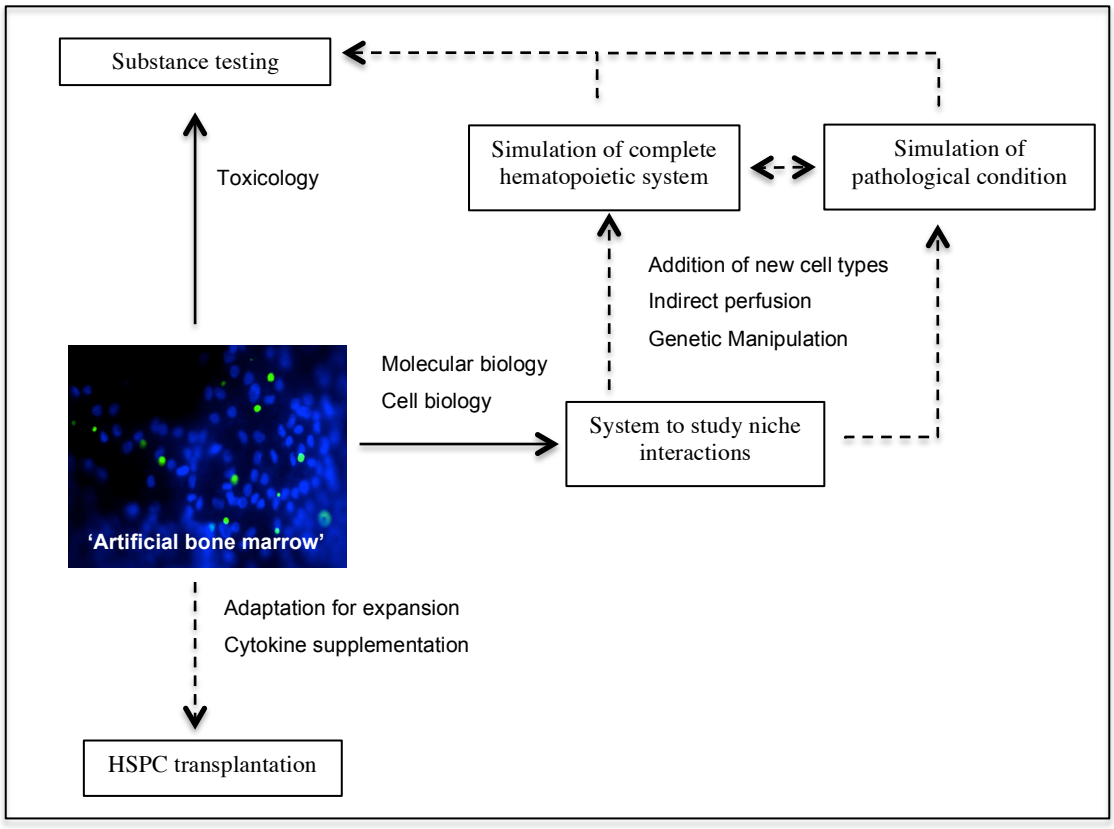

Figure 6.1. Schematic representation of the potential applications of the ceramic-scaffold 3D co-culture system. The system can be utilized as a source of long term repopulating HSPCs as well as a platform for cell and molecular biology testing, a disease model and a platform for substance testing.

As shown in fig. 6.1, The three main avenues where this co-culture system would find use are; as a model to study the molecular and cell biology of the HSPC niche, as a platform for substance testing and as an expansion strategy for HSPC transplantation.

The system described in this work has been shown to maintain HSPCs in a state closely mirroring the physiological state, and can as such be used to examine the molecular interactions, which influence HSPC engraftment and maintenance. Also, the size and structure of the ceramic scaffold make it easy to handle, manipulate and examine, and allows easy variation of media components. Although the visualization of the interior of the system, without fixation currently poses a challenge, this could certainly be circumvented by advanced microscopy techniques such as confocal or 2-photon microscopy. The relative simplicity of the system- the well-defined scaffold and the single stromal support cell type allow a variety of manipulations. For instance, the overexpression or knockdown of selected molecules implicated in niche homeostasis, for instance those investigated at the transcriptional level, in this work, would yield valuable information about their role *in vivo*. Furthermore, the role of selected surface molecules, such as ICAM1 and VCAM1 could be elucidated by simple chemical blocking studies. The ease with which substances may be added to the system also makes this system an attractive platform for substance testing. Chemotherapeutic substances, particularly those used to treat blood conditions, are usually found to be myelotoxic to varying degrees (Domellöf *et al*., 1984). Such drugs have also been observed to specifically disrupt hematopoiesis and molecular interactions directly involved in niche maintenance, such as the CXCL12-CXCR4 interaction (Georgiou *et al*., 2012). This system would provide a useful platform to exclude highly myelotoxic substances from further testing, at an early stage of testing.

In addition to these immediate potential uses, this system is highly adaptable and can be further developed or modified for several more applications. For instance, the addition of more niche-specific cell types, like endothelial cells, or the introduction of an indirect perfusion system, wherein the ceramic co-culture system is not in direct contact with, but is exposed to an actively perfused endothelialized surface, could result in a more complete simulation of the endosteal and perivascular HSPC niches, and the interaction between the two. Thus, this system potentially comprises the first stem in the development of a sophisticated and comprehensive *in vitro* model of the complete bone marrow hematopoietic system. Such a system would be invaluable for the understanding of the complex process of niche homeostasis. Additionally, this system could be manipulated to simulate a specific pathological phenotype e.g. Leukemia, and be used as a platform not only to understand the molecular mechanism pathology, but also to explore therapeutic strategies.

Finally, the ceramic-based co-culture system has been demonstrated to be capable of stably maintaining long-term repopulating HSPCs. This system therefore has the potential to be developed as a pre-transplantation expansion strategy for HSPCs. In the past several decades, hematopoietic stem cell transplantation has been the standard treatment for various hematological disorders such as severe combined immunodeficiency, congenital neutropenia and malignancies including several leukemias (Burt *et al*., 2008). The success of such transplantation therapies in the clinic is, however limited due to limited availability of HLA-matched donors, and the lack of sufficient numbers of functional HSPCs from one donor source (Lennard and Jackson, 2000). While high levels of expansion of HSPCs have been demonstrated in cytokine-supplemented media, the cells were also found to lose their primitive phenotype. There is, therefore a pressing need for a method of *ex-vivo* expansion of these cells, while simultaneously maintaining their primitive phenotype. (reviewed: Aggarwal *et al*., 2012). The co-culture system described in tis work is already capable of maintaining HSPC phenotype, and can therefore be adapted to promote expansion of these cells. If this system is adapted to serum-free media conditions, then it is likely, as demonstrated by the conditioned-media experiments described earlier, that the combination of cytokine-supplementation and the 3D niche microenvironment would support efficient HSPC expansion and phenotype maintenance, thus making this system a highly attractive possibility for clinical application.

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8. Publications

This work contributed in part or completely to the following publications:

Kanthi, D., Lauster, R., Rosowski, M.(2013) Simulation of the bone marrow endosteal niche in a ceramic-based 3D co-culture system. Manuscript submitted.

Poster presentations

Kanthi, D., Lauster, R., Rosowski, M.(2012) Simulation of the bone marrow endosteal niche in a ceramic-based 3D co-culture system. The 4th International Congress on Stem Cells and Tissue Formation, Dresden

Kanthi, D., Lauster, R., Rosowski, M.(2009) Simulation of the bone marrow endosteal niche in a ceramic-based 3D co-culture system. The International Abcam Stem Cell Conference, Antigua.

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