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Co-Culture Strategies Mimicking the Fetal Liver Niche for *In Vitro* Expansion of Hematopoietic Stem Cells

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Thesis to obtain the Master of Science Degree in

Biological Engineering

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Preface

The work presented in this thesis was performed at Stem Cell Institute Leuven (SCIL), KULeuven (Leuven, Belgium), during the period February-October 2019, under the supervision of PhD Student Samantha Zaunz. The thesis was co-supervised at Instituto Superior Técnico by Professor Cláudia Lobato da Silva.

Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Acknowledgments

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Abstract

Hematopoietic stem cell (HSC) transplantations are performed in large numbers every year for the treatment of hematological diseases, based on the ability of HSCs to reconstitute the entire blood system on an irradiated recipient. However, there is still a limiting factor to obtain enough cells to perform these therapies. The stem cell niche plays an important role in HSC self-renewal potential and their fate. During adult life, the bone marrow (BM) constitutes the main hematopoietic niche and maintains the HSC pool, whereas during development, fetal liver (FL) is the site of HSC's extensive proliferation and expansion. This project aims to apply the FL niche as a model to recreate an *in vitro* niche microenvironment to allow expansion of functional adult HSCs. After an efficient differentiation into hepatic stellate cells or endothelial cells, different medium conditions were tested individually on these differentiated feeder cells and on BM derived HSCs, to determine the most optimal medium for the co-cultures. At last, 2D co-culture systems of the previous differentiated cells with primary murine BM HSCs were established and maintained during 10 days. Using FACS analysis, it was concluded that HSCs were able to expand *ex vivo* when co-cultured with endothelial cells and stellate cells. To assess HSC functionality of the cultured HSCs, primary transplantation of the cultured cells was performed. The *ex vivo* expanded HSCs were able to repopulate the recipient mice 4 weeks after transplantation. Moreover, they were differentiated into the blood lineages such as T-, B- and myeloid cells.

Keywords: Hematopoietic Stem Cells, Niche, Fetal Liver, Co-Culture, Hepatic Stellate Cells, Endothelial Cells

Resumo

As células estaminais hematopoéticas têm a capacidade de reconstituir todo o sistema sanguíneo, sendo utilizadas em transplantação para o tratamento de doenças hematológicas. No entanto, existe um fator limitante relacionado com a obtenção de células suficientes para realizar estas terapias. O nicho, que desempenha um papel importante no potencial de auto-renovação e diferenciação das células estaminais, pode ajudar a ultrapassar esta limitação. Durante a vida adulta, a medula óssea constitui o principal nicho hematopoético e é responsável por manter o número necessário de células estaminais hematopoéticas. Por outro lado, durante o desenvolvimento, o fígado fetal é o local onde ocorre extensa proliferação e expansão destas células. Este projeto teve como objetivo recriar o microambiente do fígado fetal e utilizá-lo como um modelo para permitir a expansão de células estaminais hematopoéticas funcionais. Após uma diferenciação eficiente de células hepáticas estreladas e células endoteliais, diferentes condições de meio foram testadas individualmente nestas células e em células estaminais hematopoéticas isoladas da medula óssea de ratinhos para determinar o meio ideal a utilizar nas co-culturas. Finalmente, os sistemas de co-cultura 2D das células diferenciadas com células isoladas da medula óssea de ratinhos foram estabelecidos e mantidos durante 10 dias. Utilizando citometria de fluxo, concluiu-se que as células diferenciadas são capazes de expandir as células estaminais hematopoéticas. Para avaliar a funcionalidade das células cultivadas, foi realizado um transplante primário. As células foram capazes de repovoar os ratinhos receptores, 4 semanas após o transplante, e de se diferenciar nas linhagens sanguíneas (células T, B e mielóides).

Palavras-Chave : Células Estaminais Hematopoéticas, Nicho, Fígado Fetal, Co-Cultura, Células Hepáticas Estreladas, Células Endoteliais

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List of Abbreviations

α-SMA α-Smooth Muscle Actin.

ADRP Adipose Differentiation-Related Protein.

AGM Aorta Gonad Mesonephros.

ANGPTL Angiopoietin-Like Protein.

Angptl2 Angiopoietin-Like 2.

Angptl3 Angiopoietin-Like 3.

ATF4 Activating Transcription Factor 4.

BBB Blood-Brain Barrier.

BM Bone Marrow.

CDH5 Cadherin 5.

CLP Common Lymphocyte Progenitor.

CMP Common Myeloid Progenitor.

CXCL12 C-X-C Motif Chemokine Ligand 12.

CXCR4 C-X-C Chemokine Receptor Type 4.

DLK-1 Protein Delta Homolog 1.

E Embryonic Day.

EC Endothelial Cell.

ECM Extracelular Matrix.

EHT Endothelial-to-Hematopoietic Transition.

EPCR Endothelial Protein C Receptor.

EPO Erythropoietin.

FL Fetal Liver.

GMP Granulocyte-Macrophage Progenitor.

hESC human Embryonic Stem Cell.

HPSC Hematopoietic Progenitor and Stem Cell.

HSC Hematopoietic Stem Cell.

IGF-2 Insulin-Like Growth Factor 2.

LepR Leptin Receptor.

LILRB2 Leukocyte Immunoglobulin-Like Receptor B2.

LT-HSCs Long-Term Hematopoietic Stem Cells.

LYVE Lymphatic Vessel Endothelial Hyaluronan Receptor 1.

MEP Megakaryocyte-Erythroid Progenitor.

MPP Multipotent Progenitor.

MSC Mesenchymal Stem Cell.

NES Nestin.

NG2 Neural Glial 2.

NK Natural Killer.

PDGFRβ Platelet Derived Growth Factor Receptor Beta.

PECAM1 Platelet and Endothelial Cell Adhesion Molecule 1.

PIRB Paired Immunoglobulin-Like Receptor.

qRT-PCR quantitative Reverse Transcription Polymerase Chain Reaction.

ROS Reactive Oxygen Species.

SCF Stem Cell Factor.

SDF-1 Stromal Cell-Derived Factor-1.

Spp1 Secreted Phosphoprotein 1.

ST-HSCs Short-Term Hematopoietic Stem Cells.

STAB1 Stabilin 1.

STAB2 Stabilin 2.

TPO Thrombopoietin.

VCAM-1 Vascular Cell Adhesion Molecule-1.

VE-CAD Ve-Cadherine.

Chapter 1

Introduction

1.1 Hematopoiesis

Hematopoiesis is a hierarchical system, among which Hematopoietic Stem Cells (HSCs) (or Long--Term Hematopoietic Stem Cells (LT-HSCs)) reside at the apex of the hierarchy. [5] HSCs are single cells with the lifelong ability of self-renewing, as well as of differentiating into all mature blood cell lineages, generating the entire hematopoietic system. [8] [9] [10] [11] On Figure 1.1, a simplified schematic representation of the main lineage commitment steps of hematopoiesis is presented. LT-HSCs, the apex of the hematopoietic hierarchy, give first rise to Short-Term Hematopoietic Stem Cells (ST-HSCs) and, then, Multipotent Progenitors (MPPs), which can differentiate into all downstream hematopoietic lineages. MPPs give rise to either a Common Lymphocyte Progenitor (CLP) or a Common Myeloid Progenitor (CMP). CLPs can give rise exclusively to dendritic, B, Natural Killer (NK) and T cells, while CMPs can give rise to either a Granulocyte-Macrophage Progenitor (GMP) or a Megakaryocyte-Erythroid Progenitor (MEP). [12] [1] NK cells can be also derived from the myeloid lineage. [13]

The classical hematopoietic hierarchy roadmap was proposed to illustrate the differentiation process from HSCs to their progenies. This roadmap largely describes the stepwise differentiation process of HSCs from progenitor cells to mature blood cells, following changes in cell-surface marker expression. [9] However, recently, some studies based on single-cell transcriptomics analysis have prompted revision of this classical hematopoietic hierarchy roadmap, suggesting that hematopoiesis is a continuous and highly complex process where heterogeneity is a common feature of HSCs and their progenies. HSC heterogeneity is reflected by the fact that each individual HSC can differ in terms of molecular signature, cellular fate, and functional outcome. [14] Thus, these studies emphasize the complexity of HSC biology which is important to consider while defining and studying the process of hematopoiesis. [15] [16]

1.1.1 Hematopoietic Development

During mammalian embryogenesis, HSCs are found, successively, in multiple embryonic sites. The hematopoietic development, explained below, is schematically represented in Figure 1.2.

First, primitive hematopoiesis occurs during early embryogenesis, at Embryonic Day (E) 7.5 in the mouse, with the emergence of primitive hematopoietic progenitors in the yolk sac. [17] These progenitor cells are neither multipotent nor have self-renewal capability. They give mainly rise to early erythrocytes and macrophages supporting the embryonic development. [18] [19] [20]

Definitive hematopoiesis occurs later in development and gives rise to definitive HSCs capable of reconstituting the entire hematopoietic system. The Aorta Gonad Mesonephros (AGM) region was identified as a major initial site for the generation of definitive HSCs. The later were produced from hemogenic

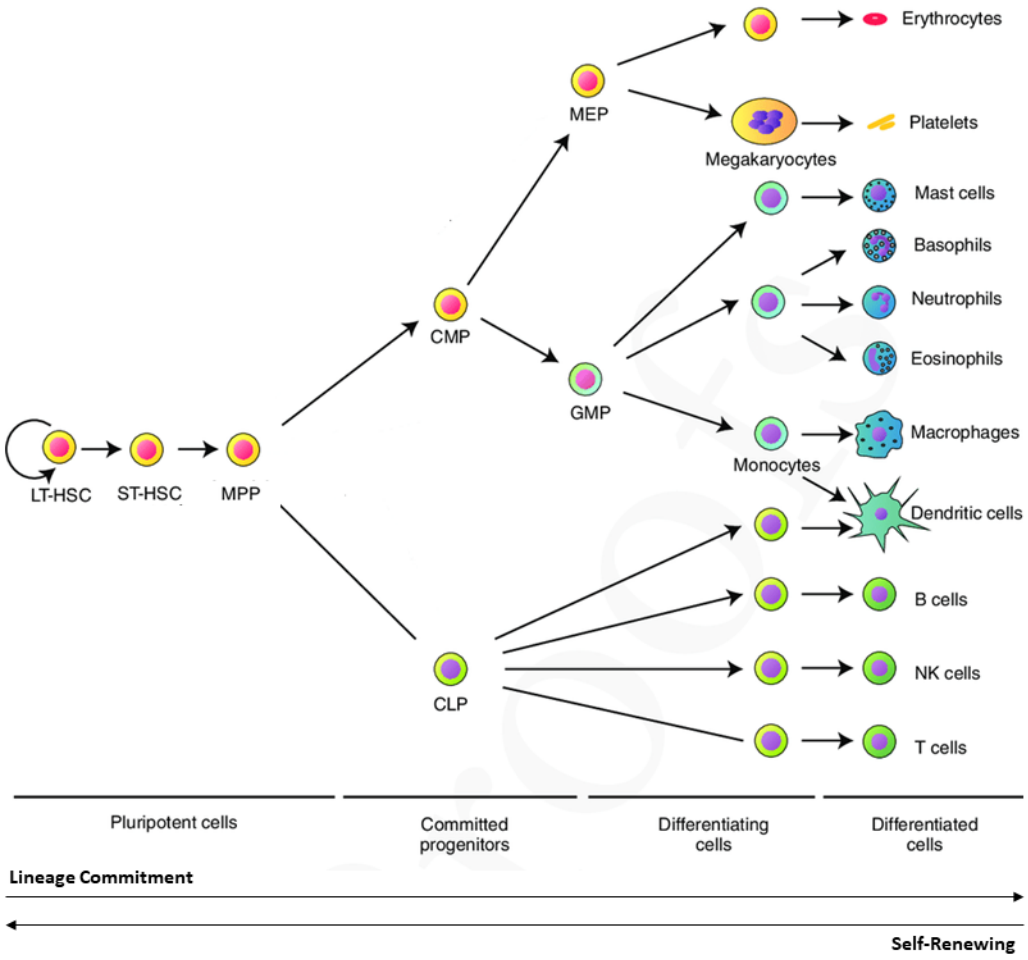

Figure 1.1: The hematopoietic tree. Schematic representation of the main lineage commitment steps of hematopoiesis. Long-Term Hematopoietic Stem Cells (LT-HSCs) are the apex of the hematopoietic hierarchy and can give rise to Short-Term Hematopoietic Stem Cells (ST-HSCs). These will, in turn, give rise to Multipotent Progenitors (MPPs), which can differentiate into either a Common Lymphocyte Progenitor (CLP) or a Common Myeloid Progenitor (CMP). CLPs can give rise exclusively to dendritic, B, Natural Killer (NK) and T cells, while CMPs can give rise to either a Granulocyte-Macrophage Progenitor (GMP) or a Megakaryocyte-Erythroid Progenitor (MEP). NK cells can be also derived from the myeloid lineage. Adpated from [1].

endothelium of the dorsal aorta via Endothelial-to-Hematopoietic Transition (EHT). [21] [22] [23] In the mouse fetus, HSCs are generated in this site around E10.5. Afterwards, they migrate to diverse embryonic sites, including the placenta and the umbilical arteries and veins, before returning to the fetus and populating the Fetal Liver (FL). [24] [25]

As no *de novo* formation of HSCs occurs in the FL, the HSCs which are generated in the AGM region, are responsible for FL colonization. [26] In the mouse, HSCs start to migrate into the FL around E11.5, which quickly becomes the unique center for Hematopoietic Progenitor and Stem Cells (HPSCs) expansion. Combined with transplantation assays, limiting dilution analysis and cells/organ culture, it was demonstrated that several HSCs will rapidly mature into definitive HSCs and expand to form a large HSC pool in the FL, since they are actively proliferative and divide symmetrically (being in cycling phase S/G2/M). This may explain why FL has a dramatic increase of HSCs at E12, where the number of competitive repopulating units increases by 38-fold. [27] [28] [5]

Between E12.5 and E16.5, HSCs not only self-renew to expand in numbers, but also undergo rapid

differentiation to generate vast numbers of hematopoietic progenitors. During this period, HSCs differentiate into myeloid and lymphoid lineage cells, very important for tissue homeostasis. Taken together, FL is a major site for the expansion of HSCs and their derivatives during embryogenesis. [5]

Around birth, HSCs leave the FL environment and migrate into the Bone Marrow (BM), where they soon become quiescent. This means they only rarely proliferate by asymmetric divisions, to replenish the ones that are lost owing to differentiation. [29] Although BM HSCs form a silent reservoir during homeostasis, they can be efficiently activated to proliferate in response to a stress or BM injury. After replenishing the HSC pool, they return to their initial dormancy state. [30] HSCs can also migrate to extramedullary sites (sites outside of the BM), like the thymus and the spleen, to bring about hematopoiesis in case of a BM cavity damage. [31]

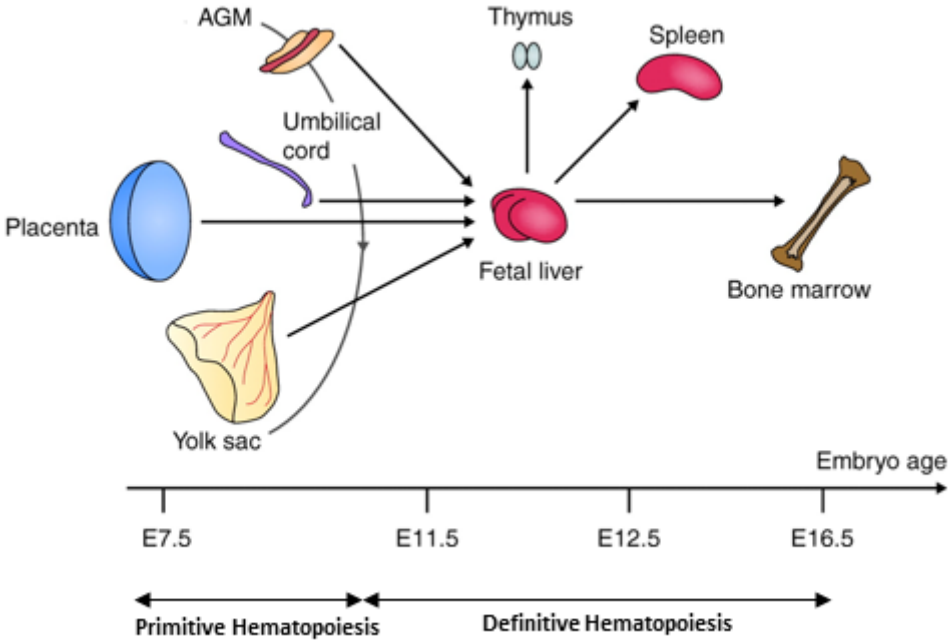

Figure 1.2: Hematopoietic Development. Primitive hematopoiesis gives rise to erythrocytes and macrophages from erythroid progenitor cells that first appear in blood islands in the extra-embryonic yolk sac at embryonic day 7.5 (E7.5). Definitive HSCs, generated in the AGM region, around E10.5, then migrate to the placenta via the umbilical arteries. Following the generation of definitive HSCs, FL quickly becomes the unique center for hematopoietic stem and progenitor cell expansion. In the mouse, HSCs start to migrate into the FL around E11.5 and, between E12.5 and E16.5, they not only self-renew to expand in numbers, but also undergo rapid differentiation to generate vast numbers of hematopoietic progenitors. Arround birth, HSCs migrate into the BM and to extramedullary sites, like the thymus and the spleen. Adapted from [2]

1.1.2 Fetal Liver HSCs and Bone Marrow HSCs Differences

In addition to the decline in the proliferative potential of the BM HSCs compared to FL HSCs during development, functionality differences in HSC self-renewal and differentiation capacity have also been reported. [32] [33] [34] FL HSCs exhibit faster rate of expansion, higher homing and reconstruction ability in transplanted recipient mice than BM HSCs. Moreover, the proportion of myeloid lineage output of transplanted FL HSCs in recipients is shown to be higher than that of BM HSCs. Obviously these functional difference between BM and FL HSCs are underlain phenotypic differences. FL HSCs exhibit

higher expression of the genes involved in cell expansion compared to the quiescent BM HSCs. Surprisingly, the FL HSCs depends on the use of oxidative phosphorylation and the citric acid cycle to fuel their metabolic demands. Thus, FL HSCs possess more active mitochondria and higher endogenous ROS compared with BM HSCs. As the use of an oxygen dependent metabolism is very unusual for stem cells, it is hypothesized that the enhanced use of oxidative phosphorylation in FL HSCs is needed to support their greater proliferation and expansion capacity as compared to the BM HSC. [35]

Collectively, these differences may be attributed to the fact that BM and FL HSCs are regulated by distinct cell-intrinsic and cell-extrinsic factors. Cell intrinsic differences have been shown to be responsible for many functional dissimilarities. For example, both BM HSCs and FL HSCs can respond to extracellular growth factors, such as Stem Cell Factor (SCF) and C-X-C Motif Chemokine Ligand 12 (CXCL12), however the different intracellular signaling pathway are triggered and may result in different cell response. [4] Aside from the intrinsic factors, the different extrinsic signals (so-called niche signals) have been identified to drive and regulate HSC maintenance, behavior, trafficking and expansion. These signals are a direct consequence of the HSC microenvironment and will be discussed in the following section.

1.1.3 HSC Transplantation

The defining property of a HSC is its ability to reconstitute hematopoiesis following transplantation. This property forms the basis of *in vivo* assays of HSC function. Transplantation assays performed in mice have proven invaluable for studying murine and human stem cell biology, facilitating an improved understanding of the immunophenotype, homing ability, engraftment properties, cytokine responsiveness and radiation sensitivity of repopulating cells. [36]

Hematopoietic reconstitution after transplantation depends on the migration and “homing” of the transplanted stem cells to the hematopoietic microenvironments of the recipient. [37] HSC “homing” is a multistep process involving sequential activation of adhesion molecules. The chemokine Stromal Cell-Derived Factor-1 (SDF-1) was the first identified chemoattractant for monocytes, lymphocytes, and $CD34^{+}$ cell homing. [38] $CXCR4^{+}$ progenitors are activated by SDF-1 and vascular ligands, which facilitate firm adhesion to Endothelial Cells (ECs). Cells expressing insufficient levels of CXCR4 detach and return to the bloodstream. Finally, migrating stem cells reach stem cell niches where they interact with supporting cells, adhesion molecules, SDF-1, and growth factors. [38]

The functionality of HSCs to be able to reconstitute the entire blood system in an irradiated recipient, is now being widely applied in clinical HSC transplantation to treat patients with hematological diseases, such as leukemia, lymphoma, and sickle cell disease. However, given the limited number of matching donors and of cord blood-derived HSCs, obtaining sufficient numbers of compatible HSCs remains a limiting factor for BM transplantation therapy. Thus, there is a major need to better understand HSCs niches and develop new strategies to expand HSCs *ex vivo* efficiently for transplantation therapies. [31]

1.2 Stem Cell Niche

The concept of the ‘niche’ was initially suggested by Schofield in 1978 to explain the variation in the self-renewal ability of apparently pure populations of HSCs following transplantation in mice. He hypothesized that the ability of stem cells to self-renew and retain their identity depended also of the environment provided by neighboring, non-HSC cells. [39] This concept has been extended to encompass other aspects of the stem cell microenvironment and has been defined as a small functional compartment with a specific anatomical position and microenvironment within an organ that homes and regulates

stem cell activity, quiescence, self-renewal and differentiation for healthy tissue maintenance and repair. [40] [41] [4] Key components of the niche include direct interactions between stem cells and neighboring cells, secreted factors, inflammation and scarring, Extracelular Matrix (ECM), physical parameters such as shear stress and tissue stiffness, and environmental signals such as hypoxia (Figure 1.3).

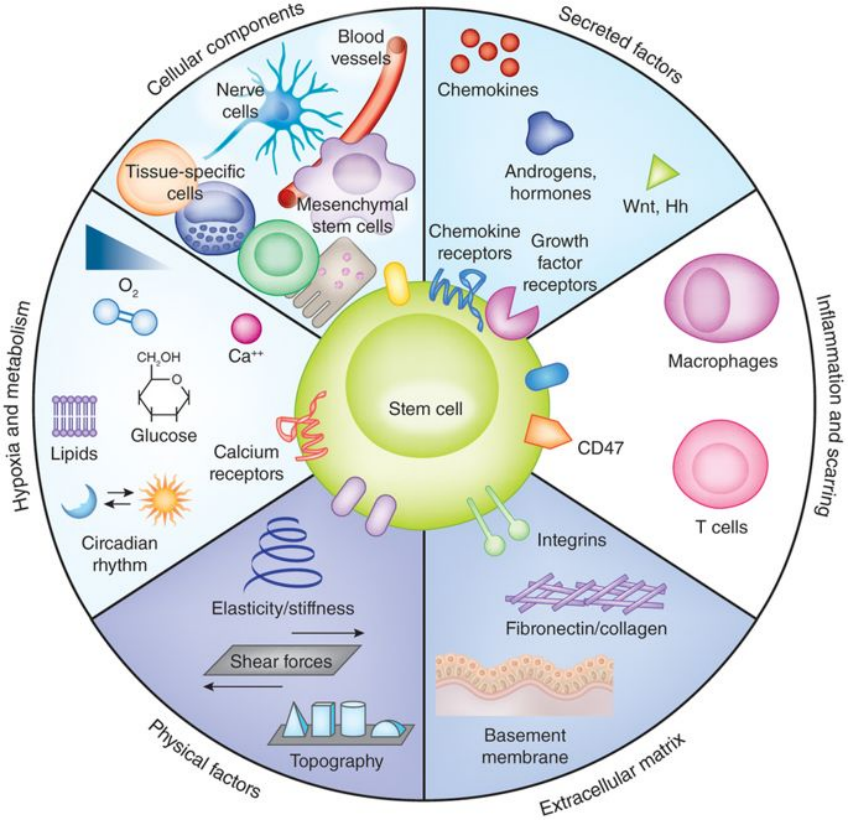

Figure 1.3: Composition of the niche. Stem cell niches are complex and dynamic structures, which include different cellular components, secreted factors, immunological control, extracelular matrix (ECM), physical parameters and metabolic control. The interactions between stem cells and their niches are bidirectional and reciprocal. Adapted from [3].

HSCs niches are present in diverse tissues throughout development, beginning in the AGM region and in the yolk sac, followed by the FL, spleen and BM. [4] Understanding how the different cell types are involved in this hematopoietic niches and whether and how the niches interact with each other and with HSCs is key to understand how the HSC function is regulated. [41]

FL and BM are two important hematopoietic organs that provide an environment where undifferentiated blood stem cells are able to self-renew, and at the same time generate offspring that differentiate into different blood cell types throughout the lifetime of the animal. [42] However, the two hematopoietic sites support different HSC functions: expansion and maintenance. The FL possesses an enhanced ability to support extensive HSC expansion during embryogenesis in order to create the entire stem cell pools needed for one's lifetime. This expansion capacity makes the FL an ideal model to investigate HSC expansion *in vitro*. In contrast to the FL, the BM is the site of adult hematopoiesis. In the BM, the lifelong HSC pool is maintained by replenishing consciously the HSCs that are lost in physiological and pathological condition. Overall, the existence of these two distinct hematopoietic niches is responsible, at least partially, for the functional differences between FL and BM HSC. Thus, further investigation of each niche and its composition could help in the establishment of novel *in vitro* models for HSC maintenance and expansion.

1.2.1 Bone Marrow Niche

The BM niche has been demonstrated to be essential for the maintenance of HSCs, where they reside mainly during adulthood. This hematopoietic niche is very complex and is composed of highly heterogeneous cell populations, including non-hematopoietic cell types such as osteoblasts, ECs, pericytes, adipocytes, Schwann cells and nerves, and other hematopoietic cell types such as macrophages, osteoclasts, megakaryocytes, lymphocytes and neutrophils. [43] [4] Nonetheless, each cell type likely resides in a distinct anatomical area in the marrow cavity, or in unique combinations, to create different niches, which complexity is not yet fully understood. [44] However, some studies have already shown the dynamic and plasticity of the niche, as it is known to adapt and change during, for example, disease and age and thereby tightly regulate HSC function. In Figure 1.4, a schematic representation of the BM niche is present.

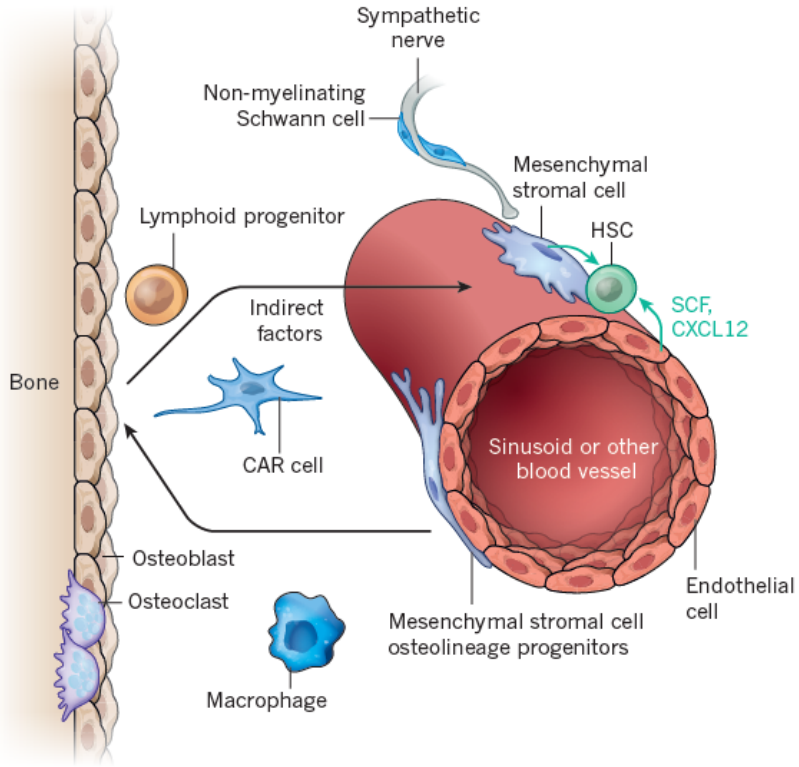

Figure 1.4: The Bone Marrow Niche. HSCs are found mainly adjacent to sinusoids throughout the bone marrow (BM), where endothelial cells and mesenchymal stromal cells promote HSC maintenance by producing SCF64, CXCL12 and other factors. Mesenchymal stromal cells and perivascular stromal cells (fated to form bone *in vivo*) are also present in the BM niche. Other cells also contribute to this niche, including cells near the bone surface in trabecular-rich areas. Other cell types that regulate HSC niches include sympathetic nerves, non-myelinating Schwann cells, macrophages and osteoclasts. The extracellular matrix and calcium also regulate HSCs. Osteoblasts also promote HSC maintenance by secreting CXCL12 and other factors. Adapted from [4].

The existence of different niches in the BM cavity has been proposed and described. These include endosteal and perisinusoidal niches, considered a quiescent and proliferative niche, respectively. Data supports the fact that both coexist in the marrow cavity and might even interact. [45] Histological and functional assays indicate that HSC and MPPs preferentially colonize the endosteal region, in close association with the bone surface. In the perisinusoidal region, HSCs can be also found, as well as committed progenitors and other differentiated cells. [46] [47]

It what concerns the endosteal niche, it was shown that slow-cycling or quiescent HSCs are found in

association with osteoblasts, the main cell type present in this niche. The role of endosteal osteoblasts on the HSC maintenance and self-renewal was first demonstrated *in vitro* [48] [49] and later *in vivo*. [50] A study in transgenic animals with increased or decreased number of osteoblasts showed an increased or decreased number of LT-HSCs, respectively, without affecting any of the others hematopoietic cells in the BM. [51] [52] [50]

Contrary to what has been proposed for the endosteal niche, it is believed that most of the fast–cycling HSCs can be found in the perivascular neighborhood of the blood vessels. In this perivascular niche, HSCs reside on the abluminal side of BM sinusoids, and are supported by the endothelial and perivascular reticular/mesenchymal cells. These cells produce SCF, which has a proliferative role, and express high levels of CXCL12, a chemokine required for HSC maintenance and lodging [53] [54] HSCs were also shown to associate specifically with other types of blood vessels, such as small arterioles. [44] So, the perivascular niche is not only limited by the sinusoids but the HSCs have also been discovered close to the arteries. The so called arteriolar niche, has been suggested to regulate the quiescent HSCs and not the cycling HSCs. [55]

1.2.2 Fetal Liver Niche

As already mentioned, the FL is the major hematopoietic organ during fetal development. The FL is an intricate and highly vascularized hematopoietic organ that can support the extensive expansion of HSCs without loss of stemness, as well as of the downstream lineages. This powerful function of the FL largely benefits from the niche, which consists of heterogeneous cell populations that are associated with the HSCs spatially and regulate their functions (Figure 1.5). [5] Compared to the BM niche, much less is known about the FL niche and its impact on HSC behavior. Only a few studies have aimed to dissect and understand the FL niche microenvironment and its niche cell types.

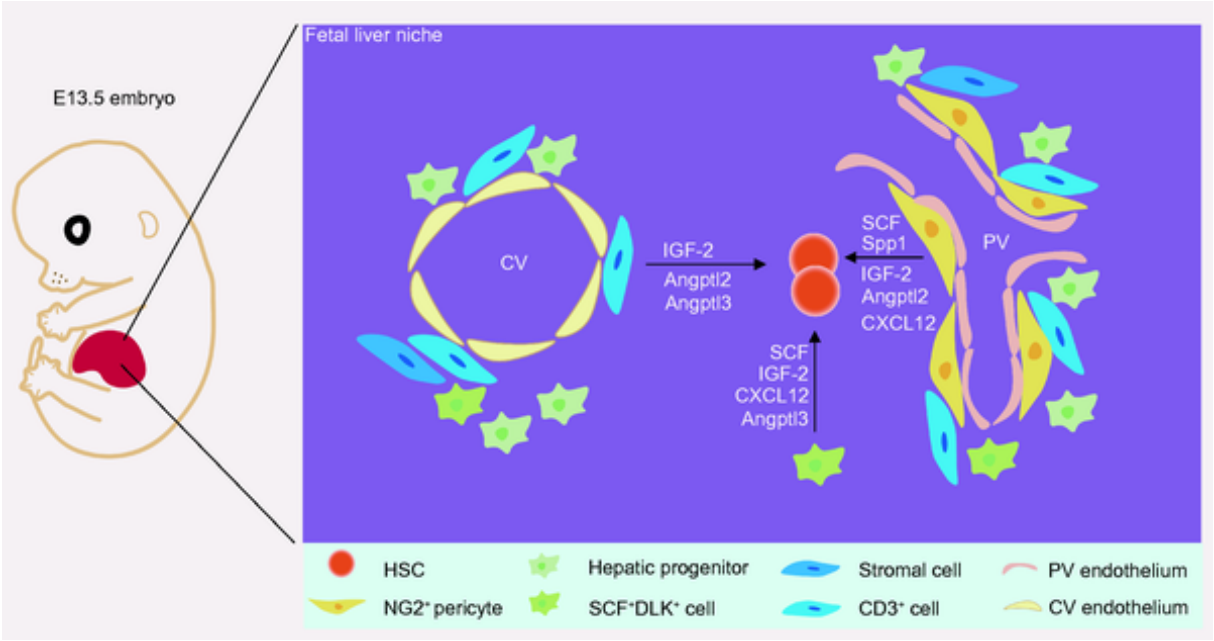

Figure 1.5: The fetal liver niche regulates HSC expansion. A schematic diagram of the fetal liver (FL) niche. Vascular endothelial cells, pericytes, stromal cells and hepatic progenitors are the major niche cells that can modulate HSC extensive expansion through production of growth factors, such as IGF-2, Angptl2/3, SCF and CXCL12. These cell-extrinsic factors will trigger intracellular signaling pathways and stimulate HSC expansion. CV: central vein; PV: portal vessel. Adapted from [5].

Recent evidence shows that the FL niche is composed of diverse cell types that have specific regula-

tory roles, working together to support HSC proliferation, differentiation and maintenance. Among them, the hepatic progenitors, stromal cells, such as pericytes, and ECs are considered to be the key niche cells for the HSC expansion in the FL. [5]

1.2.2.1 Stromal Cells

Stromal cells are a heterogeneous cell population with different differentiation potentials depending upon their environmental niche. This cell population, makes up certain types of connective tissue, supporting tissue that surrounds other tissues and organs, such as the developing FL. [56] It can include fibroblasts and pericytes, as well as Mesenchymal Stem Cells (MSCs) that have the ability to differentiate *in vitro* into osteogenic, adipogenic, and chondrogenic lineages. [57] [58]

Stromal cells constitute the primary component of the FL niche for hematopoietic cells, based on findings obtained from their co-culture with HSCs *in vitro*. Indeed, it has long been possible to demonstrate that preestablished stromal cell monolayers derived from hematopoietic tissues can support long–term hematopoiesis and HSC proliferation *in vitro*. [59] Additionally, diverse immortalized stromal cell lines derived from mouse FL were generated and found to exhibit extensive heterogeneity in their ability to support hematopoiesis *in vitro*. The ones that efficiently maintain long-term transplantable stem cells *in vitro* for prolonged intervals represent only a small fraction of the total stromal cell population. [60]

One specific FL stromal cell line, AFT024, was identified to maintain high levels of transplantable multilineage stem cell activity for extended *in vitro* culture periods (8 to 12 days). [61] [62] It has been proposed that Activating Transcription Factor 4 (ATF4) is, in part, responsible for HSC maintenance, a function of the AFT024 line. ATF4 is a basic region-leucine zipper transcription factor that is widely expressed in many tissues and cells and functions as a stress response factor and a developmental regulator. A study showed that ATF4 deletion does not affect initial HSC generation in the AGM region but markedly impairs the expansion of functional HSCs in the FL. The stromal cells from Atf4$^{-/-}$ FL exhibit abrogated capacity to support HSC repopulating activity *ex vivo*. ATF4 can control the transcription of cytokines, such as Angiopoietin-Like 3 (Angptl3). Angptl3 is a known cytokine that supports HSC activity in the FL, through extrinsic regulation and contributes to HSC maintenance and expansion. So, mechanistically, ATF4 can transcriptionally upregulate expression of Angptl3 in the niche cells, which may guarantee expansion and maintenance of functional HSCs during the unique wave of FL hematopoiesis. [63] [5]

More recently, a study cloned the hKirre gene from human FL fibroblast-like cells and established a stable overexpressing hKirre-AFT024 cell line. HKirre has 97 % of homologous with mkirre gene, isolated from OP9, a mouse BM stromal line, that was demonstrated to play an important role in supporting HPSC expansion *in vitro*. [64] Resultant cells could promote self-renewal and *ex vivo* expansion of human umbilical cord blood (hUCB) HPSCs significantly higher than AFT024-control cells. Also, the expanded hUCB cells retained the capacity of multipotent differentiation as long as 8 weeks and successfully repopulated the BM of sublethally irradiated mice, which demonstrated the expansion of long-term primitive transplantable HPSCs. [65]

Perivascular stellate cells, also known as pericytes, are a subtype of stromal cells. The next section focus on this specific subtype and its importance in the FL niche.

1.2.2.2 Pericytes

Pericytes are perivascular stellate cells that exist in close contact with ECs surrounding capillaries and microvessels. [66] They are located in the basement membrane, which allows them to communicate very efficiently with surrounding cells. (Figure 1.6) [6]

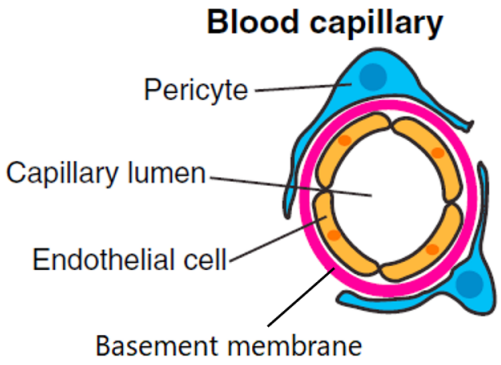

Figure 1.6: Representation of a Blood capillary. Location of pericytes, endothelial cells and basement membranes is indicated. Adapted from [6]

The embryonic origin of pericytes is heterogeneous and, in consequence, their morphology and distribution as well. Pericytes in the central nervous system, thymus, lungs, heart, liver, and gut originate from the ectoderm, while pericytes in most other organs originate from the mesoderm. [41]

Due to the heterogeneity of pericytes, several markers have been used for their identification, both in FL and adult stage. [67] Between them, it can be found markers such as CD146 (transmembrane glycoprotein that functions as an adhesion molecule), Neural Glial 2 (NG2, involved in the recruitment of pericytes during tumor vasculogenesis and expressed by immature pericytes at very early stages of vessel development) [68], Platelet Derived Growth Factor Receptor Beta (PDGFRβ), involved in the proliferation and recruitment of pericytes), α-Smooth Muscle Actin (α-SMA), Nestin (NES) and/or Leptin Receptor (LepR) (Figure 1.7) [41] [7] [26] [69] [70] It is important to mention that none of these markers are pericyte specific, since they are also expressed by other cell types. For example, PDGFRβ is a well-known marker of fibroblasts, while NG2 is expressed on macrophages as well. [71] Also, different pericytes vary in their expression of these markers which depends on the organ, development stage of the pericyte and its maturation state. [72]

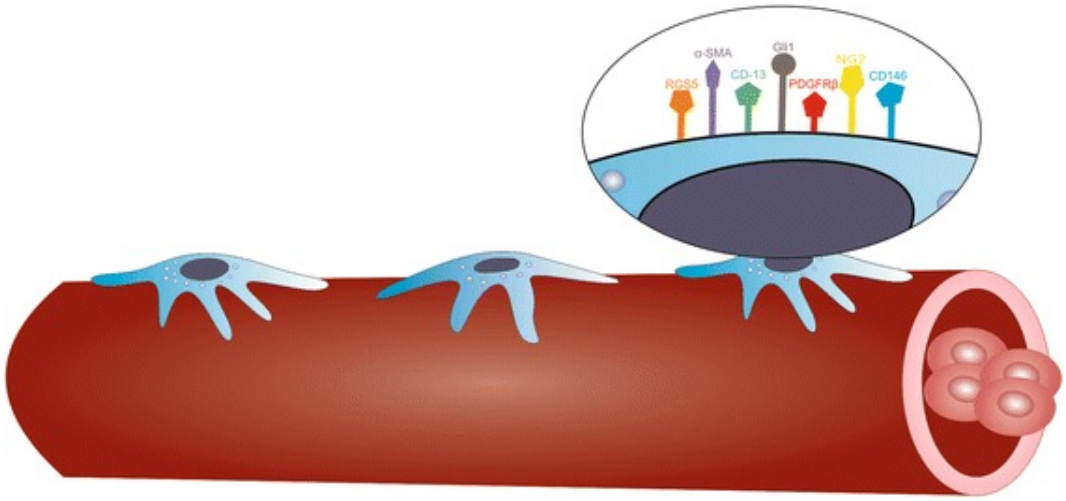

Figure 1.7: Markers of pericytes identification. Due to the heterogeneity of pericytes, several markers are usually used for their identification such as platelet-derived growth factor receptor β (PDGFRβ); neural glial 2 (NG2); CD146; G-protein signaling-5 (RGS5); α-smooth muscle actin (α-SMA); aminopeptidase N (CD-13) and glioma-associated oncogene (Gli1). Adapted from [7].

Pericyte function contribute to homeostatic and pathological physiology. For example, in what concerns the brain, they regulate the formation of the Blood-Brain Barrier (BBB) during development and act

with astrocytes to maintain its function in adulthood and aging. [73] [74] [75] Moreover they have been shown to regulate hematopoietic lineages in the blood, being able to activate lymphocytes. [76] Pericytes also promote EC survival and migration which aids in angiogenesis (the growth of blood vessels from the existing vasculature) and influence negatively tumor growth, in adults. [77] [78] Nevertheless, the major role of pericytes is vascular support through direct cell contact and cell communication via specific junctions. [79] As they are contractile cells, they can regulate vessel diameter and maintain blood vessel integrity and permeability. [68] More recently, stem cell properties have been associated with pericytes, as they act as a repair system in response to injury, by maintaining the structural integrity of blood vessels, and are able to differentiate into cell types from the mesenchymal lineage. [80]

Since not all pericytes share a common phenotype, varying between different organs and tissues, and also within a tissue or niche, different immunophenotypes can be associated with distinct functions, including hematopoietic support. In the adult BM, perivascular cells and HSCs cohabit and arteriolar and sinusoidal pericytes control HSC behavior, maintenance, quiescence and trafficking through paracrine effects. The arteriolar and sinusoidal niche are believed to have a different impact on HSC function, what might be in part due to the different subtypes of pericytes regulating them. Different groups identified and characterized hematopoietic supportive pericyte subpopulations using various markers and mouse models. [41]

Hepatic stellate cells are liver-specific pericytes residing in the space of Disse lining hepatocytes and endothelial sinusoidal cells. [69][81] Morphologically, they appear as long, slender, polymorphic cells, showing an elongated cell body and a prominent nucleus compared to the flat nucleus of ECs, which allows them to be easily distinguished from one another. They display a dendritic morphology and embrace, with thorn-like micro projections, the EC layer of the sinusoids providing physical contact not only to sinusoidal ECs but also to the cell body of the hepatocytes. [66] [7] [69] They communicate with ECs along the length of the vessel by physical contact and paracrine signaling. [66]

In a healthy adult liver, hepatic stellate cells maintain ECM homeostasis and accumulate vitamin A (retinoids) storage in the body in the form of retinyl esters in cytoplasmic lipid droplets. [81] [70] [82] They also comprise specialized functions such as hemodynamic functions, support of liver regeneration, and immunoregulation. [69] In normal liver, hepatic stellate cells are quiescent and are filled with cytoplasmic lipid droplets containing retinyl esters (especially retinyl palmitate, 37 % of the lipid droplet composition) and triacylglycerols, accounting for more than 70 % of their lipid content. Upon injury, hepatic stellate cells are activated to proliferative, contractile, myofibroblast-like cells with expanded rough endoplasmic reticulum that produce fibrotic scar tissue, participating in the wound healing response of the liver. Hepatic stellate cells activation, in which the cell changes from a quiescent to a more myofibroblastic phenotype, is characterized by the progressive loss of lipid droplets and retinoid content, along with an increase in α-smooth muscle actin (α-SMA) expression. [70] [83] If injury persists, activated hepatic stellate cells increase the production of ECM components and reduce degradation, becoming the main fibrogenic cell type of the liver. [81] Cytoplasmic lipid droplets are coated by lipid droplet-associated proteins, mostly members of the PAT domain family, whose founding members are perilipin, Adipose Differentiation-Related Protein (ADRP) (or adipophilin), and tail-interacting protein of 47 kDa (TIP47). [83]

Embryonic development of hepatic stellate cells is not well understood. In mice, at E9.5, mesenchymal cells in the septum transversum mesenchyme specify to mesenchymal progenitors that give rise to mesothelial liver cells. [84] During liver morphogenesis at E11.5, submesothelial and mesothelial cells migrate inward from the liver surface to generate hepatic stellate cells and perivascular mesenchymal cells. [84] [72]

Interestingly, a group analysed the spatial association between FL HSCs and niche cells and found that FL HSCs have a close physical association with portal vessel and Nestin+ NG2+ pericytes, and

about 40 % FL HSCs reside within 20 μm of portal vessels. The availability of niche cells to sustain proliferating HSCs may thus be tied to the innate growth of the portal vascular tree. So, expansion of HSCs and Nestin+ cells during fetal development is governed by fractal-like geometries of the portal vessel niche. [4] The rapid loss of HSCs in the postnatal liver can be explained by the dramatic postnatal changes in portal vessels that lead to a loss of niche cells and, thus, the migration of HSCs away from the portal niche. In fact, portal vessels expression levels of arterial markers Neuropilin-1 and Ephrin-B2, is greatly reduced after birth. At postnatal day 8, portal vessels, were shown to be devoid of Nestin+ cells, in part due to Nestin+ cell apoptosis, and expressed Ephrin-B4, suggesting that they transited into a vein phenotype. It was found that, in contrast to the FL, few HSCs ($<$ 3 %) were located within 20 mm of postnatal portal vessels, after birth, and mean HSC distances to portal vessels increased significantly. These results thus further underscore the critical role for the arterial portal vessels in forming a FL niche. [24]

Additionally, Nestin+ pericytes can produce growth factors such as SCF, Secreted Phosphoprotein 1 (Spp1), Insulin-Like Growth Factor 2 (IGF-2), Angiopoietin-Like 2 (Angptl2) and CXCL12 to support HSC expansion. The authors showed that these cells are responsible for HSC expansion *in vitro* by using co-culture systems and *in vivo* by depleted these niche cells, showing a decrease of HSC functionality in the FL. This group also dissected the intricate vascular network and generated the three-dimensional (3D) reconstruction of FL vasculature. In addition, they found that the expression of growth factors (SCF, Angptl2, IGF-2), presents similar levels from E12.5 to E14.5, but the vascular structure extends obviously accompanied with the HSC expansion in FL. Using this model, they further emphasized that the extension of niche structure may govern the HSC expansion. [24] [5] [31]

In vitro, adult hepatic stellate cells can maintain HSCs in a similar manner to BM mesenchymal stem cells. Stellate cells may therefore play an important role in maintaining the liver microenvironment and expanding the HSC pool. [85]

1.2.2.3 Hepatoblasts

Hepatoblasts are hepatic progenitor cells that expand and give rise to either hepatocytes or cholangiocytes (epithelial cells that line the bile ducts) during liver development. [86] The analysis of FL samples has revealed the presence of FL hepatoblasts, that express liver-specific markers and exhibit cellular bipotency, with an ability to generate progeny of a hepatocytic or biliary fate. [87] [88]

Murine hepatoblasts have been characterized by their expression of Protein Delta Homolog 1 (DLK–1) and a range of important hematopoietic cytokines, such as Erythropoietin (EPO), TTP, IL-6, SCF, and Flt3 ligand. Hepatoblasts also express high levels of ECM molecules, including vitronectin, fibronectin and tenascin C that are expressed under the control of TGFb1 signaling. [26]

These cells have been shown to have an important role in the FL HSC niche. The importance of hepatoblasts in expanding HSCs was demonstrated by the co-culture of hepatocyte cell lines (MMH from Met Murine Hepatocyte) with FL HSCs, which resulted in a large increase in its number (40-fold to 80-fold expansion of total hematopoietic cells). [89] Their importance was further underscored by the analysis of mutant mouse embryos, lacking hepatoblasts, where hematopoiesis was strongly affected following a decrease in cytokine expression in the FL, such as EPO and SCF, relative to wild-type mice. Accordingly, these embryos displayed a strong decrease in the number of HSCs. [90]

More recently, a co-culture system established that DLK+ fetal hepatic progenitors are the authentic supportive cells for expansion of HPSCs. This is supported by the fact that DLK- cells are incapable of expanding HSCs. Physical contact between these DLK+ cells and HSCs was shown to be crucial for maintaining this long-term expansion. [29]

More specifically, SCF+ DLK+ FL hepatic stem and progenitors cells have been identified as an

important cell population that supports the maintenance of HSCs in *ex vivo* culture. These cells were reported to express growth factors important for HSC expansion, including Angptl3, IGF-2, SCF and Thrombopoietin (TPO). [31] [91]

Ex vivo co-culture of these primary FL hepatoblasts with BM HSCs leads to enhanced HSC long–term repopulation and HSC expansion. Treatment of these FL cells with anti–IGF-2, abrogated their HSC supportive activity what suggests that IGF-2 is a key molecule produced by these cells that stimulates HSC expansion. [92] Moreover, it was also found that Angptl2 and Angptl3 secreted by these cells can facilitate 24- and 30-fold expansion of HSCs after culture *in vitro*. [93] It was further suggested that Angptl2 could mediate its effects through the Leukocyte Immunoglobulin-Like Receptor B2 (LILRB2) receptor. [26]

Another study discovered a unique CD34(lo)CD133(lo) cell population in the human FL that gives rise to cells in the hepatic lineage. The authors showed that CD34(lo)CD133(lo) cells express growth factors that are important for human HSC expansion: SCF, IGF-2, CXCL12, and factors in the angiopoietin-like protein family. Co-culture of autologous FL HSCs and allogenic HSCs derived from cord blood with this cell population showed that these cells support and expand both types of HSCs (18-fold increase in the 7th day of culture compared to 3-fold in the control group). [94]

1.2.2.4 Endothelial Cells

ECs are the main regulator of vascular homeostasis, interacting with circulating blood cells and cells present in the vascular wall, like the pericytes. As they are the interface between blood and tissue, ECs are the main responder to all changes in blood composition and flow and play a central role in the control of vascular function. Besides participating in all aspects of vascular homeostasis, ECs also have a role in pathological processes like thrombosis, inflammation, or vascular wall remodeling. [95]

Endothelial and hematopoietic cells are both originated from a common precursor, the hemogenic endothelium, which is present in the yolk sac, the AGM region, and the placenta. [96]

Between the processes where ECs participate, the support of hematopoiesis is of great importance for both BM and FL ECs. Murine ECs were shown to support hematopoiesis *ex vivo* and promote FL HSC proliferation and differentiation. When EC lines were cultured, hematopoietic colonies expanded in close contact with the ECs, supported through the expression of SCF/KitL. [26] [97] In human, it was described that direct physical contact between HSCs and ECs may be mediated by E-Selectin and Vascular Cell Adhesion Molecule-1 (VCAM-1) that are selectively expressed on both ECs of adult and fetal hematopoietic organs. [98]

The molecular mechanism of HSC maintenance in mouse FL using HSCs expressing Endothelial Protein C Receptor (EPCR), was also investigated. EPRC, expressed by murine FL HSCs, can bind the activated protein C (expressed on the surface of the ECs) that will induce protease-activated receptor 1 signaling, which inhibits apoptosis and maintains HSC self-renewal activity. [99]

More recently, human FL sinusoidal endothelial cells (FLSECs), engineered to express adenoviral E4 or F1 genes (hFLSECs-E4orF1), were shown to be capable of efficient expansion *ex vivo* for human cord blood HSPCs, with a 3.15-fold increase of CD34+ cells compared to control group. Since primary FLSECs have limited expansion capability and lose their phenotype rapidly in culture, making physiologic application as *in vitro* hematopoiesis microenvironment challenging, the authors transfected them with the E4 or F1 gene of adenoviruses, which have been demonstrated to maintain long-term survival and facilitate organ-specific purification of ECs by providing an “antiapoptotic” signal. [100] Long–term co–culture, during 14 days, of these cells with HSCs resulted in the generation of substantially more total nucleated cells, without loss of functionality after transplantation. In addition, HSCs exhibited Notch signaling activation, important to regulate HSC maintenance and homeostasis. [101]

1.3 *Ex Vivo* Culture Approaches for the Expansion of HSCs

Although researchers aim to develop various strategies to culture HSCs, the field is still facing a major challenge when it comes to truly expand functional HSCs *in vitro*. In order to define the better culture conditions for *ex vivo* HSC expansion, it is imperative to understand the molecular signals and the signaling pathways provided by the HSC niches.

1.3.1 Cytokines Regulating HSC *Ex Vivo* Expansion

So far, the majority of the published studies that involve HSC culture, employ cell culture medium supplemented with one or more cytokines. These cytokines have multiple actions, mediated by receptors whose cytoplasmic domains contain specialized regions, initiating various responses — survival, proliferation, differentiation commitment, maturation, and functional activation. [102]

The most commonly used cytokines in HSC culture medium are SCF, TPO, Flt3 ligand and interleukin family. Those have first been identified to be produced by BM niche cells. [103] Data showed that with the addition of these growth factors, alone or a combination of them, affects BM HSC maintenance and proliferation *ex vivo*. [104] [93] SCF is a widely-used growth factor, added in nearly all HSC cultures, to promote HSC expansion. Its receptor, c-Kit, encoded by the c-kit proto-oncogene, is a cell surface protein that is critical for proliferation, survival and differentiation in the hematopoietic system. [105] The binding of SCF to the c-kit receptor triggers downstream signaling cascades, such as MEK/ERK and PI3K/Akt kinase signaling pathways, and further upregulate the expression of expansion–associated regulators. [106] The cytokine TPO is the chief regulator of megakaryocyte and platelet production, signaling via its receptor, Mpl. JAK2 and TYK2, proteins from the Janus kinase (JAK) family, are phosphorylated upon TPO binding to Mpl, regulating platelet production and maintenance and expansion of HSCs. [107] FLT3 is a receptor tyrosine kinase expressed by immature hematopoietic cells and is important for the normal development of stem cells and the immune system. The ligand for FLT3 is expressed by marrow stromal cells and other cells and synergizes with other growth factors to stimulate HPSC proliferation. [108] Finally, from the interleukin family, IL-2, IL-3, IL-5, IL-6, IL-7 and IL-11 stand out for having an action in the regulation of hematopoiesis. [102]

As already mentioned earlier, the FL niche is much better specialized in functional HSC expansion compared to the BM, which mainly preserves the dormant HSCs. Therefore, it is not only necessary to identify the FL niche cells surrounding HSCs, but also to elucidate which signaling pathways impact HSC expansion in the FL. Previous studies have already indicated that HSCs are able to expand when co-cultured with FL cells. [91] Further analyses unravel that growth factors produced by the co-cultured cells are the major cause of HSC expansion. Similar to the BM niche, SCF, TPO and some interleukins have been proposed to be secreted by some FL niche cells to support hematopoiesis during development. On the top of those, other growth factors such as IGF-2 have been discovered to play an important role in the FL HSC proliferation. IGF-2, segregated by FL cells can promote HSC expansion. A high concentration of this growth factor binds to the receptors, IGF1R, IR and IGF2R, and activates the downstream signaling cascades. Along with SCF and TPO, IGF-2 activates mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI-3K) pathways to facilitate HSC expansion. [92]

Moreover, Angiopoietin-Like Proteins (ANGPTLs), a family of seven secreted glycoproteins, are also known to support the activity and expansion of HSCs *in vitro* and *in vivo*. [93] However, the downstream signaling pathways triggered remain to be discovered. Recently, the receptor of ANGPTL proteins, immune-inhibitory receptor human LILRB2 and its mouse orthologue Paired Immunoglobulin-Like Receptor (PIRB) were identified and its activation can support HSC expansion *ex vivo*. [109] Gene expression studies showed that Angptl2 can increase NOTCH activation through binding of LILRB2, and

then activates intranuclear target genes, including MYC and RUNX1 (known to support HSC expansion). The activation of MYC expression could explain why HSCs expand *ex vivo* with the addition of Angptl2. [93]

Also, Notch signaling molecule was verified to regulate HSC maintenance and homeostasis, in both FL and BM. [110] [111] Notch receptors (NOTCH1-4) are single-pass type I transmembrane receptors implicated in various developmental and disease processes including the activation of the hematopoietic program. They are synthesized as $\sim$ 300 kD full-length precursor proteins that undergo a series of proteolytic cleavages in order to become fully activated. Notch signaling pathway is mediated by the cell-cell contact and is engaged in diverse biological processes. In mouse FL, it was revealed that the balanced level of Notch signaling regulated by BLOS2 is indispensable for HSC homeostasis. Over activated Notch signaling will augment the frequency of HSCs, whereas disturb their self-renewal ability. [110]

1.3.2 Fetal Liver Models for Expansion of HSCs

Extensive researches have been performed to determine the optimal conditions critical for *ex vivo* HSC expansion, leading to various expansion techniques development. In contrast to the BM niche mimicking culture strategies, only few studies have tried so far to establish FL-like culture systems. These studies focus, mainly, on the establishment of co-culture systems with either cell lines such as AFT024 [61], MMH [89] and hFLSECs-E4orF1 [101] or primary cells including Nestin+ pericytes [24], DLK+ cells [29] and CD34(lo)CD133(lo) cells [94]. [112] These studies were already described previously for each FL cell type.

Thus, to recapitulate the FL organ *in vivo* and to establish FL-like culture systems for HSC expansion for clinical applications, further investigation is required. The help of evolving views and emerging techniques can greatly facilitate the understanding on the crosstalk between the FL niche and HSC expansion and further help on the reconstruction of a FL organoid *in vitro* to acquire sufficient functional HSCs for clinical use.

An important advancement in the tissue engineering field is the design of bioscaffolds. The ECM, including the components of structural and functional proteins, has become the popular three-dimensional scaffold for tissue reconstruction. Moreover, a significant advancement in the field of bioscaffold design has been the utilization of decellularized tissue as the three-dimensional scaffold in tissue engineering strategies, which allows the protection of the macrovascular skeleton and the niche cells to easily enter. Liver cells, ECs and stromal cells can enter the scaffold and reseed the vascular network to realize the functional artificial FL organoid. [88] The Verfaillie lab is currently working on these area, which promise to reconstruct FL organoids that will play an important role in HSC expansion and drug discovery.

Chapter 2

Aims

Although HSC transplantation has been used in clinics since decades to treat diverse hematological diseases, the lack of matching donors and the inability to expand HSCs *in vitro*, while preserving HSC functionality, are still a major burden in the field. It is believed that the latter is due to the fact that we do not understand key HSC-intrinsic and -extrinsic factors that regulate their functions. The extrinsic molecular signals are provided by the so-called stem cell niche that supports the self-renewal potential and tightly regulates HSC fate. During adult life, the BM constitutes the main hematopoietic niche and maintain HSC pool, whereas during development the FL is the site of HSC's extensive proliferation and expansion. [113] [31]

This project aims to apply the FL niche as a model to recreate an *in vitro* niche microenvironment that allows expansion of functional adult HSCs. We hypothesize that culturing BM HSCs with the cell population(s) composing the *in vivo* FL, will enable *ex vivo* self-renewing divisions of HSCs without ageing, such as seen during developmental hematopoiesis in the FL.

Therefore the objectives of my thesis are:

- establishment of 2D co-culture systems for primary murine BM HSCs using the different FL niche cells (endothelial cells and pericytes), derived from an inexhaustible pluripotent stem cell source;

- determination of the regulatory effect of the co-culture systems on primary murine HSC maintenance and expansion;

- determination of the functionality of the *in vitro* cultured HSCs in the engineered FL-like microenvironment.

Chapter 3

Results and Discussion

The main goal of this project was to establish co-culture systems using human Embryonic Stem Cells (hESCs)-derived hepatic stellate cells or Endothelial Cells (ECs), both part of the Fetal Liver (FL) niche, for *in vitro* expansion of Hematopoietic Stem Cells (HSCs). To fulfill this thesis' goal, hESCs were differentiated into fetal hepatic stellate-like cells and EC-like cells. The differentiated niche cells were characterized for cell type specific expression markers at various time points throughout the differentiation. To set up the best medium condition for the co-culture systems, the effect of different medium conditions was first evaluated on the previously differentiated cells and on HSCs isolated from mice Bone Marrow (BM). Lastly, the co–culture systems were established between HSCs and hepatic stellate-like cells or EC-like cells. The influence of each niche cell type on the *ex vivo* maintenance and expansion of HSCs was assessed.

3.1 Generation of Fetal Liver "Niche" Cells from Pluripotent Stem Cells for *Ex Vivo* HSC Expansion

3.1.1 Differentiation of Fetal Liver Stellate-Like Cells

hESCs were differentiated into hepatic stellate cell-like cells following the protocol developed previously in the laboratory. [81] These cells resemble human fetal liver hepatic stellate cells, at the transcriptional, cellular and functional levels.

To assess the efficiency of the differentiation, cells were characterized based on their morphology and on their expression profile of various stellate cell-specific markers, through quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR), at various time points throughout differentiation (D0, D2, D4, D6, D8 and D10). These results are present on Figure 3.1 (A) and (B), respectively.

In what concerns cell morphology, it was observed an evolution from hESC colonies to an almost homogeneous hepatic stellate cell-like cell layer (Figure 3.1 (B)). Some fibroblast islands, composed by some stellate cells that got activated during differentiation and aquired a fibroblastic morphology, could also be observed (indicated with a black arrow on day 8 of differentiation). The hESC colonies were composed of small round cells and presented jagged edges. Cells began to change their morphology to a fusiform one, while expanding, and later, around day 6, began to acquire a stellate (star) shape (oval shape with scattered edges).

The qRT-PCR results indicate that the cells display an increased expression of ACTA2, COL1A1, PDGFRβ and LOXL2, however they did not express higher levels of ALCAM compared to undifferentiated cells. P75NTR expression levels decreased as it was expected. [81] In addition, DES expression

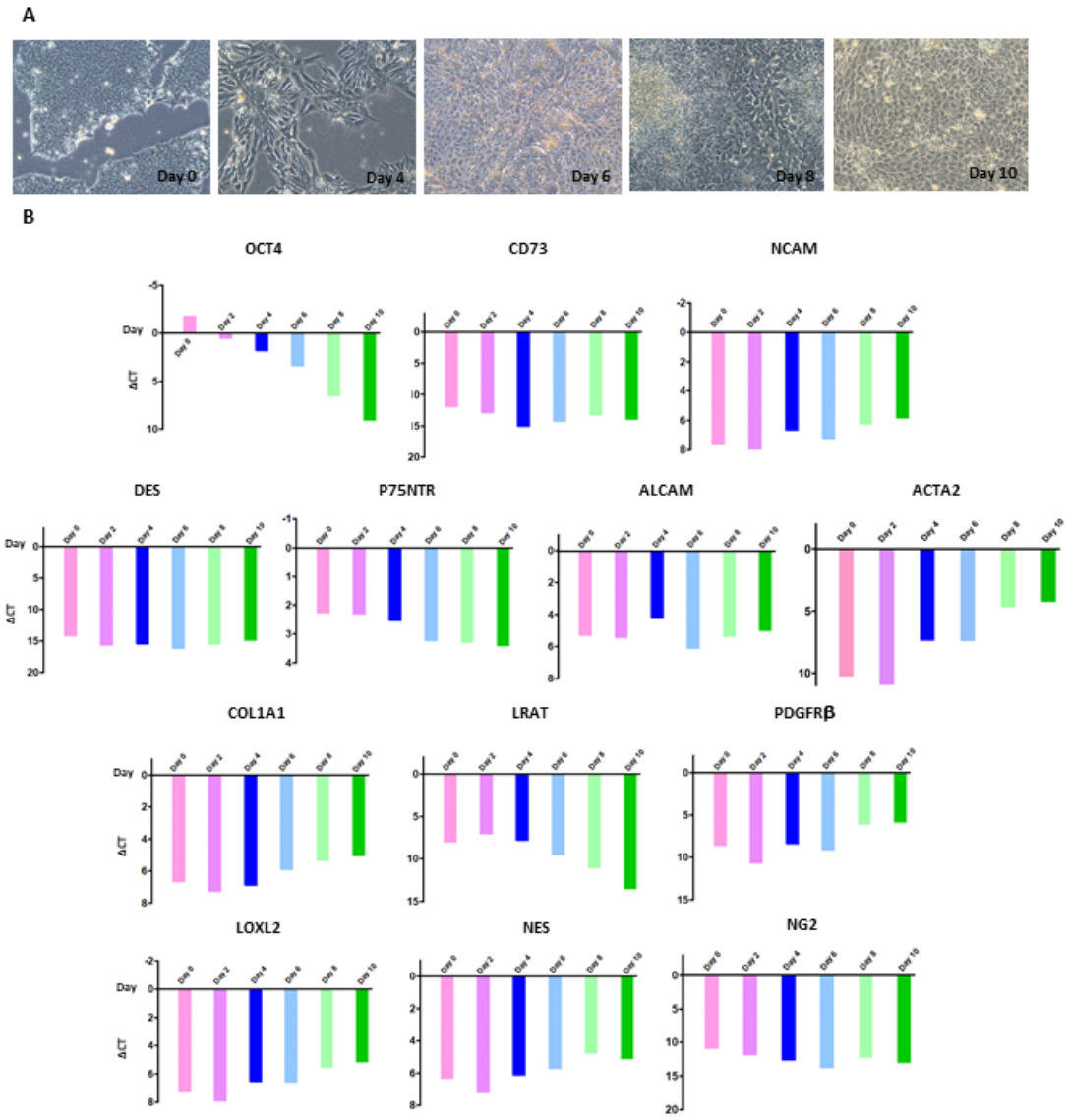

Figure 3.1: Generation of Hepatic Stellate-Like Cells from a hESC Line. (A) Representative images of the cell morphology on days 0, 4, 6, 8 and 10 of differentiation (10x magnification). **(B)** Gene expression of pluripotency (OCT4), mesodermal and submesothelial phenotype markers (CD73 and NCAM) and hepatic stellate cell markers (DES, P75NTR, ALCAM, ACTA2, COL1A1, LRAT, PDGFRβ, LOXL2, NES and NG2) along differentiation. mRNA levels were assessed by qRT-PCR and expressed as the arithmetic mean $\pm$ standard deviation of the mean (SD). n = 1 independent differentiations.

was more or less constant throughout differentiation, and LRAT expression even decreased compared to day 0 cells.

The main limitation of the qRT-PCR results is that they were only performed once with one sample. For these results to be more reliable, the analysis of more independent differentiations (at least 3) should take place, as well as more biological replicates should be included to consider the variation of the differentiation between wells. This way, the qRT-PCR results would be confirmed, in particular CD73 and ALCAM expression. If the CD73 expression is indeed not enhanced, this would be in contrast to what has been published by Sancho-Bru and his group. [81] To clarify in which stage of development the differentiated stellate cells were along differentiation, additional markers known to be expressed in embryonic development could have been checked for. Between them, there are Platelet-Derived Growth Factor Receptor Alpha (PDGFRα) and Kinase Insert Domain Receptor (KDR) that are mesodermal and mesothelial phenotype markers. Reelin (RELN) and Protocadherin 7 (PCDH7) could have been checked as mature hepatic stellate cell markers. [81] However, a similar morpholgy of the differentiated stellate-like cells was observed on other differentiations along this project.

Additionally, qRT-PCR has some drawbacks like bulk analysis of the cells, where there might exist different subpopulations, and the fact that it assesses RNA and not actually protein expression. [114] Immunophenotyping, like flow cytometry, could be an alternative to confirm cell phenotype and overcome these drawbacks. Immunostaining could also be performed to detect specific proteins, like, for example, alpha Smooth Muscle Actin (α-SMA or ACTA2), in the sample.

3.1.2 Differentiation of Fetal Liver Endothelial-Like Cells

hESCs were differentiated towards endothelial-like cells during, at least, 8 days, following the protocol optimised in the Verfaillie Lab. Similar evaluation of the differentiated ECs was done as for the hepatic stellate-like cells, by bright field microscopy and qRT-PCR, at various time points throughout differentiation. Some representative images of the cell morphology and the qRT-PCR gene expression results are also present on Figure 3.1 (A) and (B), respectively.

In what concerns to cell morphology, it was observed an evolution from hESC colonies to a homogeneous EC-like cell layer (Figure 3.2 (B)). Similar to what was observed for hESC colonies, these colonies were composed of small round cells presenting jagged edges. Over the time of differentiation, the typical hESC colonies disappeared and cells began to proliferate and become more spherical. Later, around day 6, cells began to acquire a more elongated shape, characteristic of ECs. If the cells did not present the typical endothelial appearance, they were discarded.

After the up-regulation of Endo-TF, it was observed a significant expression of transcripts for well known specific endothelial marker genes, such as CD31 (also known as Platelet and Endothelial Cell Adhesion Molecule 1 (PECAM1)) and Ve-Cadherine (VE-CAD) (also known as Cadherin 5 (CDH5)) in the hESC derived cells. The expression of Stabilin 1 (STAB1) and Stabilin 2 (STAB2) genes, which encode a large, transmembrane receptor protein expressed on sinusoidal ECs of liver, spleen, and lymph node was also analysed. [115] [116] Both of these markers did show the highest expression on day 8 differentiated cells. Lymphatic Vessel Endothelial Hyaluronan Receptor 1 (LYVE) and CD32B marker expression was not detected. Both markers seemed to be barely expressed by our EC-like cells. Since LYVE is considered a selective marker for lymphatic ECs, this observation was expected. [117] [118] However, a small increase of its expression level from day 0 to day 14 was observed. For CD32B marker, high expression levels at the beginning of the differentiation were expected, followed by a downregulation over time (pattern conserved across several species). [119] Instead, this marker was only slightly expressed at day 8, showing a imperceptibly downregulation from day 12 onwards. Interestingly, all these endothelial markers showed a slightly decrease in the expression level on day

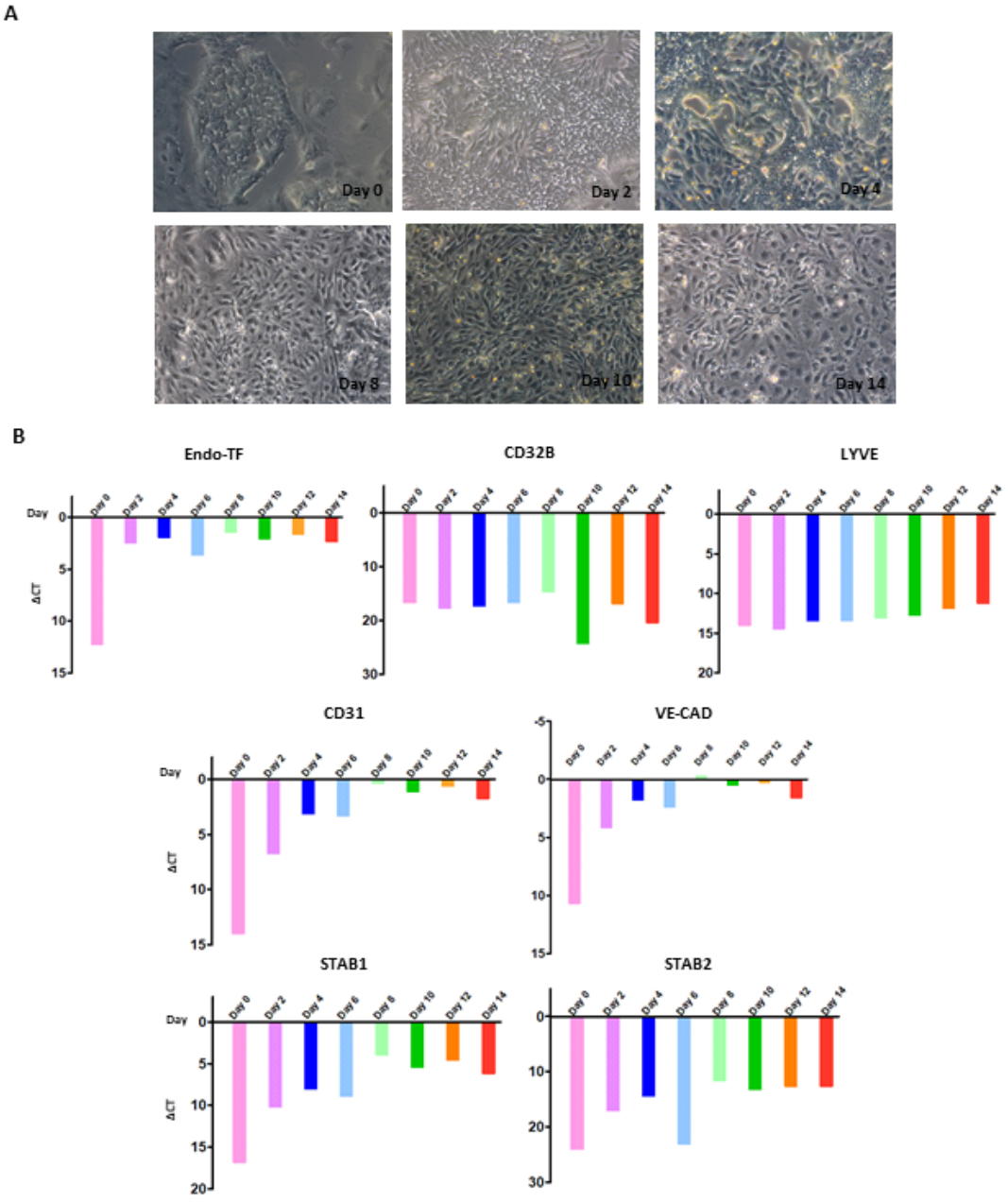

Figure 3.2: Generation of Endothelial-Like Cells from a hESC Line. **(A)** Representative images of the cell morphology on days 0, 2, 4, 8, 10 and 14 of differentiation (10x magnification). **(B)** Gene expression of Endo-TF, CD32B, LYVE, CD31, VE-CAD, STAB1 and STAB2. mRNA levels were assessed by qRT-PCR and expressed as the arithmetic mean $\pm$ standard deviation of the mean (SD). n = 1 independent differentiations.

14 compared to day 8. This might indicate that differentiated cells start to lose their endothelial-like properties. Therefore, day 8 EC-like cells were used for all the subsequent experiments.

Similar to what was stated in the previous Section (Section 3.1.1, Differentiation of Fetal Liver Stellate-Like Cells), the analysis of more independent differentiations (at least 3) as well as more biological replicates, to consider the variation of the differentiation between wells should have taken place, for these results to be confirmed and more reliable. However, a similar behavior of the cells was observed on other differentiations along this project. Additional markers, like Receptor Tyrosine Kinase (TEK or Tie2) and KDR, could have been checked. [120] FACS analysis could be an additive technique to confirm cell phenotype and overcome qRT-PCR drawbacks.

3.2 Defining Cell Culture Medium Conditions for Fetal Liver Co-Culture Strategies

In order to establish co-culture systems for HSCs with either hESC-derived stellate or ECs, different medium conditions were first tested individually on these differentiated feeder cells and on BM derived Hematopoietic Progenitor and Stem Cells (HPSCs). To determine the most optimal medium for the co–cultures, phenotype of the cultured cells was assessed based on their morphology and gene expression profile.

3.2.1 Effect of the Co-Culture Medium Conditions on Hepatic Stellate-Like Cells

The effect of different medium conditions was evaluated on the differentiated cells. In the first part of this experiment, we tested if the hematopoietic medium, supplemented with stellate growth factors, could support the survival and maintenance of the stellate phenotype. Therefore, day 10 NS and day 11 S stellate cells were cultured in either the stellate or hematopoietic medium, both supplemented with stellate cytokines (control S and condition S1, respectively). In addition, a third condition was included in our experiment, to test the potential effect of the hematopoietic cytokines, on the stellate cells (condition S2). These cytokines are secreted *in vivo* by adjacent niche cells and are needed for HSC *ex vivo* culture to ensure their survival and proliferation, being usually added in standard HPSC culture.

In Figure 3.4, the results obtained in this experiment are present, including some representative images of the cell morphology at specific days and qRT-PCR results for pluripotency (OCT4), mesodermal and submesothelial (CD73 and NCAM) and hepatic stellate cell markers (DES, P75NTR, ALCAM, ACTA2, COL1A1, LRAT, PDGFRβ, LOXL2, NES and NG2) along the 10 days of culture.

The cells looked the same in all three conditions and did not change their typical stellate shape over the culturing period. The cells were spherical and part of a layer of confluent stellate-like cells containing some fibroblast islands (identified with arrows). The viability did not change significantly overtime.

Next, our qRT-PCR results showed that the stellate medium can be replaced by the hematopoietic medium (control S and condition S1) to culture the differentiated stellate-like cells. Moreover, we did not observe any indications that the presence of the hematopoietic cytokines (condition S2) has any negative influence on the growth and viability of the stellate-like cells, nor on their stellate marker expression.

S stellate cells were also tested since they correspond to a more pure population. While the NS stellate cells appear like a very confluent cell layer with some fibroblast islands, day 11 S stellate-like cells appear as a less dense layer of cells with a different morphology. Instead of a more spherical shape as seen for the NS stellate cells (zoomed in Figures), day 11 S cells presented an elongated shape, closer to the morphology that has been described for activated stellate cells (Figure 3.4 (A)). [70]

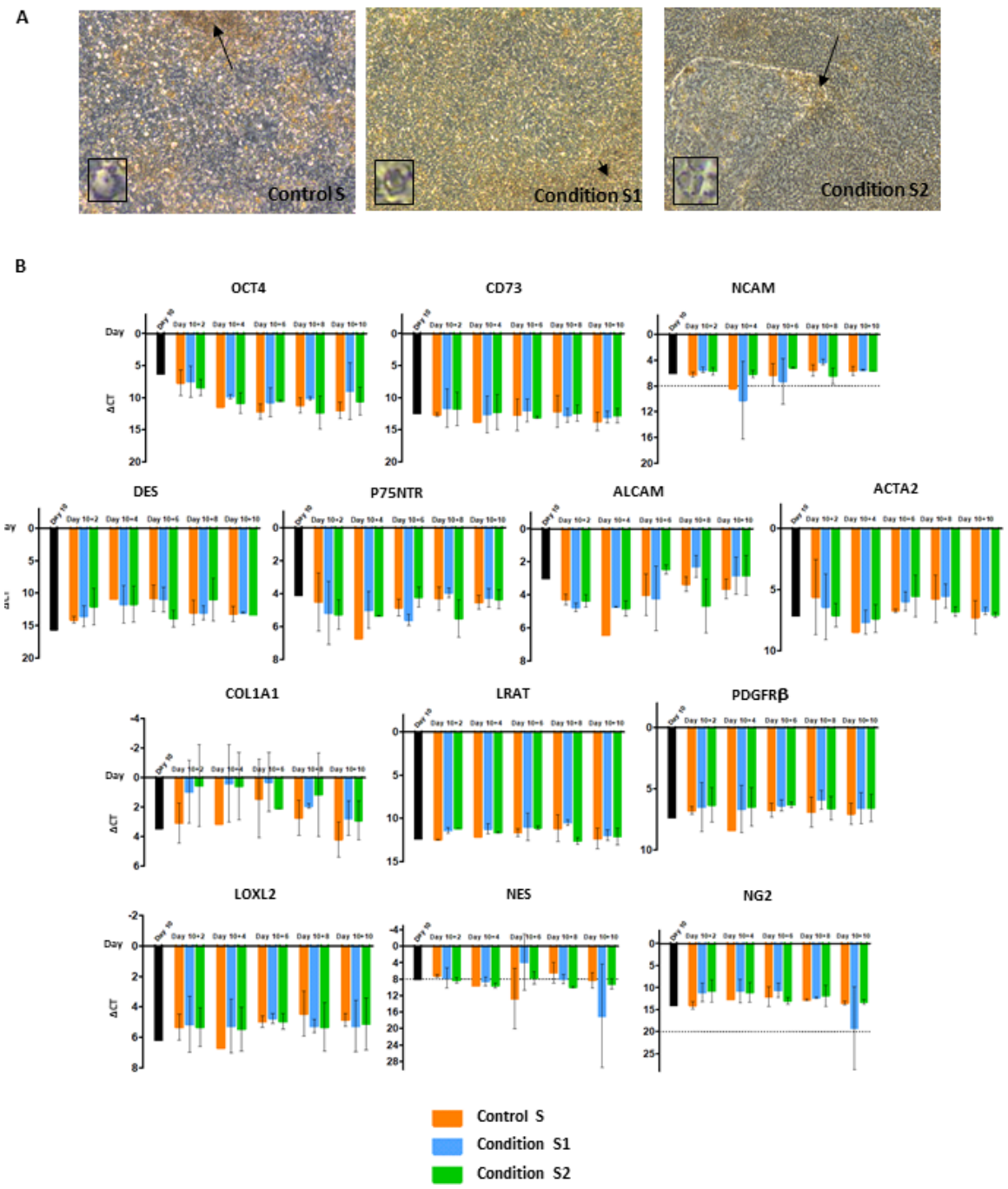

Figure 3.3: Medium Testing on NS Stellate Cells. (A) Representative images of the cell morphology of differentiated day 10 NS stellate cells cultured during additional 8 days (Day 10+8) for the different conditions (5x magnification). From left to right: Control S, Condition S1, Condition S2. **(B)** Gene expression of pluripotency (OCT4), mesodermal and submesothelial phenotype markers (CD73 and NCAM) and hepatic stellate cell (DES, P75NTR, ALCAM, ACTA2, COL1A1, LRAT, PDGFRβ, LOXL2, NES and NG2) along the 10 days of the experiment. mRNA levels were assessed by qRT-PCR and expressed as the arithmetic mean $\pm$ standard deviation of the mean (SD). n = 2 independent experiments. Day 10 - black; Control S - orange; Condition S1 - blue; Condition S2 - green.

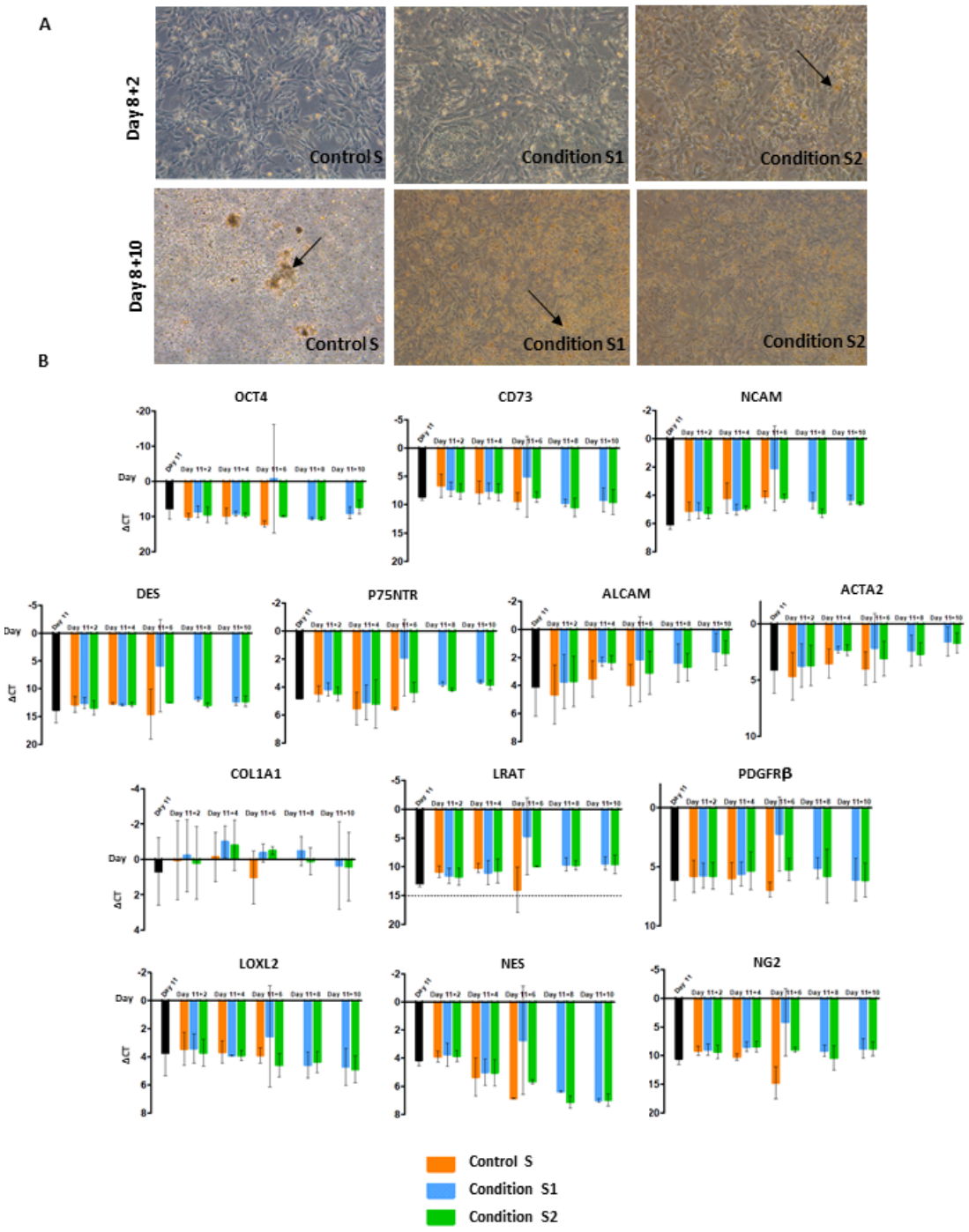

Figure 3.4: Medium Testing on S Stellate Cells. (A) Representative images of the cell morphology of differentiated day 11 S stellate cells cultured during additional 2 days (Day 11+2) (10x magnification) and during additional 10 days (Day 11+10) (20x magnification (Control S) and 5x magnification (Condition S1 and S2)). From left to right: Control S, Condition S1, Condition S2. **(B)** Gene expression of pluripotency (OCT4), mesodermal and submesothelial phenotype markers (CD73 and NCAM) and hepatic stellate cell (DES, P75NTR, ALCAM, ACTA2, COL1A1, LRAT, PDGFRβ, LOXL2, NES and NG2) along the 10 days of the experiment. mRNA levels were assessed by qRT-PCR and expressed as the arithmetic mean $\pm$ standard deviation of the mean (SD). n = 3 independent experiments. Day 10 - black; Control S - orange; Condition S1 - blue; Condition S2 - green.

In what concerns the medium testing on S stellate cells, a clear difference between cell media was observed. In fact, in control S condition using stellate medium, an increased celular death of the S stellates was noticed. At day 8 of culture (Day 11+8), barely any viable cells were detectable in the stellate medium condition. This was in contrast to the two tested hematopoietic medium conditions (condition S1 and condition S2), in which a decrease in cell viability was also noticed over the culture period, however much less compared to the stellate medium cultured cells. Thus, compared to the stellate medium (control S), the hematopoietic medium (condition S1 and condition S2) is much more suitable to maintain the differentiated S stellate cells. Moreover, no morphological dissimilarities between condition S1 and condition S2 were observed. Both cultures appear to have a similar influence on cell morphology and viability.

The S stellate cells of the tested media show similar expression levels of the analysed markers, as well as the same evolution of expression of the gene over culture time. This indicates that culture condition does not influence the stellate phenotype of the S stellate like cells, but seems to enhance their survival.

One explanation for the clear difference between control S condition and conditions S1 and S2 in cell survival observed in the medium testing experiment with the S stellate cells, might be related to the different formulation of the stellate medium (control S condition) and the hematopoietic medium (condition S1 and condition S2). Although stellate medium is the one used by the laboratory in the improved differentiation protocol and stellate cell maintenance, it seems that these cells can survive in hematopoietic medium for more days. Unfortunately, hematopoietic medium complete formulation is not available to the consumers, which makes it difficult to elucidate which component of the medium might be responsible for the enhanced cell survival of the S stellate cells. However, further experiments could unveil if any component of the stellate medium is responsible for the increased cell death observed among the S stellate-like cells.

In summary, we can conclude from this experiment that hematopoietic medium seems to be slightly better for the NS stellate cells and is definitely better for the cell survival of the S stellate cells, compared to the regular stellate medium. Moreover, the hematopoietic cytokines did not affect the NS and S stellate cells in any conditions. Just a small remark in the fact that hematopoietic medium was not tested during the optimization of the stellate differentiation protocol.

Since condition S1 and condition S2 present similar results both in NS and S stellate cells and taking into account that condition S2 has important growth factors for HSCs, this will be the condition used in the co-culture systems.

3.2.2 Effect of the Co-Culture Medium Conditions on Endothelial-Like Cells

Similar to the stellate medium evaluation, we assessed if the hematopoietic medium could replace the endothelial medium to maintain the EC-like cells, as the latter one impairs HSC functions and thereby cannot be used for the co-cultures. The impact of six different medium conditions was tested on day 8 differentiated ECs. In the same manner as in the previous medium testing experiment, endothelial medium was referred as control E, and all the other conditions E1, E2, E3, E4 were hematopoietic medium supplemented with different additional cytokines. Moreover, similar to the stellate medium testing experiment, the effect of the hematopoietic cytokines on the hESC derived ECs was elucidated. The combined effect of the hematopoietic cytokines was assessed in condition E2, whereas the individual effect on the EC-like cells was also tested in conditions E3 and E4, respectively.

Similar to the previous experiment with the stellate-like cells, we evaluated the effect of the different media on the morphology of cells over the culture period of 10 days. A first interesting observation was made in condition E2, in which the hematopoietic stem cells growth factors were added. In this

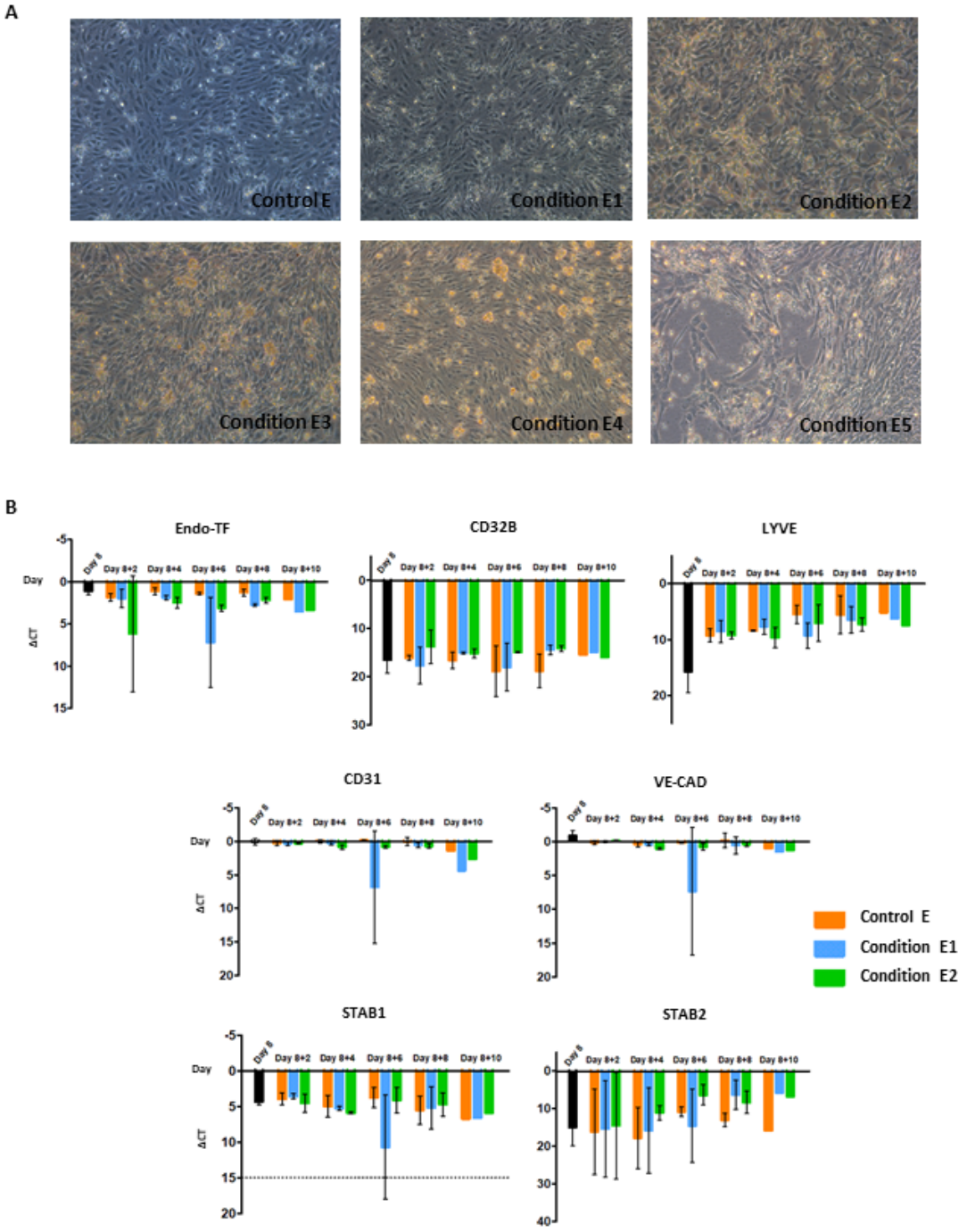

Figure 3.5: Medium Testing on ECs. (A) Representative images of the cell morphology of differentiated day 8 ECs cultured during additional 10 days (Day 8+10) for the different tested conditions (10x magnification). **(B)** Gene expression of Endo-TF, CD32B, LYVE, CD31, VE-CAD, STAB1 and STAB2 along the 10 days of culture. mRNA levels were assessed by qRT-PCR and expressed as the arithmetic mean ± standard deviation of the mean (SD). n = 2 independent experiments. Day 8 - black; Control E - orange; Condition E1 - blue; Condition E2 - green.

condition, a small decrease in cell viability was seen on the EC-like cells compared to the endothelial and hematopoietic medium without any hematopoietic cytokines present. It was also noticed that there was slightly more cell death in the hematopoietic medium based condition compared to the control endothelial medium one. Nevertheless, the alone hematopoietic medium did not negatively affect the endothelial morphology. The cells presented an oval elongated shape, similar to what was observed in control condition (Figure 3.5 (A)).

While the microscopic observation indicated a negative influence of the hematopoetic growth factors on the EC-like cells, the qRT-PCR results, for the endothelial specific markers such as Endo-TF, CD31 and VE-CAD, did not reveal any major changes between the distinct media (Figure 3.5 (B)). It was observed that CD31 expression levels decreased over the culture time in all the conditions (control E, condition E1 and E2). In contrast, LYVE, a selective marker for lymphatic ECs, expression goes up from day 2 to day 10, especially for E1 and E2 condition. For STAB2, the data suggest that its lowest expression is in the control E.

It is also important to take note of the high standard deviation for delta CT value ($\triangle$CT), that was obtained for some specific sample groups. This actually indicates a high heterogeneity of the differentiation efficiency between the wells and implies that the experiment should be repeated with more technical replicates of the differentiation.

As our initial results of this medium testing experiment on the EC-like cells suggested an negative effect of the hematopoietic cytokines on the cell viability, we further investigated the individual influence of the presence of each hematopoietic cytokine respectively on cell morphology. Therefore, conditionE3 and condition E4 were implemented, each one of these containing hematopoietic medium and the endothelial growth factors with either one of the suppoting hematopoetic cytokines. Observing Figure 3.5 (A) conditionE3 and condition E4 (first and second figures of the second row, respectively), no significant difference between conditions is observed.

As our qRT-PCR results did not underlined the morphologic changes that we observed during the experiment, the EC-like cells were further analysed at protein level. Therefore, flow cytometry was performed on the day 8 cultured cells for the two main EC surface markers, CD31 and VE–CAD. Indeed, qRT-PCR and flow cytometry assess completely different cellular endpoints. qRT-PCR measures mRNA, thus gene expression strength, whereas immunological methods, like flow cytometry, quantify protein amounts. Numerous post-transcriptional and translational regulation mechanisms of the transcribed RNA can take place inside the cells and result in a different protein expression. This means that an increased or the presence of mRNA transcripts does not necessary correlates with the amount of protein present in the cells. [121]

In Figure 3.6, the results obtained by FACS analysis are present. The gating strategy used for ECs is present in Figure A.2 (B). A quantification of the double positive CD31+ VE-CAD+ cells was performed (Figure 3.6 (B)). First, we observed that not 100 % of the differentiated EC-like cells were double positive (CD31+ VE-CAD+).

The FACS data showed that the negative effect of the hematopoietic cytokines is not that obvious as it seemed based on the morphology pictures. Although at the end of culture, less CD31+ VE-CAD+ are present in the condition E2 than in condition E1 with no hematopoietic cytokines, the amount of double positive ECs were not always higher in condition E1 compared to E2 during the other days of culture. Same was observed for the individual incubations of the hematopoietic cytokines with the ECs, we could not clearly distinguish which one of the hematopoietic cytokines was responsible for a major decrease of CD31 and VE-CAD expression. This experiment needs to be repeated and should each time include at least technical triplicates for the FACS staining to reduce the overall variability. Lastly, similar to the previous data, the withdraw of an endothelial supporting growth factor impaired the endothelial phenotype. After 8 days in culture (D8+8), less then 5 % of the live cell present were still

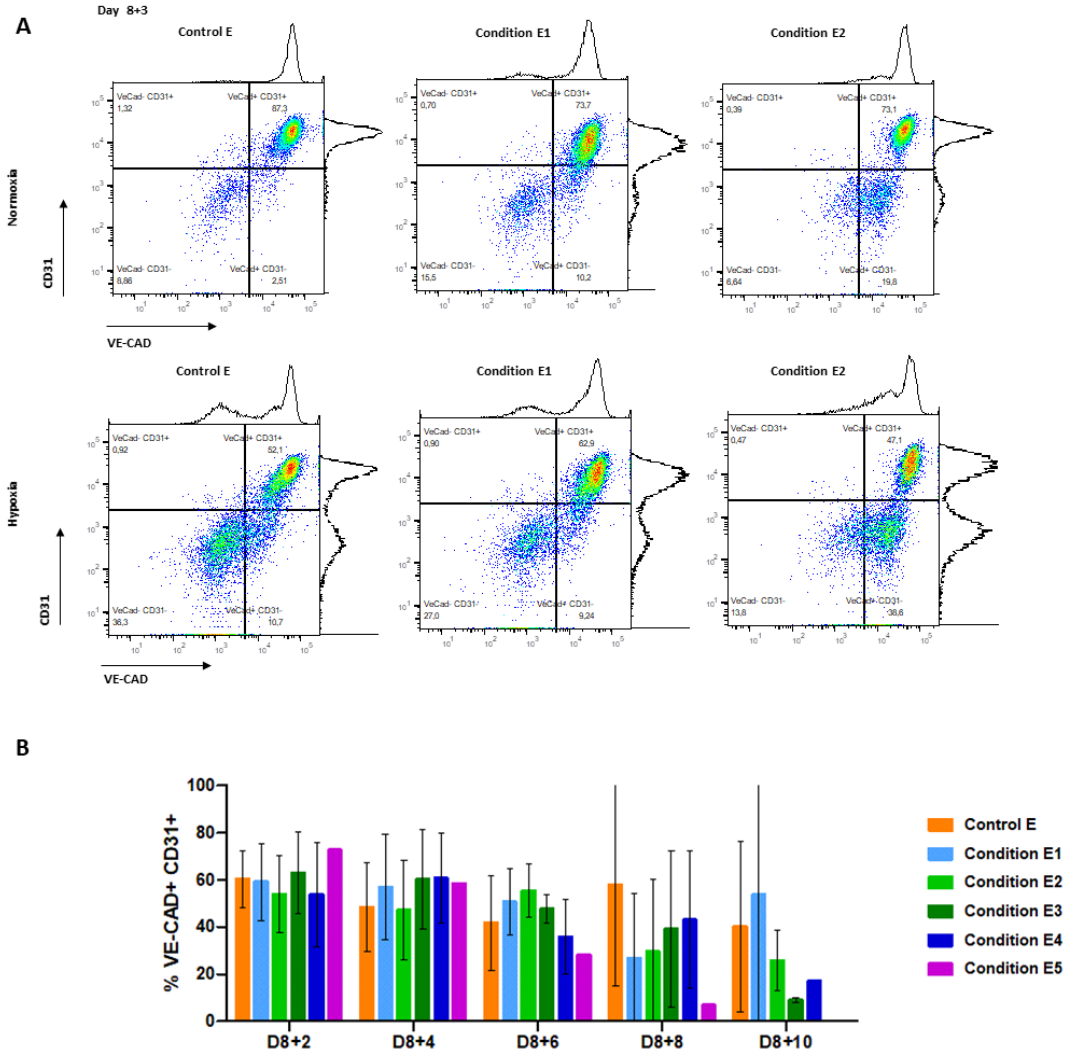

Figure 3.6: FACS Results of the Medium Testing on ECs. (A) Representative dot plots of the differences for Control E, Condition E1 and Condition E2. Results obtained from ECs cultured for additional 3 days (Day 8+3) in the different medium conditions tested. **(B)** Evolution of the percentage of CD31+ VE-CAD+ cells during the 10 days of culture for each condition tested. FACS results expressed as the arithmetic mean $\pm$ standard deviation of the mean (SD). n=4 independent experiments for control E, condition E1 and E2; n=3 independent experiments for condition E3 and E4; n=1 independent experiments for condition E5.

CD31+ VE–CAD+.

In summary, we can conclude from this experiment that endothelial medium seems to be the better medium, compared to hematopoietic one, for the endothelial culture. Control E maintains the highest viability and percentages of CD31+ VE-CAD+ EC-like cells. Also, the data of this thesis suggest a negative effect on the survival and phenotype of the hematopoietic cytokines on the ECs. Further investigation is needed to shed light on the exact outcome of culturing the ECs in hematopoietic based medium. These questions could not be fully answered in this experiment, in part due to the high variation of the endothelial samples. The FACS and qRT-PCR should be repeated with more technical replicates (at least 3) and more differentiation batches to take better into account the heterogeneity of the differentiation.

Despite our indications that the hematopoietic cytokines might also negatively affect the endothelial cells, we decided to use the complete supplemented hematopoetic medium for our co-culture settings. Apart from the fact that the endothelial medium was previously shown to impair HSC expansion and function, the presence of the hematopoietic cytokines is crucial for the HSC survival and maintenance *in vitro*. Moreover, the effect of the cytokines on the EC-like cells might be attenuated in presence of the HSCs, as the latter ones will respond to the hematopoetic growth factors.

3.2.3 Effect of the Co-Culture Medium Conditions on HSCs

In this medium testing experiment, the two co-culture media were tested on the HSCs, and compared to their regular culture medium. Each condition is based on the hematopoietic medium with hematopoietic cytokines, with or without the growth factors required for the maintenance of the differentiated FL niche cells. Condition 1 corresponds to the medium used in the co-culture of HSCs with ECs (endothelial co-culture) and condition 2 corresponds to the medium used in the co-culture of HSCs with stellate cells (stellate co-culture).

As it can be observed in Figure 3.7 (A), all conditions look similar at day 7 of the medium experiment. In fact, no major morphological differences were visible between the cells in suspension cultured in the distinct conditions during the 10 days experiment. Cells were highly proliferative from day 2 onwards and a typical heterogeneity among the culture could be observed, reflecting the differentiation process of the $Lin^{-}c-kit^{+}$ population seeded initially. These cells presented a small and round shape and were of different sizes.

In Figure 3.7 (B)-(E), the FACS results for each tested media condition on the $Lin^{-}c-kit^{+}$ cells are presented. The purified BM $Lin^{-}c-kit^{+}$ cells seeded at day 0 in the different media are considered an enriched HPSC population. Cells that are identified as LSK ($Lin^{-}Sca1^{+}c-kit^{+}$) correspond to more pure HPSCs with higher differentiation ability than the $Lin^{-}c-kit^{+}$ cells. HSCs are also characterized by the LSK markers, however in addition they are positive for CD150 and negative for the CD48 expression (SLAM markers). Thus, we distinguish HSCs on FACS as being LSK SLAM cells ($Lin^{-}Sca1^{+}c-kit^{+}CD150^{+}CD48^{-}$). The LSK SLAM cells have the highest repopulation capacity, as they are able to differentiate into all the downstream progenitors (such as the LSK) and able to self-renewal themselves. The gating used to identify the LSK and LSK SLAM populations by FACS is depicted in Figure A.2 (A) (Appendix).

First, the effect of endothelial growth factors (condition 1, represented by the blue bars) in the hematopoietic medium on the LSK and LSK SLAM was assessed. Although the endothelial growth factors support the expansion of the LSK cells (increase in the number of LSK from day 0 till day 10 of culture), the expansion is 2-fold less as compared to the control culture. Moreover, the percentage of LSK decreases over the culture period, reflecting that the non-LSK cells also expand and that they even proliferated more than the LSK ones in presence of endothelial growth factors. When looking at the LSK SLAM population, a decrease in the cell number can be observed for the HSC compartment

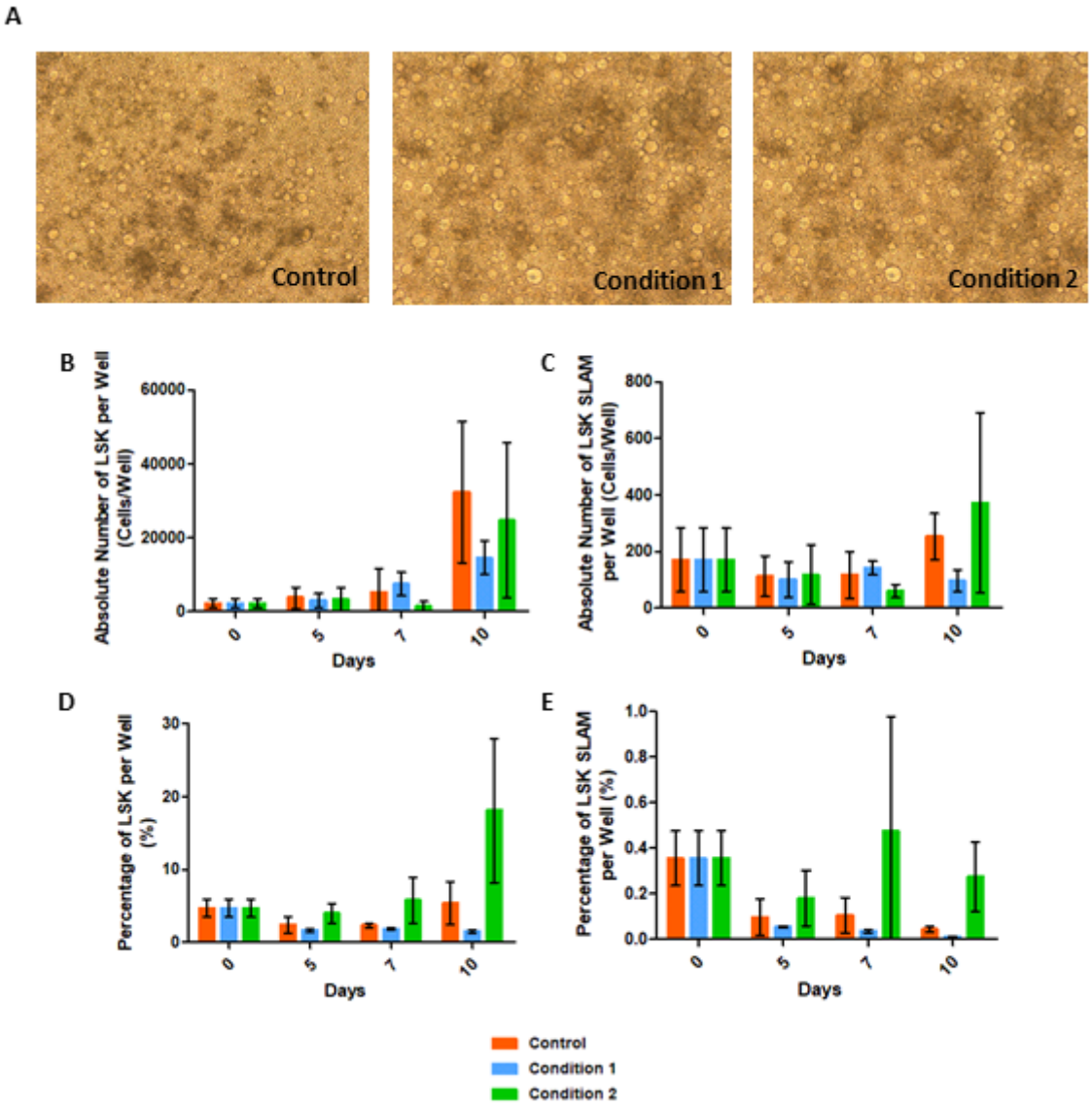

Figure 3.7: Medium Testing on Hematopoietic Stem Cells. (A) Representative images of the cell morphology on each condition at day 7 of culture. From left to right: Control, Condition 1, Condition 2 (10x magnification). **(B)-(E)** FACS Results expressed as the arithmetic mean ± standard deviation of the mean (SD). n = 2 independent experiments with two biological replicates each. Control - orange; Condition 1 - blue; Condition 2 - green. **(B)** Evolution of the absolute number of LSK per well (cells/well) during the 10 days of culture. **(C)** Evolution of the absolute number of LSK SLAM per well (cells/well) during the 10 days of culture. **(D)** Evolution of the percentage of LSK per well (%) during the 10 days of culture. **(E)** Evolution of the percentage of LSK SLAM per well (%) during the 10 days of culture.

in condition 1 after 10 days in culture. This is not the case for the control medium, which support not only the maintenance but also a small expansion of the HSCs *ex vivo* (slight increase of the LSK SLAM number from day 0 onwards in the control). However, both the control and the condition 1 seems to better allow the proliferation of the more differentiated cells (non-LSK SLAM cells) in culture (decreased percentage of LSK SLAM overtime).

Next, the influence of the stellate supplements (condition 2, represented in green) was tested on the LSK and LSK SLAM cells. Our data shows that the presence of stellate growth factors in the medium supports the proliferation of the LSK cells in a similar way than the control condition (higher LSK number at day 10 versus day 0). For the first time, the percentage of LSK cells increased from day 0 to day 10, suggesting that the addition of stellate growth factors enable the medium in particular to expand the HPSCs. On top of that, condition 2 also expand the HSC cells, and this even better than the control medium. Interestingly, it appears that the medium with the stellate supplements is the best in maintaining the HSC cells, as the LSK SLAM percentage is more or less stable after 10 days of culture.

In summary, this experiment suggests that medium supplemented with the stellate growth factors has a positive effect on the maintenance and the expansion of HPSCs and HSCs *ex vivo*. Nevertheless, further experiment would need to evaluate due to which one of stellate supplements the observed outcome is due to. The second important finding of our medium testing experiment on the HSPCs, is that medium containing endothelial growth factors seems to impair the maintenance and the expansion of HSCs *ex vivo*.

The main limitation of this medium testing experiment is that it does not exactly reflect the effect of the medium to be used in the co-culture experiments. For example, the negative influence of the endothelial growth factors on HSCs might reduce in the co-culture where the EC-like cells are close by the HSCs. It is possible that the effect of the medium is being overestimated, not compromising its use in the actual co-culture system where both cells types (HSCs and FL niche cells) are present. Another disadvantages of this experiment is that it only took into account the phenotype (expression of cell surface markers) as a read-out of the effect of different media on the HSCs. Therefore, a functional assessment of the cultured HSCs in these different conditions is being included in the last section of the results.

3.3 Establishment of Fetal Liver Niche Mimicking Co-Cultures Experiment for HSC Expansion

In the previous experiments (Section 3.2, Defining Cell Culture Medium Conditions for FL Co-Culture Strategies), we show that the hematopoietic medium can be used as co-culture medium instead of the stellate based medium and that it can be supplemented with the cytokines needed for HSCs and the FL niche like cells. The results from the medium testing are less promising for the endothelial co-culture as it seems that the endothelial medium have a negative effect on the HSCs. However, for the co-culture of HSCs with ECs (endothelial co-culture), the medium used was still hematopoietic medium supplemented with both the hematopoietic and endothelial cytokines. For the co-culture with stellate cells (S co-culture and NS co-culture, with S and NS stellate cells, respectively), the medium used was hematopoietic medium supplemented with both hematopoietic and stellate cytokines.

3.3.1 Co-Culture of HPSCs with Stellate-Like Cells

3.3.1.1 Co-Culture of HPSCs with NS Stellate Cells

Day 10 NS stellate cells were co-cultured with HSCs. The gating for the analysis of the results of the FACS analysis for each co-culture was performed in a similar way to the one mentioned in Section 3.2.3 and is present on Figure A.2 (A). The co-cultured HSCs could be observed along the experiment. No morphological differences were observed between the co-cultured HSCs and the control ones. Similar to the images of the previous experiment, they are characterized by being in suspension and by being bright, round cells of different sizes.

To sum up, this experiment shows that a co-culture of day 10 NS stellate cells and BM HPSCs is not suitable for the *ex vivo* culture of HSCs. Therefore, the next strategy of this thesis aims to use a more pure population of stellate-like cells (day 11 S stellate cells) for the HSCs culture.

3.3.1.2 Co-Culture of HPSCs with S Stellate Cells

In this experiment, day 11 S stellate cells were used to co-culture BM HPSCs for 10 days.

In summary, this experiment showed that co-culturing the BM HPSCs with a relatively purer population of stellate-like cells during 10 days, enable expansion and maintenance of HPCs and HSCs *in vitro*. Despite the fact these results are very favorable for future application, it is important to keep in mind that this co-culture experiment with the day 11 S stellate cells was only performed once during this thesis. In order to validate our primary results, it is necessary to perform this experiment at least 2 more time. Moreover, further investigation is needed to understand the underlying mechanism of the enhanced expansion of HSCs and, most importantly, the functionality of the HSCs still needs to be assessed in primary and secondary transplantations.

3.3.2 Co-Culture of HPSCs with Endothelial Cells

For this experiment, ECs were used to co-culture BM HPSCs for 10 days.

In conclusion, the EC-like cell co-culture experiment supported the expansion of HPS and HSCs *ex vivo*. Even though this experiment, as well as the medium testing experiment on the HSCs, indicate that the hematopoietic medium supplemented with the endothelial growth factors does not allow the expansion of HSCs, the differentiated EC-like cells provided eligible feeder support for the expansion of HSCs during 10 days. The main limitation of this endothelial co-culture is that the feeder is only composed of about 25 % CD31+ VE-CAD+ cells after 10 days of culture. Thus, further investigation needs to evaluate if the HSC expansion is indeed mainly due the presence of the EC-like cells (CD31+ VE-CAD+) or if there is another sub population present in the feeder layer that positively affects the HSCs. Next, the functionality of these expanded HSCs in the endothelial co-culture needs to be assessed.

3.4 Endothelial Co-Culture and Tested Medium Culture Conditions Support HSC Functionality *In Vivo*

Our previous co-culture results seem very promising for HSC *ex vivo* expansion. Nevertheless, the previous read-out of those experiment was only based on the number of phenotypical HSCs that were detected after culture. Therefore, we further investigate if those cultured HSCs are also able to repopulate an irradiated recipient after transplantation. 5×10^5 total cells (CD45.1) of the progeny of the initial seeded cells were transplanted together with CD45.2 competitor cells into lethally irradiated

mice (CD45.2). In order to assess their ability to regenerate the hematopoietic system, peripheral blood was analysed by FACS for the CD45.1 and CD45.2 antigens at 4 weeks post-transplantation. In this experiment, $Lin^{-}c-kit^{+}$ cells that were co-cultured during 5 days with the EC-like cells, as well as the $Lin^{-}c-kit^{+}$ cells that were cultured for 5 days in the different medium condition, from Section 3.2.3, were used. Due to time restriction of this thesis, the HPSCs that were co-cultured with the NS and S stellate-like cells could not have been evaluated functionally in a transplantation assay.

Observing the results obtained after 4 weeks of transplantation, present in Figure 3.8 (A), it can be seen that the total percentage of donor cells in the recipient mice are similar, approximately 60 %, for all the tested culture conditions. The cells cultured with hematopoietic medium supplemented with the endothelial and hematopoietic cytokines (condition 1), present in the recipient mice a slightly less percentage. However, these data suggest that the HPSCs are maintained functional *in vitro* in the hematopoietic medium supplemented with endothelial cytokines and in the hematopoietic medium supplemented with the stellate cytokines (condition 2) in a similar way than in the control hematopoietic medium medium (control). Moreover, the same repopulation capacity was observed for the HPSCs that were cultured together with the EC-like cells during 5 days.

Next, the differentiation capacity into the different blood lineages was assessed at 4 weeks after infusion. In Figure 3.8 (B), it can be seen that the cultured HPSCs are able to give rise to T Cells, B Cells and Myeloid Cells. Also similar to the repopulation percentages, the percentages of the different lineages are similar for the different cultures. Just a little highlight to the fact that the cells co-cultured with ECs transplanted, originated a slightly less percentage of B Cells and a slightly bigger percentage of Myeloid Cells. This might indicate some first differences of the co-cultured HPSCs compared to the other cells transplanted. This normally happens when HSCs age and become dysfunctional. Aging causes a marked shift in the representation of these HSC subsets and long-lived myeloid-biased HSCs accumulate, producing more myeloid cells. [122] However, since results do not show a significant difference, we would need to reassess this values after a longer transplantation period.

One important fact when considering this transplantation data is that the analysis is at 4 weeks, meaning it is only showing the short-term repopulation capacity of the cultured cells. The functional LSK and LSK SLAM will both be able to home in the recipient BM niche, and can both contribute to the repopulation and the different lineages of the blood 4 weeks after transplantation. In the contrary to their short-lived progenitors, the true stem cells divide slowly *in vivo*, avoiding exhaustion by limiting expansion and reverting to a dormant state when mature compartments are fully reconstituted. Therefore, it is indispensable to analyse the blood chimerism at 16 weeks post-transplantation to have an idea about the functionality of the long-lived HSC (LSK SLAM). Moreover, to prove the long-term functionality of true HSCs, secondary transplantation needs to be performed after the 16 weeks primary transplantation. [38][123]

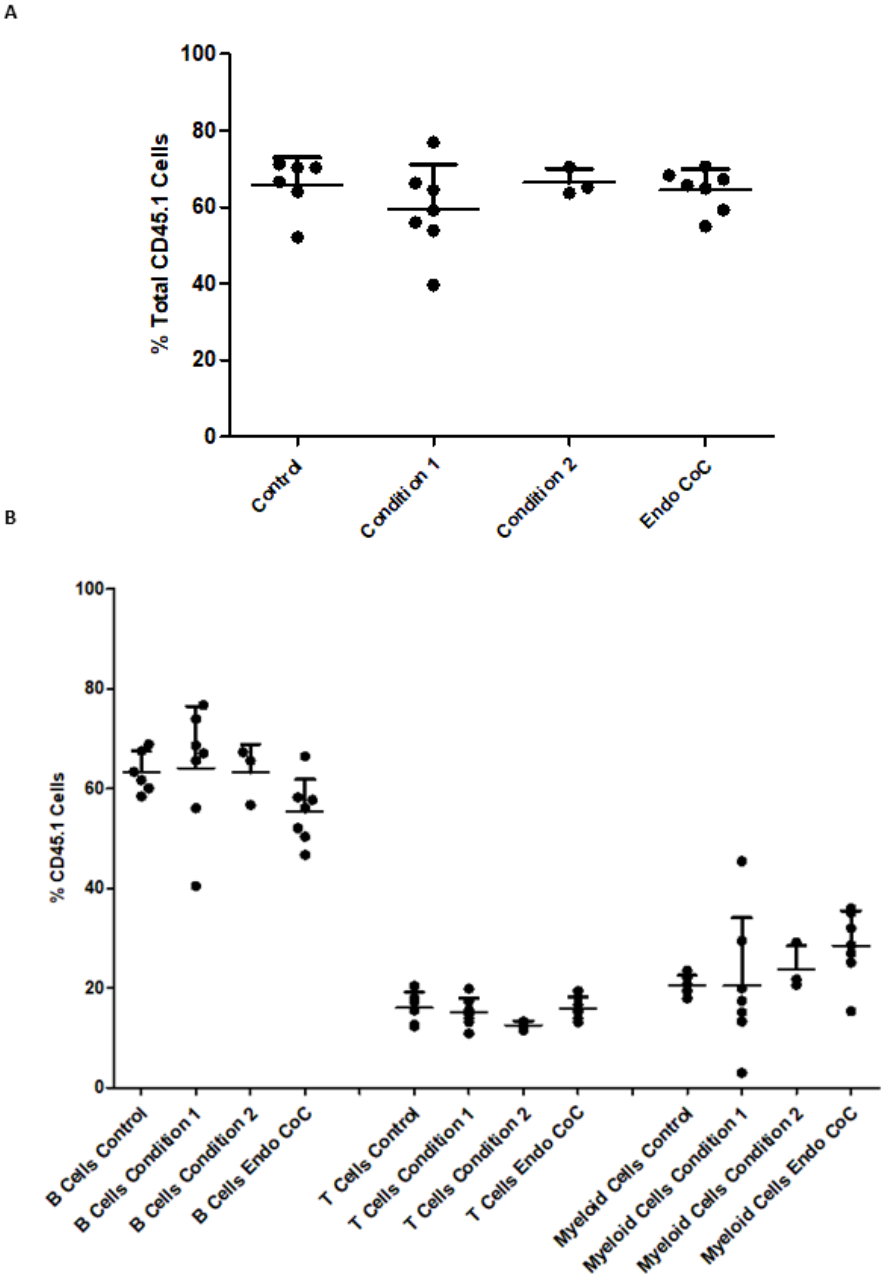

Figure 3.8: Primary Transplantation Results. (A)-(B) FACS results of the peripheral blood collected after a period of 4 weeks from primary transplanted mice. Primary transplantation of *in vitro* cultured HSCs was performed into irradiated congenic recipients. Cells were cultured in different medium conditions (Control, Condition 1 and Condition 2) or co-cultured with ECs (Endo CoC) during 5 days. Condition 1 was used in the endothelial co-culture. Each dot represents a biological replicate. **(A)** Percentage (%) of Total Donor Cells (CD45.1 Cells) in the Recipient Mice per Condition (Control, Condition 1, Condition 2, Endo CoC). **(B)** Percentage (%) of Each Donor (CD45.1) Cell Type (B Cells, T Cells and Myeloid Cells) in the Recipient Mice per Condition (Control, Condition 1, Condition 2, Endo CoC).

Chapter 4

Conclusion

HSC transplantations are performed in large numbers every year for the treatment of hematological diseases such as leukemia, lymphoma and sickle cell disease. [38] These clinical treatments are based on the functionality of HSCs to reconstitute the entire blood system in an irradiated recipient. However, there is still a limiting factor related with obtaining enough cells to perform these transplantation therapies, since there is a limited number of matching donors and the inability to expand HSCs *in vitro*, while preserving its functionality. To overcome this obstacle, it is essential to develop new strategies to expand HSCs *ex vivo* efficiently. The stem cell niche plays an important role and it is able to support HSC self-renewal potential and regulate HSC fate. During adult life, the BM constitutes the main hematopoietic niche and maintains the HSC pool, whereas during development the FL is the site of HSC's extensive proliferation and expansion.

This project aimed to apply the FL niche as a model to recreate an *in vitro* niche microenvironment to allow expansion of functional adult HSCs. The main novelty of this work is precisely the establishment of a 2D co-culture system for HSC expansion through the usage of specific FL niche cells, differentiated from an inexhaustible source.

This thesis focused on the use of immature endothelial and stellate cells to recreate the FL niche environment. First, human pluripotent stem cells were differentiated into hepatic stellate cells or endothelial cells, for later use in the co-culture system. Both cell types, revealed a downregulation of pluripotency genes, as expected, and an upregulation of specific stellate or endothelial markers during differentiation protocol.

Next, the 2D co-culture for the mouse BM HSCs and the two differentiated FL niche cells was established. Therefore, the effect of different co-culture medium compositions was first tested individually on the previously differentiated cells and on HSCs isolated from mice BM.

For the stellate-like cells, we used two different subtypes of stellate, the NS and the S cells. The latter one being a more pure stellate population. Our results indicate that the hematopoetic medium was the better co-culture medium in maintaining the stellates than their regular medium.

This same positive effect of the co-culture medium was not observed for the EC-like cells. The highest percentage of phenotypical ECs was found in the regular endothelial medium without any hematopoietic cytokine present. However, these settings cannot be used for the culture of the primary HSCs, as they have been previously shown to impair the HSCs.

In addition, our evaluation of the effect of the co-culture media on the HSCs showed that the medium with stellate cell growth factors was enhancing HPC and HSC expansion compared to the control routine HSC culture. Further investigation is needed to unravel which stellate cytokine is the key driver of the increased expansion. When the HPSCs were cultured in the endothelial co-culture medium, a slight decrease in the HSC expansion was noticed. In what concern the functionality of these HSCs cultured

in the endothelial or stellate factors supplemented medium, all the HSCs were able to reconstitute the blood of an irradiated host, and contribute to the different blood lineages.

In conclusion, this thesis showed two distinct feasible co-culture approaches to expand primary HSCs *in vitro*. In contrast to most published papers, that attend to recreate a BM environment for the HSC culture, this project aimed to create the FL niche and thereby its extensive expansion capacity. To our knowledge, this is the first study that intent to develop co-culture strategies for HSCs using differentiated cells from a pluripotent source. However, this research also emphasize on the difficulties encountered when it comes to define the proper medium condition for the co-culture of different cell types.

Chapter 5

Future Work

Taking into consideration the ultimate goal of this project to establish co-culture systems, mimicking the FL niche, for *in vitro* expansion of HSCs, it is evident that the obtained results are still very preliminary. There is still a long path to go before achieving high efficient and safe systems that can be used to expand patient HSCs in a clinical setting. Although this thesis showed some interesting results, further validation is needed.

As addressed beforehand, one of the major shortcomings of this project was the lack of enough independent experiments for the various project parts, most importantly of enough technical replicates. With regard to our results, this seems in particular needed for the assessment of the differentiated FL–like cells. In general, our results suggested high variability between different cells coming from the same differentiation and from different batches of differentiation. Moreover, this high variability rise the question about the heterogeneity of the differentiated cells. Indeed, more characterization by single cells techniques should be done to identify the different cell subtypes generated by the differentiation protocols. Especially regarding safety for potential future clinical applications, it is necessary to assess that not a single pluripotent cells remains inside the differentiated feeder.

Besides that aspect, there are still a lot of parameters that could be optimized to improve the performance of our co-culture systems.

One of them, is the use of matrigel and mTeSR medium when we culture our hESC cells. Both of these materials contain animal components and are therefore suitable not for clinical use.

This study as well as others, have emphasized again on how difficult it is to find a common medium that support the growth and the function of two different cell type. One undeniable way to make progress in this field is to understand the precise metabolism of the different cell in co-culture. This might then also help to avoid undifferentiation or unwanted differentiation of cells and to keep a more homogeneous cell population as niche cells for the HSCs

As mentioned, further validation is also needed for our co-culture systems. Not only the long-term repopulation capacity of the co-cultured needs to be evaluated *in vivo*, but the expansion capacity of co-culture systems also needs to be assessed with human HSCs.

Finally, in order to better recreate the organ-like structure and the niche of FL, to mimick its *in vivo* characteristics, the establishment of co-culture or bioreactor systems with more than one cell type might be the next steps. However, there is the disadvantage that this co-culture systems are highly complex and that a broad range of parameters need to be taken into account. Additionally, the future transposition of the 2D co-cultures systems into a 3D set may also be one of the paths to follow. This will better mimick the FL niche and lead to the obtainment of improved results for HSC expansion, since 3D cultures take into account the biophysical properties, providing an environment similar to the *in vivo* condition.

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Appendix A

FACS Analysis Gating Strategy

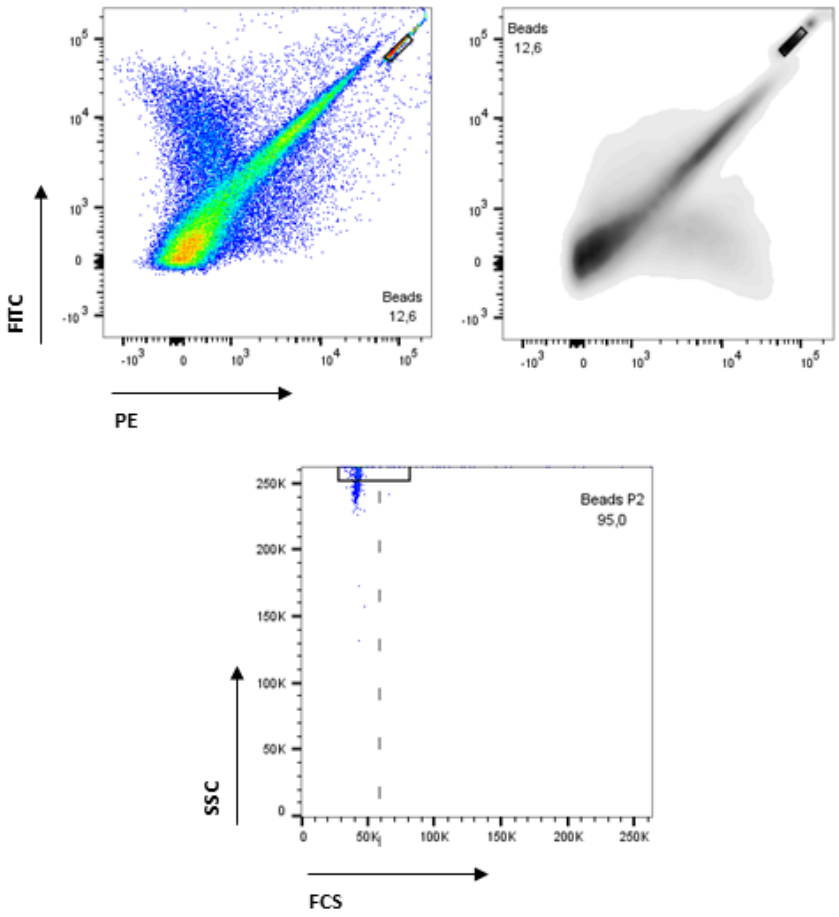

Figure A.1: FACS Analysis, Beads Gating Strategy First, beads are gated according to their FITC and PE flourescent properties (first row). After, beads are gated based on the forward and side scatter (FSC-A and SSC-A) characteristics. The beads will fall off-scale for SSC-A.

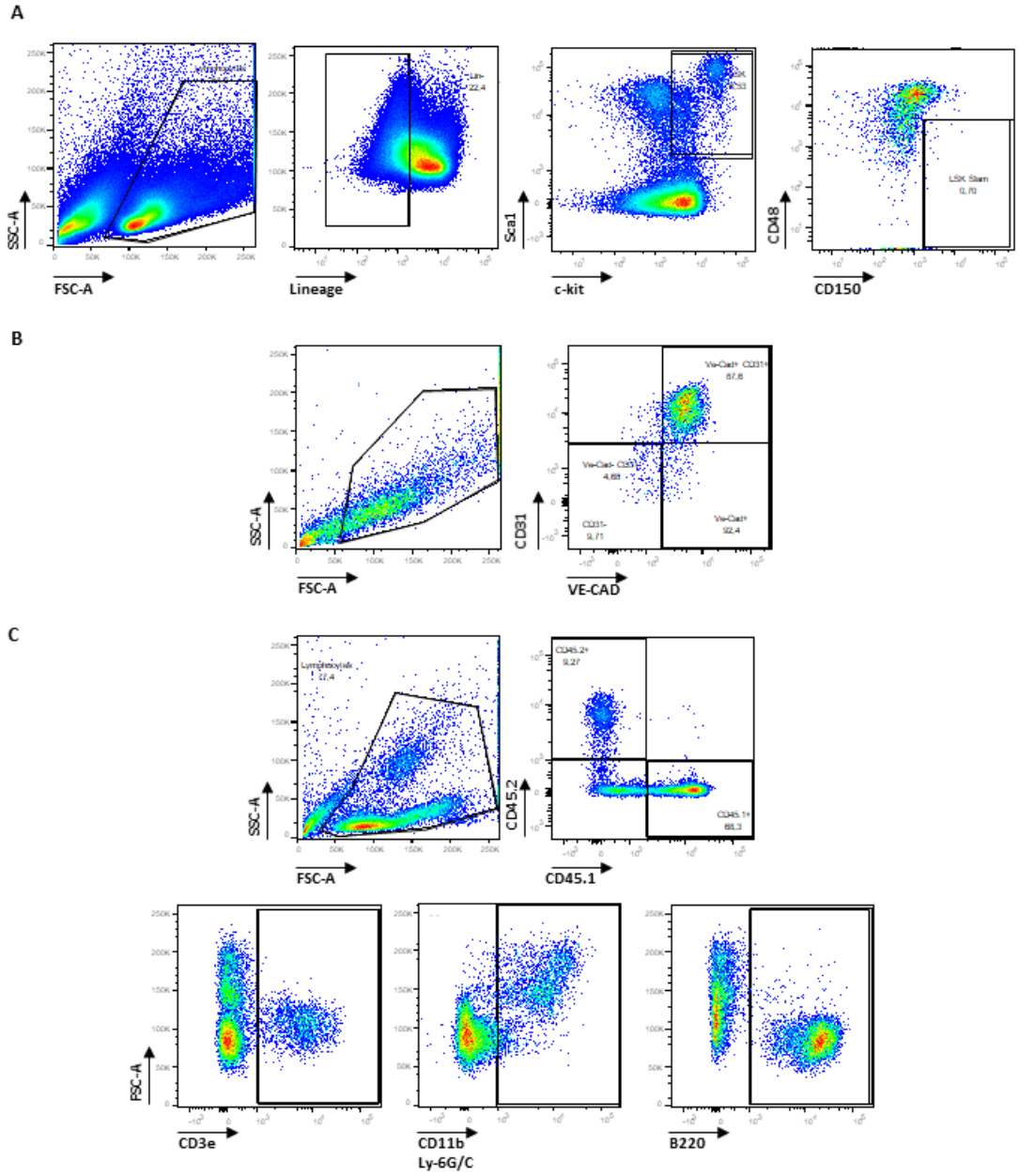

Figure A.2: FACS Analysis Gating Strategy. (A) LSK Slams. After gating the total BM cells based on the forward and side scatter (FSC-A and SSC-A) characteristics of each single cell, cells were gated acording to the Lineage marker in which Lin- cells were selected. After, Sca1+ and c-kit+ cells were selected. So, the gated population becomes Lin-, Sca1+and c-kit+ (LSK). Lastly, as SLAM markers consist of CD48- and CD150+, LSK cells that exhibit such marker expression were selected to be LSK SLAM cells. **(B)** Endothelial Cells. After gating the total BM cells based on the FSC-A and SSC-A characteristics of each single cell, endothelial cells expressing Ve-Cad+ and CD31+ were selected. Other cell population like Ve-Cad- CD31-, Ve-Cad+ and CD31- were selected to facilitate comparison and discussion. **(C)** Peripheral Blood. After gating the total BM cells based on the FSC-A and SSC-A characteristics of each single cell, positive cells to CD45.1 and CD45.2 markers were selected separately. From there and to each one of the previous selected cell population, three different cell populations were selected: CD3e+ (T cells), CD11b+ Ly-6G/C+ (myeloid cells) and B220+ (B cells).