Mimicking the Fetal Liver Niche

Ex Vivo Approaches for Hematopoietic Stem Cell Expansion

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Para ser grande, sê inteiro: nada
Teu exagera ou exclui.
Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.
Assim em cada lago a lua toda
Brilha, porque alta vive.

Ricardo Reis

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Aqueles que passam por nós, não vão sós, não nos deixam sós.
Deixam um pouco de si, levam um pouco de nós. – Antoine de Saint-Exupéry

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Inês

Abstract

From their generation in the earliest stages of embryonic development to the course of life, hematopoietic stem cells (HSCs) play a remarkable role in nourishing and replenishing the hematopoietic system. Ergo several highly fatal hematological diseases rely on HSC transplantation as definitive treatment. However, the limited numbers of this scarce cell population in some of the widely used sources of HSCs pose a significant obstacle to achieving successful clinical outcomes. The development of strategies to efficiently expand HSC are thus pivotal for the improvement of this therapy. Even though HSCs remain quiescent on the adult bone marrow (BM), significant expansion of this pool occurs during its brief passage through the fetal liver (FL), the major hematopoietic organ across development. Growing evidence positions the FL microenvironment as the source of cellular and molecular cues that drive HSC proliferation while sustaining their characteristic properties. Therefore, understanding and mimicking the biological processes taking place in this organ might translate to controlling HSC expansion *ex vivo*. To that end, multiple culture approaches were implemented in an attempt to emulate the unique FL niche environment, from its composition to its hematopoietic supportive ability. Traditional two-dimensional (2D) cultures, a microfluidic system, and three-dimensional (3D) cell aggregates were studied systematically to identify the best cultures conditions to test the effect of FL stroma on hematopoietic stem and progenitor cells *ex vivo*.

Albeit the observed sharp decrease in the expression of important structural proteins and cytokines following *in vitro* culture, cultures with multiple FL stromal populations seem to improve cell maintenance and hematopoietic supportive ability. Although hepatoblasts and combinations of stromal populations could support the development of different mature hematopoietic lineages, no evidence for expansion or robust maintenance of the progenitor compartment was observed. The examined culture methods proved the important role of molecular and physical cues in hematopoietic support and made clear the necessity of further dissecting the unique FL microenvironment.

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List of Abbreviations

AGM	Aorta-gonad-mesonephros
BM	Bone marrow
CLPs	Common lymphoid progenitors
CMPs	Common myeloid progenitors
E	Embryonic day
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetate acid
EHT	Endothelial to hematopoietic transition
EMP	Erythro-myeloid progenitor
EPO	Erythropoietin
ESC	Embryonic stem cell
FCS	Fetal calf serum
FL	Fetal liver
GMPs	Granulocyte-monocyte progenitors
HE	Hemogenic endothelium
HS	Hematopoietic system
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
IL	Interleukin
iPSC	Induced pluripotent stem cell
LMPPs	Lymphoid-primed multipotent progenitors
LSK	Lin^{-} Sca-1^{+} c-Kit^{+} cell
LT-HSC	Long-term hematopoietic stem cell
MEPs	Megakaryocyte-erythrocyte progenitors
MFI	Mean fluorescence intensity
MPPS	Multipotent progenitors

P-Sp	Para-aortic splanchnopleura
PBS	Phosphate-buffered saline
PDMS	Polydimethylsiloxane
PGC	Primordial germ cell
PI	Propidium iodide
qPCR	Quantitative polymerase chain reaction
RT	Room temperature
SCF	Stem cell factor
ST-HSC	Short-term hematopoietic cell
STM	Septum transverse mesenchyme
TPO	Thrombopoietin
TRM	Tissue-resident macrophage
YS	Yolk sac
2D	Two-dimensional
3D	Three-dimensional

INTRODUCTION

Significance – HSC expansion *ex vivo*

Hematopoiesis, the process by which blood cells are formed, occurs during embryonic development and throughout adulthood to produce and replenish the blood system. Hematopoietic stem cells (HSCs) sustain this process by their ability to not only differentiate into all mature blood lineages but also to regenerate themselves, which is referred to as self-renewal activity. The mechanisms conveying these properties have been under investigation over the last 60 years, however, to date, they remain poorly understood.

HSC transplantation is a widely used cell-based therapy intervention in the treatment of hematologic, autoimmune, and genetic disorders (**Tanaka** *et al.*, 2016). The most common sources of HSCs, either in autologous or allogenic transplants, are the bone marrow (BM), umbilical cord blood, or mobilized circulating cells. Thus far, this therapy is still associated with high mortality rates, mainly due to infection, graft-versus-host-disease, and organ dysfunction, pointing forth the necessity for its improvement. In addition, the number of transplanted HSCs correlates with successful engraftment and patient survival (**Kumar & Geiger**, 2017). As high numbers of this scarce cell population are required, protocols that result in HSC expansion or generation *ex vivo* surface as highly desirable tools to further increase positive outcomes in the clinic. Current strategies include media supplementation, transgene overexpression, the use of culture matrices, and small-molecule agonists, among others (as reviewed in **Wilkinson** *et al.*, 2020). Despite extensive investigation, HSCs have long proved difficult to maintain and expand *ex vivo*.

Understanding in depth the mechanisms of HSC self-renewal and differentiation *in vivo* is the holy grail for the development of new strategies for *ex vivo* manipulations of these cells. There is growing evidence that not only cell-intrinsic factors regulate the self-renewal *versus* differentiation switch, but also extrinsic cues from the local and systemic environment impact HSC decisions. Therefore, unraveling the molecular and physical cues of hematopoietic niches is of

utmost importance. Even though HSCs reside in the BM throughout adulthood, during embryonic development they are transiently present in the fetal liver (FL). These two distinct microenvironments induce distinct cell behaviors as the BM supports maintenance and differentiation, while the FL promotes the rapid expansion of newly formed HSCs. Yet, in contrast to the BM niche, much remains unknown regarding the complex interplay among distinct cellular and non-cellular elements of the supportive FL microenvironment.

There is a clear need for a new viewpoint on hematopoiesis. Current strategies for HSC expansion focus on HSC function in the BM, a site where expansion does not occur physiologically, or on specific cell populations, neglecting the possibility that distinct cellular subsets might need to interact to induce HSC expansion. Herein, the establishment of the hematopoietic system (HS) will be reviewed, the latest dogmas on the hematopoietic hierarchy discussed, and the different environments that host the HS in the embryo mouse described.

Ontogeny of the Hematopoietic System

In the adult organism, hematopoiesis is settled in the BM and is guaranteed by a rare and largely quiescent cell population, the HSCs (**Morrison & Weissman**, 1994). The balance between self-renewal and differentiation of this population allows the maintenance of a stem cell pool and the replenishment of lineage-restricted progenitors, either in homeostatic or imbalanced conditions (**Wilson** *et al.*, 2008). However, during embryonic development hematopoiesis is anything less than settled as it is characterized by waves of blood development, sprouting in distinct anatomical sites (**Figure 1**).

The first hematopoietic wave emerges at around 7.5 days of gestation (further referred to as embryonic day (E)) within the blood islands that line the extraembryonic yolk sac (YS) (**Moore & Metcalf**, 1970) and consists of unipotent precursors that give rise to either primitive erythrocytes, megakaryocytes, or macrophages (**Palis & Yoder**, 2001). Primitive erythroid progenitors synthesize embryonic (εy- and βH1-) globins and give rise to large, nucleated erythrocytes, which become the predominant blood cells in circulation, responsible for oxygenating the developing embryo (**Kingsley** *et al.*, 2006; **Palis** *et al.*, 1999). From E12.5, they continue to circulate as enucleated cells throughout gestation and even into the postnatal period (**Kingsley** *et al.*, 2004). Myeloid progenitors, such as macrophage and megakaryocyte colony-forming cells, are also represented during these early stages (**Palis** *et al.*, 1999). Opposite to what is found in the adult BM, primitive macrophages do not differentiate through a monocyte stage, but directly from unipotent macrophage precursors (**Bertrand** *et al.*, 2005; **Takahashi** *et al.*, 1989). These primitive

progenitors mature in the embryo bloodstream, unlike definitive precursors which require FL and post-natal BM maturation (**Kingsley** *et al.*, 2004).

The second wave marks the onset of definitive hematopoiesis at around E8.5 in the recently formed vascular bed of the YS and is characterized by the emergence of erythro-myeloid progenitors (EMPs) (**Bertrand** *et al.*, 2005; **Migliaccio** *et al.*, 1986) that can differentiate into erythrocytes, megakaryocytes, macrophages, and other myeloid lineages such as neutrophils, granulocytes, and mast cells (**McGrath** *et al.*, 2015; **Palis** *et al.*, 1999). EMPs stem from a specialized endothelial subpopulation known as hemogenic endothelium (HE) by a process termed endothelial to hematopoietic transition (EHT) (**Frame** *et al.*, 2016; **Kasaai** *et al.*, 2017). These early progenitors proliferate extensively and are found in the bloodstream shortly after their emergence, moving towards the nascent FL, which is colonized by E10.5 (**Palis** *et al.*, 1999). EMPs are identifiable by their surface expression of c-Kit, CD16/32, and low levels of CD45 *(**McGrath** et al.*, 2015). Even though HSC-independent hematopoietic cells are transient, they serve an extremely important role as the major producers of erythrocytes throughout embryonic life (**Soares-da-Silva** *et al.*, 2020). Definitive erythrocytes can be found in circulation at around E11.5-E12.5, resembling adult erythrocytes as they express embryonic βH1- and adult β1- but lack εγ-globins, alongside their smaller size and absence of a nucleus (**McGrath** *et al.*, 2011). Albeit the initial thought that YS hematopoiesis was only required to bridge the gap of HSC activity, it has become clear that these cells also contribute to embryonic organogenesis and to the adult hematopoietic system. Of note, the initial seeding of tissue-resident macrophages (TRMs) in the developing tissues is attributed to YS-derived hematopoietic cells, which acquire specific signatures and functions. Some maintain their self-renewal ability and support the population throughout life (prominently brain, but also in liver, lung, and epidermal TRMs), while others are replaced or co-exist with HSC-derived cells (**Gomez Perdiguero** *et al.*, 2015).

The third and final wave of embryonic hematopoiesis occurs between E9.5 and E11, now on an intraembryonic structure, the aorta-gonad-mesonephros (AGM) region, and gives rise to HSCs, also through EHT (**Cumano** *et al.*, 1996; **Dignum** *et al.*, 2021; **Medvinsky & Dzierzak**, 1996; **Müller** *et al.*, 1994). HSCs can be defined as the hematopoietic precursor cells that harbor the ability to generate all types of blood cells and to maintain hematopoiesis *in vivo* throughout life by means of self-renewal. They can be experimentally identified by the ability of a single cell to engraft, self-renew and regenerate all blood lineages when transplanted to lethally irradiated mice and further repopulate secondary recipients (**Morrison** *et al.*, 1995). After being formed, they rapidly enter circulation and colonize the FL, where they mature, expand extensively, and differentiate into erythroid, myeloid, and lymphoid lineages (**Ema & Nakauchi**, 2000; **Kieusseian** *et al.*, 2012; **Taoudi** *et al.*, 2008). HSCs are found within the $Lin^{-}CD45^{+}Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSK) compartment and can

be further divided based on their reconstitution ability into long-term (LSK CD150^{+}CD48^{-}, LT-HSCs) or short-term (LSK CD150^{-}CD48^{+}, ST-HSCs) reconstituting cells (**Kim** *et al.*, 2006). This wave is also characterized by a surge of multipotent progenitors (MPPS), biased towards specific lineages but lacking long-term self-renewal capacity, wherefore they can only regenerate blood lineages for a short period of time. This population arises from progenitor-restricted HE cells, present in both extra- (YS) and intraembryonic (para-aortic splanchnopleura, P-Sp) tissues, prior to HSCs emergence (**Cumano** *et al.*, 1996; **Frame** *et al.*, 2016). MPPs can generate lymphoid-primed multipotent progenitors (LMPPs), common lymphoid progenitors (CLPs), and common myeloid progenitors (CMPs), that further differentiate into either granulocyte-monocyte progenitors (GMPs) or megakaryocyte-erythrocyte progenitors (MEPs) (**Akashi** *et al.*, 2000).

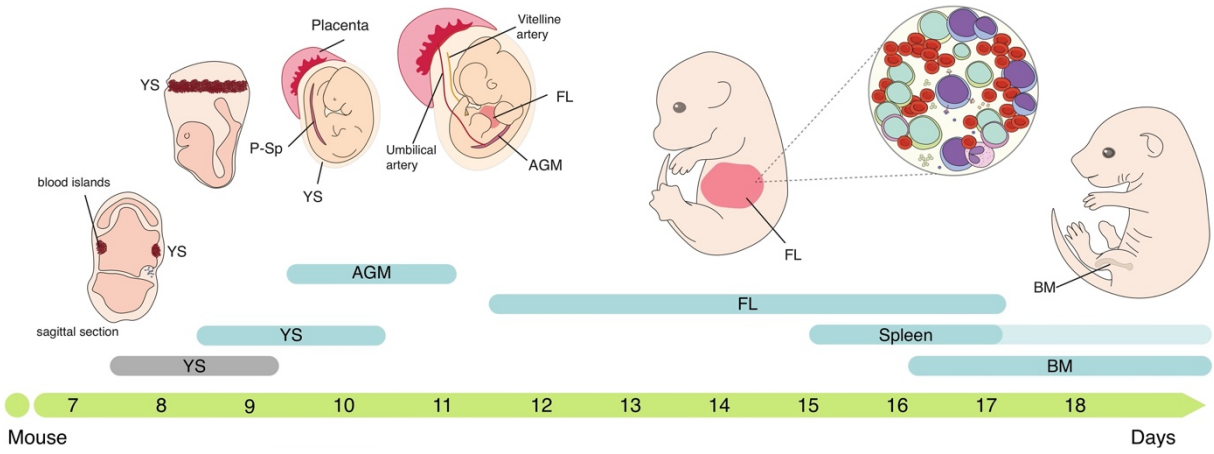

Figure 1 | Ontogeny of the hematopoietic system

Embryonic hematopoiesis is established in three distinct waves. The first wave sprouts in YS blood islands, giving rise to primitive erythrocytes, macrophages, and megakaryocytes. The second wave emerges in the vascular plexus of the YS, generating EMPs. The latter are the origin of TRM that can persist throughout life. The third and final wave occurs in the AGM, from where HSCs emerge to then migrate to the FL, the major hematopoietic organ during embryonic development. Small numbers of hematopoietic progenitors also colonize the fetal spleen and are still found a few weeks after birth. Migration to the BM occurs as a continuous process. Grey lines correspond to primitive waves, while blue ones represent definitive waves. Adapted from (**Soares-da-Silva** *et al.*, 2020).

Hematopoietic progenitors from all waves circulate via the blood vessels and seed the FL to support homeostasis until LT-HSC-derived hematopoiesis is established in the BM between E16.5 and E18 (**Christensen** *et al.*, 2004). At this stage, the BM becomes the main organ responsible for hematopoiesis, where HSCs become largely quiescent but still retain the capacity of unlimited self-renewal, albeit only dividing to maintain the stem cell pool. The replenishment of blood lineages appears to be guaranteed by downstream MPPs. In the adult, all blood lineages (except microglia) arise from the differentiation of HSCs and although adult and fetal HSCs share similar lineage potentials, some lymphoid lineages are only produced during embryonic development, namely dendritic epidermal T cells (**Ikuta** *et al.*, 1990), lymphoid tissue inducer cells (**Eberl** *et al.*, 2004) and a subset of IL-17-producer γδT cells (**Haas** *et al.*, 2012).

Hematopoietic hierarchy

HSCs have historically been defined based on two essential properties: self-renewal and multipotency. By contrast, progenitors lack extended self-renewal ability and present a restricted lineage differentiation capacity. With the characterization of progenitor populations downstream of HSCs in the year 2000, the hematopoietic hierarchy was postulated as a tree-like branched roadmap that describes the stepwise differentiation process – the HSC population sat at the apex of the hierarchy and generates a lineage-balanced differentiation outcome. In this model, the first branch segregates lymphoid potential from all other lineages (myeloid, erythroid, and megakaryocytic), followed by several further branching steps on either side, from multi- to bi- and finally to unipotent progenitor cells. However, this classical model was based on the premise that purified HSCs were homogenous due to their identical cell surface markers and that each cell possessed identical differentiation ability.

Upon the subsequent refinement of surface marker characterization, the classical tree was modified, and the lymphoid and myeloid fates remained associated until further down the tree (**Adolfsson** *et al.*, 2005; **Doulatov** *et al.*, 2010), megakaryocyte branching happened earlier (**Sanjuan-Pla** *et al.*, 2013; **Yamamoto** *et al.*, 2013) as well as subdivisions of the multipotent progenitor compartment into distinct subpopulations (**Cabezas-Wallscheid** *et al.*, 2014; **Pietras** *et al.*, 2015). Additionally, the HSC pool itself was discovered to be functionally and molecularly heterogenous, which further intricated the tree (**Sanjuan-Pla** *et al.*, 2013; **Yamamoto** *et al.*, 2013). Regarding self-renewal, this heterogeneity seems to be directly correlated to the time HSCs spend in quiescence, as the most dormant HSCs display the most robust and longest repopulation capacity (**Bernitz** *et al.*, 2016; **Qiu** *et al.*, 2014). Moreover, distinct cell cycle properties, namely a wide range of division frequencies, result in substantial variations in the contribution of distinct HSC subsets to blood formation *(**Bernitz** et al.*, 2016; **Takizawa** *et al.*, 2011; **Wilson** *et al.*, 2008).

Recently, with the development of multiple omics analysis tools at single-cell resolution, new insights revealed that each HSC is different in terms of molecular signature, cell fate, and functional outcome. This suggests that the differentiation process of a particular blood cell lineage has been already segregated at the HSC stage, and its lineage outcome is principally derived from HSCs. At the molecular level, HSCs retain their common signature gene expression, but possess different chromatin architecture at lineage-specific gene loci and exhibit lineage-specific priming (**Buenrostro** *et al.*, 2018; **Tang** *et al.*, 2017). Hematopoiesis is, therefore, a continuous process that lacks obvious boundaries between cell populations located at different hierarchical levels in the classic hierarchy model. It was found that megakaryocytes are predominantly and directly

differentiated from HSCs, which indicates that this cell population can bypass the stages of MPPS, CMPs, and MEPs (**Notta** *et al.*, 2016).

A summary of the changes in the hematopoietic hierarchy paradigm can be found in **Figure 2**.

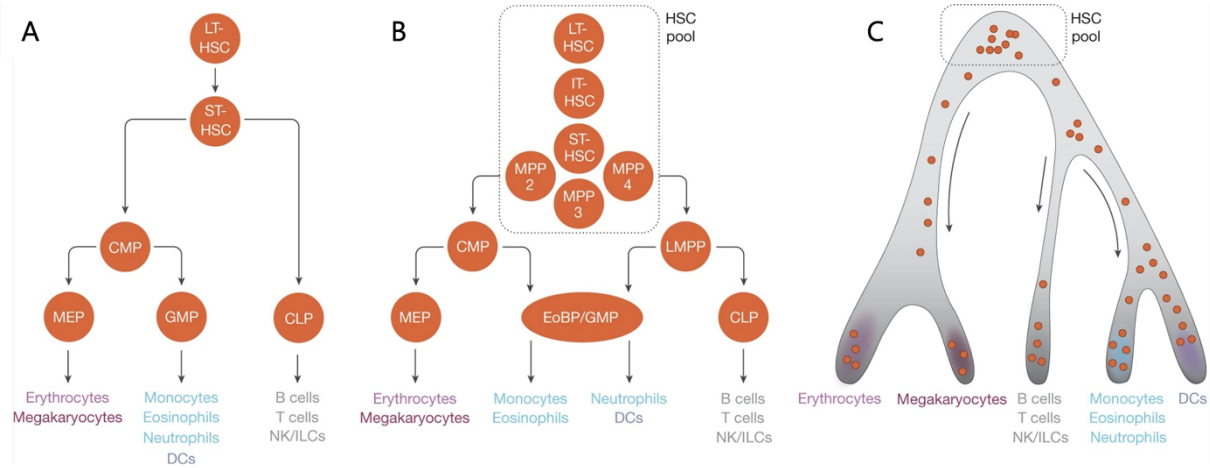

Figure 2 | Timeline of hierarchical models of hematopoiesis

A First representation of a hierarchical model, where HSCs are represented as a homogeneous population from where myeloid and lymphoid lineages bifurcate via the CMPs and CLPs; **B** The model was revised as the HSC pool was accepted as heterogeneous in terms of self-renewal (vertical axis) and differentiation properties (horizontal axis), and the myeloid and lymphoid branches remain associated further down in the hierarchy; **C** Revised hierarchy tree indicating a continuum of differentiation, as each dot represents a single cell and its location along a different trajectory. Adapted from (**Laurenti & Göttgens**, 2018).

Interestingly, hematopoiesis undergoes distinct paths under different physiological conditions. Native hematopoiesis encompasses processes that occur under unperturbed physiological conditions, while stress hematopoiesis encompasses non-homeostatic conditions, including transplantation, cell culture, and injury (**Zhao & Baltimore**, 2015). Even though HSCs exhibit differentiation tendency biases toward particular lineages at steady state, it was reported that transplanted HSCs can generate all blood lineages to reconstitute the impaired hematopoietic system. This means that, in stressed conditions, impaired hematopoiesis is largely replenished by HSCs.

The complexity of the HSC fate decision, especially on the control of the self-renewal *versus* differentiation switch, increases the challenge of cell fate manipulation *in vitro*. Aside from cell-intrinsic factors, is of extreme importance to understand what kind of external signals are influencing hematopoietic cells.

Development of embryonic hematopoietic sites

YS

The YS is an extraembryonic structure that consists of two layers, a visceral endoderm layer, and an extraembryonic mesodermal layer. The importance of this structure in normal embryonic development is mirrored in its functions since its endoderm layer transports and metabolizes maternally derived molecules, while its mesoderm layer is the first site of hematopoiesis during mouse ontogeny (**McGrath** *et al.*, 2011). Between E7 and E7.5, cells from the latter proliferate locally and produce thickened regions called mesodermal masses, that continue to rapidly divide and form more distinct solid structures, the angioblastic cords (**Ferkowicz & Yoder**, 2005). These are the first morphological indications of blood island formation, which is completed by E8.25 and defined by a cluster of primitive erythroblasts that accumulate hemoglobin surrounded by an endothelial covering (**Haar & Ackerman**, 1971; **Palis** *et al.*, 1995; **Silver & Palis**, 1997). Angioblasts further divide and differentiate into endothelial cells that migrate and form the primitive capillary plexus, where the newly generated erythroblasts continue to divide for several days (**Drake & Fleming**, 2000; **Palis & Yoder**, 2001). The vascular network of the YS continues to go through rapid morphological changes (**Udan** *et al.*, 2013) and by E8.5 EHT occurs in the remodeled vascular plexus. The understanding of YS' niche properties on cell differentiation and proliferation is scarce. It was shown that E9.5 YS-derived cell lines could support *in vitro* growth of hematopoietic cells and induce an *in vivo*-like differentiation (**Ferkowicz & Yoder**, 2005).

Placenta

During gestation, the placenta serves as a transient extraembryonic support organ that connects the embryo to the mother. Adequate placental function is instrumental for development progression throughout intrauterine development since it is needed for hormone production that sustains pregnancy, induction of an immune-privileged environment, nutrient supply, and gas and product waste exchange between the fetal and maternal environments (**Ander** *et al.*, 2019). As such, the placenta arbitrates development outcomes and embryogenesis cannot progress without a functional placenta. Defects in placentation can result in fetal growth restriction and other pregnancy-associated disorders (**Hemberger** *et al.*, 2020). Its complex vascular network, the placenta labyrinth, is where maternal blood vessels lined by layers of trophoblast cells interact with the fetal vascular bed that arises from the embryo allantois (pre-umbilical tissue), having, therefore, a mesodermal origin (**Adamson** *et al.*, 2002; **Solomon** *et al.*, 2014). The placenta had not been recognized as a hematopoietic organ until it was shown to be a source of multipotent hematopoietic progenitors before their circulation in blood or FL colonization (**Gekas** *et al.*, 2005). The onset of HSC activity parallels that of the AGM region starting at E10.5-E11, expanding rapidly

until E12.5-E13.5, and disappearing thereafter (**Gekas** *et al.*, 2005; **Ottersbach & Dzierzak**, 2005). Additionally, it was suggested that the placenta could generate *de novo* hematopoietic cells with multilineage potential in a mouse model that lacked a functional circulatory system (**Rhodes** *et al.*, 2008). However, direct proof of HSC emergence from this organ has not yet been attained. Nonetheless, the placental microenvironment exerts an important influence on protecting HSCs from premature differentiation (**Chhabra** *et al.*, 2012).

AGM

The AGM region is derived from the splanchnopleura (of mesodermal origin) and generally extends from the forelimbs to the hindlimbs of the E9.5-E12 mouse embryo, containing the dorsal aorta, the genital ridge, and the mesonephros. At E9, the paired dorsal aortas are linked to the YS vasculature through the vitelline artery and fuse to form a single midline dorsal aorta **(Garcia-Porrero** *et al.*, 1995). The dorsal aorta and placenta are connected via the umbilical artery. In parallel, the urogenital system begins to form with the differentiation of the pronephric and mesonephric ducts, which will originate definitive kidney and the gonads, being populated by primordial germ cells (PGCs) at E10 (**Kaufman**, 1992; **Rugh**, 1968). HSCs are produced *de novo* by the AGM and the major embryonic vessels (**De Bruijn** *et al.*, 2000) through an EHT mechanism (**Cumano** *et al.*, 1996; **Medvinsky & Dzierzak**, 1996; **Müller** *et al.*, 1994). It was shown that hematopoietic activity does not come from urogenital ridges and mesonephros, but primarily from the dorsal aorta (**Godin** *et al.*, 1999). The first HSCs bear a distinctive dual hematopoietic-endothelial phenotype, expressing both VE-cadherin and CD45 markers (**North** *et al.*, 2002; **Taoudi** *et al.*, 2005). Cells of the VE-cadherin^{+}CD45^{+} fraction are intimately associated with the endothelial lining of the dorsal aorta, either integrated into it or forming discrete intra-aortic clusters protruding into the lumen, suggesting a polarity in HSC development (**Taoudi** *et al.*, 2008).

FL

The FL is the major hematopoietic organ throughout embryonic development. Its architecture comprises numerous cell types and tissue interactions, orchestrated from the earliest stages of embryonic development. Around E7, morphogenetic movements fold the endoderm into a hollow structure surrounded by mesoderm, the primitive gut tube (**Zorn**, 2008). Differential Wnt and FGF signaling from the adjacent mesoderm along the anterior-posterior axis patterns this structure into foregut, midgut, and hindgut domains, distinguishable by the expression of *Hhex*, *Pdx1*, and *Cdx* respectively (**Grapin-Botton**, 2005; **Moore-Scott** *et al.*, 2007). At E8.5, hepatic specification is induced in the ventral domain of the foregut (**Calmont** *et al.*, 2006; **Gualdi** *et al.*, 1996; **Rossi** *et al.*, 2001), which thickens to form the hepatic diverticulum (**Bort** *et al.*, 2006). This

monolayer of cuboidal cells begins to express liver genes (*Albumin*, *Afp*, and *Hnf4α*), and transitions to a pseudostratified columnar layer of cells, which proliferate, delaminate, and invade the adjacent septum transverse mesenchyme (STM) to form the liver bud by E9.5 (**Bort** *et al.*, 2006). Of note, at this stage endothelial cells (identified by CD31 and Flk1) lay between the hepatoblasts and the STM, a close association that persists and is necessary for hepatoblasts to migrate (**Matsumoto** *et al.*, 2001). A process of extensive proliferation follows and around E13 bipotent hepatoblasts start to differentiate into either hepatocytes or biliary epithelial cells (cholangiocytes), depending on their location. Within this pool and until around E16.5, there is a subset of hepatoblasts that exhibit a stem cell-like signature, expressing *Dlk1*, continuously self-renew, and differentiating *in vitro* into both hepatocytes and cholangiocytes (**Tanimizu & Miyajima**, 2004). Hepatoblasts in contact with the portal vein begin to adopt a biliary epithelium fate by increasing *CK19* expression and down-regulating hepatic genes, while the majority of hepatoblasts in the parenchyma gradually differentiate into mature hepatocytes.

In addition to the hepatic lineage, endothelial, mesothelial, and mesenchymal cells are the major cell types identified to constitute the FL (**Soares-da-Silva** *et al.*, 2020). Liver endothelial cells are the main regulators of vascular homeostasis as they serve as the interface between blood and tissue. Vitelline veins sprout throughout the STM and by E10 give rise to the hepatic sinusoids, the first vessels to appear in the developing liver. Sinusoidal endothelial cells strongly express CD31 and Flk1 in the early stages of liver development and are downregulated throughout gestation. The portal vein also arises early in liver development, being formed between E10.5 and E12.5 (**Swartley** *et al.*, 2016). Portal vessels express the arterial markers Ephrin-B2 and Neuropilin-1, but not the venous marker EphBB4. This expression profile is inverted at the end of gestation, with the transition into a venular phenotype (**Heng** *et al.*, 2018). Of note, lymphatic vessels were only reported after birth (**Swartley** *et al.*, 2016).

Mesothelial cells compose the liver parenchyma's epithelial lining on the lobes' surface. From E12.5, these cells are characterized by the expression of *Gpm6a*, *podoplanin* (Gp38), *PODXL*, and *mesothelin* (**Lua & Asahina**, 2016). The mesothelium proliferates during liver development, expressing growth factors essential for hepatic development, and remains quiescent after birth (**Onitsuka** *et al.*, 2010). Underneath this layer lays a population often referred to as sub-mesothelial cells or capsular fibroblasts, distinguishable by their expression of *Desmin*, *NGFR*, and *PDGFRα* (CD140a).

The mesothelium and mesenchymal cells share a common ancestry as they both originate from the STM. At approximately E11.5, mesothelial and sub-mesothelial cells migrate inward from the liver surface and give rise to hepatic stellate and perivascular mesenchymal cells (**Lua & Asahina**, 2016). Although these terms have been used interchangeably in the literature, it is not consensual they represent the same population. Hepatic stellate cells can be found in the space of Disse

lining hepatocytes and endothelial sinusoidal cells and are characterized by the expression of *Desmin* and their storage function of vitamin A in the healthy adult liver. Perivascular cells express *NG2*, *Nestin*, and *Vimentin* and are found around periportal but not pericentral blood vessels nor within the space of Disse. These cells exhibit high osteogenic and myogenic, but low adipogenic and chondrogenic, differentiation potential in *in vitro* differentiation assays.

From E10 to E18, the FL accommodates the HS, being the major hematopoietic organ during embryonic development. After arising from the YS and AGM, hematopoietic stem and progenitor cells (HSPCs) eventually migrate to the FL where they undergo proliferation and differentiation before homing to the BM, where they reside in the adult **(Ema & Nakauchi**, 2000; **Palis** *et al.*, 1999). The organization of FL cells changes as the frequency of hematopoietic cells increases. At E13.5, the most frequent FL population are primitive nucleated erythrocytes, located throughout the liver parenchyma, but after E14.5 enucleated erythrocytes can be found within the vessels (**Ayres-Silva** *et al.*, 2011). Concomitantly, megakaryocytes and macrophages are also distinguishable and B cell progenitors can be found interspersed in the tissue by E12.5 and forming perivascular aggregates by E18.5. Additionally, granulocytes appear from E16.5 onward, concentrating around central veins and in the periphery by E17.5, correlating with the presence of mesenchymal cells, which therefore suggests crosstalk between these cell types (**Ayres-Silva** *et al.*, 2011). By this time point, hematopoietic cells exit the organ and migrate to the BM.

BM

Long bones of the mouse embryo begin to develop at approximately E14.5 and arise from mesodermal progenitors. Cartilaginous bone templates are avascular until E16 when they start to be perfused in the periosteum region (the layer surrounding the developing bone) and in the epiphyseal plates found at each end of the long bones (**Coşxkun** *et al.*, 2014). Calcification and vascularization of the BM cavity then begins, primarily in the middle region of the long bone. At this stage, collagen-expressing osteoblasts emerge in the periosteum and by E17.5 they occupy the middle BM cavity and expand alongside endothelial cells, both proliferating toward the epiphyseal plates, thus the pattern of calcification over time parallels that of the forming vascular network (**Coşxkun** *et al.*, 2014). The first fetal BM hematopoietic activity is detected between E15.5 and E16.5, restricted to the vascularized region of the developing bones, and throughout the tissue from E17.5 (**Coşxkun** *et al.*, 2014). Homing, adhesion, and retention of hematopoietic cells in the BM niche are controlled by chemotactic factors, particularly by CXCL12, as proved by that the inactivation of either the chemokine or its receptor CXCR4 led to a significant decrease of HSC frequency in E17.5 fetal BM (**Zou** *et al.*, 1998).

Although cell cycle quiescence is a hallmark of adult BM HSCs (**Wilson** *et al.*, 2008), during the first 3 weeks after birth HSCs are still actively cycling and only become quiescent thereafter (**Bowie**

et al., 2006). The shift to a quiescence state seems to be dependent on the cellular composition of the microenvironment, however very little is known about the immediate changes in the neonatal niche despite the profound shift in its physiological activity (**Coşxkun** *et al.*, 2014). Fast forward to the adult BM, HSCs reside within perisinusoidal niches, which comprise Leptin receptor-expressing mesenchymal stromal cells and endothelial cells synthesizing factors required for HSC maintenance (**Ding & Morrison**, 2013).

The FL niche

HSCs usually reside in the special microenvironment referred to as niche, which supports their self-renewal ability and retention of their identity (**Schofield**, 1978). Once HSCs leave the niche, they easily lose the ability for self-renewal and either start differentiation upon cytokine signaling or undergo apoptosis. As such, decades of laboratory studies in trying to recapitulate an environment that permits HSCs self-renewal *in vitro* have proven exceedingly difficult with limited success. While most studies of the HSC niche involve those residing in the BM, a better understanding of the FL niche is essential to recapitulate it *in vitro* and may provide clinically relevant information about expanding HSCs *ex vivo* for transplantation.

The FL provides HSCs with a unique microenvironment to support their expansion, maturation, and differentiation towards distinct lineages. The crosstalk between the hepatic and hematopoietic systems, mediated through cell-cell interactions, either directly, or through cytokine signaling, makes the FL a unique organ in embryonic hematopoiesis. HSPCs, and also EMPs, colonize the FL after bud formation at E10.5. Its privileged anatomical location in the architecture of fetal circulation makes the FL the first intraembryonic organ circulating hematopoietic cells encounter. Distinct mechanisms of homing to the FL have been proposed, mostly relying on cell adhesion-mediated processes and/or chemoattraction by cytokines, chemokine signaling, and growth factors. It has been shown that HSPCs lacking β1 integrin were unable to colonize the FL, even though they were still present in the circulation and capable of generating all blood progeny, suggesting a role for integrin-mediated FL colonization (**Hirsch** *et al.*, 1996). Additionally, progenitors bind to extracellular matrix (ECM) components such as fibronectin, collagen, and laminin through integrins. In the FL, hepatoblasts are the major producers of ECM components, which might explain the premature death of embryos lacking hepatoblasts, even though they can still form the liver bud (**Nishina** *et al.*, 1999). Regarding cytokine and chemokine signaling, the SDF-1, commonly known as CXCL12 and acting through binding to its receptor CXCR4 present in HSPCs, has been extensively studied in the adult BM **(Yu & Scadden**, 2016). In the FL, CXCL2 is expressed by hepatoblasts and pericytes, and its role in FL

colonization has been proved using CXCL12$^{-/-}$ embryos. Although HSCs' numbers were similar at the early stages of development, by E16.5 FL HSCs were reduced by more than two-fold, and an abnormally high number of these cells was found in circulation. Therefore, CXCL12 might be an important factor for HSC retention in the FL, although not for its initial colonization (**Ara** *et al.*, 2003). The colonization step is crucial for the normal progression of hematopoiesis, as the FL provides signals necessary not only for the maturation of newly formed HSCs but also for the differentiation of committed progenitors towards distinct lineages. Immature HSCs cultured with the OP9 BM stromal cell line in the presence of thrombopoietin (TPO) or with E10.5 FL rudiments acquire a mature HSC phenotype, proving the FL's role in maturation (**Kieusseian** *et al.*, 2012). Regarding differentiation, for instance, IL7-producing hepatoblasts control the survival and proliferation of lymphoid progenitors that develop into the B-cell lineage (**Berthault** *et al.*, 2017). Moreover, erythropoietin (EPO) produced by the same cell population is required for the proliferation and terminal differentiation of erythroid progenitors (**Inoue** *et al.*, 2011). Additionally, TPO-expressing hepatoblasts are the main regulators of megakaryocyte differentiation and platelet production (**Kaushansky** *et al.*, 1995). Other cytokines, chemokines, and growth factors that play important roles in HSC proliferation, maintenance, and survival are also present in the FL, expressed by hepatoblasts and other stromal cells, as reviewed in **Soares-da-Silva** *et al.*, 2020.

Ex vivo mimicking of the FL niche

Given the clinical importance of HSCs in transplantation, numerous studies have attempted to develop conditions that stimulate HSC self-renewal *ex vivo*. As the FL niche holds the unique capacity of expanding HSCs *in vivo*, recapitulating its environment might be the key to achieving so. So far, most of the attempts involving HSC cultures utilize cytokines and growth factors that were proven to impact hematopoietic function, such as stem cell factor (SCF), TPO, and FLT3L, as well as IL11 and IL6 (**Wilkinson** *et al.*, 2020; **Zhang & Lodish**, 2008). A recent breakthrough was reported with culture conditions supporting between 236- and 899-fold expansion of functional HSCs. High levels of TPO synergized with low levels of SCF and polyvinyl alcohol was proposed as a replacement for serum albumin. However, considerable heterogeneity in the self-renewal capacity of HSCs was found under these conditions (**Wilkinson** *et al.*, 2019). Additionally, attempts to the establishment of co-culture systems have been developed and tested for the maintenance or expansion of HSCs. That is the case for several stromal cell lines such as AFT024, MMH, and hFLSECs-E4orF1 which were proven to offer a supportive *ex vivo* microenvironment for HSCs (**Aiuti** *et al.*, 1998; **Li** *et al.*, 2019; **Moore** *et al.*, 1997). Importantly, the common ground of successful stromal cell lines appears to be correlated with the expression of *Dlk1*, a factor sufficient for

hematopoietic support (**Moore** *et al.*, 1997). Nevertheless, these cell lines only maintain HSC potential and do not promote their expansion. Co-culture systems with primary cells have also been recently reported in studies devoted to understanding the role of specific FL populations that might be implicated in this mechanism. It is the case of $DLK1^+$ hepatoblasts that can expand LT-HSCs around 20-fold after a 3-week co-culture period, in what appears to be a contact-dependent manner as conditioned medium and transwell cultures did not result in the same expansion levels (**Chou** *et al.*, 2013). However, it has been challenging to assess the role of hepatoblasts *in vivo* since mice with hepatoblast deficiencies die between E12 and E14 and studies of liver development fail to look at the hematopoietic system (**Nishina** *et al.*, 1999). *Dlk1* is also expressed in $Nestin^+$ cells surrounding portal vessels, a cell type that appears to have a close physical association with HSCs and, therefore, an implication in the FL niche (**Khan** *et al.*, 2016). Upon $Nestin^+NG2^+$ pericytes selective elimination, HSCs become less proliferative, and their numbers are reduced. Additionally, upon *Scf* deletion from both hepatic stellate and endothelial cells, populations also related to the hepatic vasculature, FL HSCs were reported to be lost (**Lee** *et al.*, 2021). This evidence suggests that tight control of the location of HSCs relative to other cells in culture might be an important parameter in future *in vitro* models.

With the help of evolving views and emerging bioengineering techniques, novel systems might greatly facilitate the understanding of the crosstalk between the FL niche and HSC expansion. Fundamental biological questions that could not be investigated using traditional culture plates can now begin to be answered. Engineered niches should be easy to produce and scale up with the capacity for high-throughput analysis. In addition, it should afford niche elements serving as essential inducers of HSC fate and offer the possibility to examine combinations of cues (**Choi** *et al.*, 2015). Recent advances in biopolymers and hydrogels have allowed the incorporation of ECM into cell culture systems containing cells and immobilized soluble factors to maintain a higher level of control over cells without overcomplicating the system (**Gilchrist** *et al.*, 2019). Moreover, a growing number of studies have revealed crucial roles in the mechanical properties of the hematopoietic niche (**Adamo & García-Cardeña**, 2011; **Zhang** *et al.*, 2019). The effects of flow, matrix stiffness, and other mechanical properties on HSC homing and expansion remain entirely unexplored in the FL, in part due to the inaccessibility of the organ and difficulty in manipulating mechanical properties *in vivo*. It was recently reported the design of a biomimetic *ex vivo* microfluidic model of the FL that enables monitoring of HSC homing to and interaction with FL niches under flow, using embryonic matrix elasticity conditions (**Mohammadalipour** *et al.*, 2021). As it is highly customizable, this model enables the study of the molecular and mechanical cues that impact HSC trafficking and the self-renewal behaviors that occur within the FL niche. Significant advances are also being made in the design of bioscaffolds. The ECM, including its structural and functional constituents, has become a popular 3D scaffold for tissue reconstruction.

Moreover, the use of decellularized tissues has been also proposed, as it allows the harnessing of the preserved macrovascular skeleton to aid cell seeding and proliferation throughout the scaffold. This and other approaches have been used in an attempt to create a functional artificial FL organoid *(**Salmon** et al.*, 2022). Another possibility for niche establishment is through the differentiation of induced pluripotent stem cells (iPSCs) or embryonic stem cells (ESCs) into a complex multilineage hepatic culture. Overexpression of GATA6 followed by guided hepatic differentiation resulted in engineered iPSC cultures that expressed markers for FL cells and also produced hematopoietic cells, likely through EHT of residual mesodermal populations (**Guye** *et al.*, 2016). Such systems provide a potential model for unraveling novel interactions and pathways between hepatic and hematopoietic cells.

Despite all the above-mentioned efforts, a robust model for HSC expansion *ex vivo* is still yet to be attained. Nonetheless, many efforts with promising results have been arising in modeling the adult BM niche. As an example, a BM-on-a-chip method was implemented by inducing the development of bone tissue in a device implanted *in vivo*, which was later inserted into a microfluidic system for *in vitro* culture (**Torisawa** *et al.*, 2014). Blood cell populations could be maintained in normal proportions for at least one week, even in the absence of exogenous cytokines. In another attempt, the BM niche's structure, composition, and organizational features were recapitulated using a perfused bioreactor system that combined scaffolds with structural and compositional features of bone with cell-deposited ECM, which resulted in an increase in phenotypic HSC numbers in culture (**Bourgine** *et al.*, 2018). However, vascular and neuronal networks, which are known to be regulators of HSC activity (**Katayama** *et al.*, 2006), were not included in this model.

MATERIALS AND METHODS

Mice management

C57BL/6J mice were purchased from Charles River Laboratories. Timed pregnancies were generated after overnight mating and females with vaginal plugs detected the following morning were considered to be at E0.5. All animal manipulations were performed according to the ethics charter approved by the French Agriculture ministry and to the European Parliament Directive 2010/63/EU.

Isolation of FL stromal and hematopoietic cells

FLs were dissected under a binocular magnifying lens and recovered in Phosphate-buffered saline (PBS) solution (Gibco) supplemented with 1% fetal calf serum (FCS) (Gibco) and cut into 1 mm^2 pieces. For stromal cell isolation, enzymatic digestion was performed by incubation with 0.025 mg/mL Liberase (Roche) and 0.2 mg/mL DNAse (Roche) for 6-8 minutes at 37ºC. Enzymatic treatment was stopped by the addition of cold PBS + 1% FCS solution followed by centrifugation. For hematopoietic cell isolation, mechanical digestion was performed by passing FLs through a 26-gauge needle of a 1 mL syringe (BD Biosciences). Before staining, cell suspensions were filtered using a 100 µm cell strainer (BD Biosciences). For stromal isolation, cell suspensions were depleted of hematopoietic cells ($Ter119^+CD45^+c\text{-}Kit^+CD71^+$), and for hematopoietic isolation depleted of lineage-committed hematopoietic cells ($Ter119^+CD71^+CD19^+Gr\text{-}1^+$) using MACS columns (Miltenyi Biotec) according to manufacturer instructions. Briefly, cells were incubated with biotinylated antibodies listed in **Supplementary Table 1** (or **Supplementary Table 2** for depleted spheroids) for 20-30 minutes at 4ºC, washed twice with PBS + 1% FCS solution, and incubated with anti-biotin microbeads (Miltenyi Biotec) for 20 minutes at 4ºC and passed through an LS column (Miltenyi Biotec). The flow-through was collected and used for cell surface staining.

Flow cytometry and cell sorting

Cell suspensions were stained for 20-30 min at 4ºC in the dark with antibodies listed in **Supplementary Table 1**. Dead cells were excluded by incorporation of Propidium Iodide (PI) (Sigma-Aldrich). Unstained, single stained, and before depletion samples were taken as controls. Single stains were performed using UltraComp eBeads (Invitrogen). Stained cells were analyzed on ID7000 Spectral Analyzer (SONY) and were sorted with a BD FACSAria III (BD Biosciences) according to the guidelines for the use of flow cytometry and cell sorting (**Cossarizza** *et al.*, 2019). Data were analyzed with SONY ID 7000 Analyzer and FlowJo software (v.10.8.1, BD Biosciences).

Cell culture

Irrespectively of the culture system, E14.5 FL and hematopoietic cells were cultured immediately after sorting in Opti-MEM (Gibco) supplemented with 5% FCS (Gibco), 1% Penicillin Streptomycin (Biowest), and 0,1% b-Mercaptoethanol (Invitrogen). For plate cultures, stromal cells were seeded at a density of 3 000 or 10 000 cells per well in 96 or 48-well plates, respectively, coated with a 5% collagen solution (PureCol™, Advanced BioMatrix). When hematopoietic cells were co-cultured with stromal populations, the already existing media was not replaced, and cells were seeded at a density of 1 000 cells per well in 48-well plates. Cultures were kept in a humidified atmosphere at 37ºC with 5% CO_2 throughout the desired culture time.

Microfluidic Chip (Emulate Inc.)

The design and fabrication of the microfluidic chip were based on previously described protocols. Succinctly, the chip consists of a polydimethylsiloxane (PDMS) block with two parallel microchannels (a 1 x 1 mm top channel and a 1 x 0,2 mm bottom channel) separated by a thin (50 μm), porous membrane (7 μm diameter pores with 40 μm spacing). Flow can be induced to each channel independently to continuously provide fresh media to the culture, as effluent containing secretion and waste components is collected from each channel separately. Prior to cell seeding, chips were functionalized using Emulate's protocols and reagents. Briefly, ER-1 and ER-2 (Emulate reagents 10461 and 10462, Emulate Inc.) are added to the top and bottom channels as a solution of 0,5 mg/mL. The chip is then irradiated with high-power UV light having a peak wavelength of 365 nm and intensity of 100 $\mu J/cm^2$ for 20 minutes using a UV oven. After surface functionalization, both channels were coated with a collagen solution for 4 hours (100 μg/mL, Corning). Cells were seeded at a density of 2 x 10^6 cells/mL in both channels and incubated overnight in a static

environment. The day after, chips were connected to the Pod-1® and placed in the Zoë® Culture Module (Emulate Inc.) and both channels were maintained under constant perfusion (60 μL/h) at 37ºC with 5% CO_2 throughout the desired culture time. Cells were either fixed with Paraformaldehyde (4% w/v) for 20 minutes for imaging or collected to lysis buffer (RLT+, Qiagen) for qPCR analysis, using Ethylenediamine tetraacetate acid (EDTA) (100 mM, Fisher BioReagents).

Spheroid formation

Hanging drop

Depleted or freshly sorted cell suspensions were prepared considering the desired cell density and the different cell population combinations. Drops of 20 μL of media containing cells were done on the lid of a Petri dish, which was then inverted and kept in a humidified atmosphere at 37ºC with 5% CO_2 throughout the desired culture time. Spheroids were collected by aspiration flowed by further washing of the lid with PBS to ensure all spheroids were collected.

U-bottom plates

Freshly sorted cell suspensions were prepared considering the desired cell density and the different cell population combinations and seeded on an ultra-low attachment round bottom plate (Costar) containing media. The plate was centrifuged at 300g for 5 minutes to promote cell contact and then kept in a humidified atmosphere at 37ºC with 5% CO_2 throughout the desired culture time.

Immunofluorescence

FL sectioning, immunostaining, and optical clearing of thick sections

FLs from time-pregnant females were collected directly into freshly prepared 2% Formaldehyde fixative solution (Thermo Scientific) and kept at 4°C overnight under rotation. After washing in PBS and removing surrounding tissues, FLs were shortly stored at 4ºC until sectioning. Following FLs embedding in 4% Agarose (Invitrogen), 100 μm sections were performed using a Leica VT1200 vibratome. Sections were permeabilized and blocked with PBS containing 0.05% Tween-20 (Sigma-Aldrich) or 0.5% Triton-X 100 (Sigma-Aldrich) for nuclear staining and 10% donkey serum (Sigma-Aldrich) for 1 hour at room temperature (RT). This solution was also used to dilute primary and secondary antibodies. Sections were incubated with primary antibodies for 2 hours at RT followed by overnight at 4ºC, and with secondary antibodies for 2 hours at RT, both in the dark

and with rotation (see **Supplementary Table 3**). Between incubations and after secondary antibody incubation, sections were washed repeatedly (minimum of 4 times, 15 minutes each) with PBS containing 0.05% Tween-20 and 3% NaCl (Sigma-Aldrich). Sections were counterstained with DAPI (D9542, Sigma-Aldrich) for 2 hours at RT and kept overnight in RapiClear 1.52 (SunJin Lab Co.) for optical clearing. Sections were mounted in RapiClear and imaged on a Leica TCS SP8 using a PL APO 63x /1.40 oil immersion lens (zoom factor 0.75). 10-20 µm tile scans were acquired at 200/400Hz, bidirectional mode, with z spacing of 0.3 µm, at 12-bits and 1024×1024 resolution.

Spheroid imaging

Formed spheroids were collected to Eppendorf tubes, centrifuged (300g, 5 min), and immediately incubated in freshly prepared 2% Formaldehyde fixative solution (Thermo Scientific) for 15 min at 4°C. All the below-mentioned steps were interspersed with thorough centrifuging (300g for 5 minutes) to minimize spheroid loss. After washing the fixative in PBS, spheroids were incubated with primary antibodies diluted in blocking solution (PBS containing 0.05% Tween-20 (Sigma-Aldrich) or 0.5% Triton-X 100 (Sigma-Aldrich) for nuclear staining and 10% donkey serum (Sigma-Aldrich)) overnight at 4ºC. Secondary antibodies were incubated for 1 hour at RT, in the dark and with rotation (see **Supplementary Table 3**). Between incubations, spheroids were washed with PBS containing 0.05% Tween-20 and 3% NaCl (Sigma-Aldrich) and centrifuged. Spheroids were counterstained with DAPI (D9542, Sigma-Aldrich) for 20 min at RT and kept overnight in RapiClear 1.52 (SunJin Lab Co.) for optical clearing. Spheroids were mounted in RapiClear and imaged on a Leica TCS SP8 using a PL APO 40x /1.30 oil immersion lens (zoom factor 1.8). Z-stacks were acquired at 200Hz, bidirectional mode, with z spacing of 0.3 µm, at 12-bits and 1024×1024 resolution. For viability assessment, spheroids were collected and incubated with Propidium Iodide (Sigma-Aldrich) for 2 hours at RT and representative images of different z-positions of the spheroid acquired.

Gene expression

qPCR

Cells were either sorted directly or collected into lysis buffer (RLT+, Qiagen), and mRNA was extracted (RNeasy Micro Kit Cat# 74004, Qiagen), reverse transcribed (PrimeScript RT Reagent Kit Cat# RR037A, Takara Bio), and quantitative PCR (qPCR) was performed with Power SYBR Green PCR Master Mix (Cat# 4304437, Applied Biosystems) (**Supplementary Table 4**). qPCR reactions were performed on a Quantstudio3 thermocycler (Applied Biosystems). Gene expression was normalized to that of β-actin, and relative expression was calculated using the $2^{-\Delta Ct}$ method.

Culture samples were collected by scratching the content of each well and spheroids were collected directly into lysis buffer.

Statistics

All results are shown as mean ± SD. Statistical significance was determined using Sidak's multiple comparisons test or one-way ANOVA followed by Tukey's multiple comparison test where a P value of <0.05 was considered significant.

CONTEXT

The work herein stems from an ongoing project shared between Ana Cumano's laboratory at Institut Pasteur (Paris, France) and Perpétua Pinto-do-Ó's group at Instituto de Investigação e Inovação em Saúde (Porto, Portugal) which aims to unravel the mechanisms of HSC expansion in the FL during embryonic hematopoiesis by thoroughly characterizing this organ. A comprehensive approach was designed to portray the cellular organization of the FL as a way to identify the spatial relationship between hematopoietic and stromal cells. The latter were further characterized into distinct lineage-affiliated cellular subsets by flow cytometry and gene expression. At E14.5, the major non-hematopoietic populations (that represent around 3% of the FL's composition) are the hepatoblasts, endothelial, mesenchymal, and mesothelial cells. However, a population of $CD54^{-}CD31^{-}CD140a^{-}Gp38^{-}$ remains to be affiliated. PI and Hoescht staining revealed that most of these cells are either dead or deficient in DNA content, and therefore might result from the drastic changes in the cellular composition of the FL that occur during its ontogeny (**Figure 3**).

The gathered knowledge set the base to aim for the establishment of an *ex vivo* FL niche that would mimic the expansion-prone environment of this organ using its native cell populations. In this regard, much effort has been devoted to understanding how the different hepatic populations behave in culture and which conditions result in the best *in vitro* maintenance. After testing different media and coating solutions, it was evident that the more elaborated (i.e., cytokine-supplemented) culture conditions did not result in significant improvements. Therefore, simpler conditions were maintained throughout this work.

The work herein intends to explore different culture approaches to establish an *ex vivo* FL niche capable of supporting hematopoietic activity.

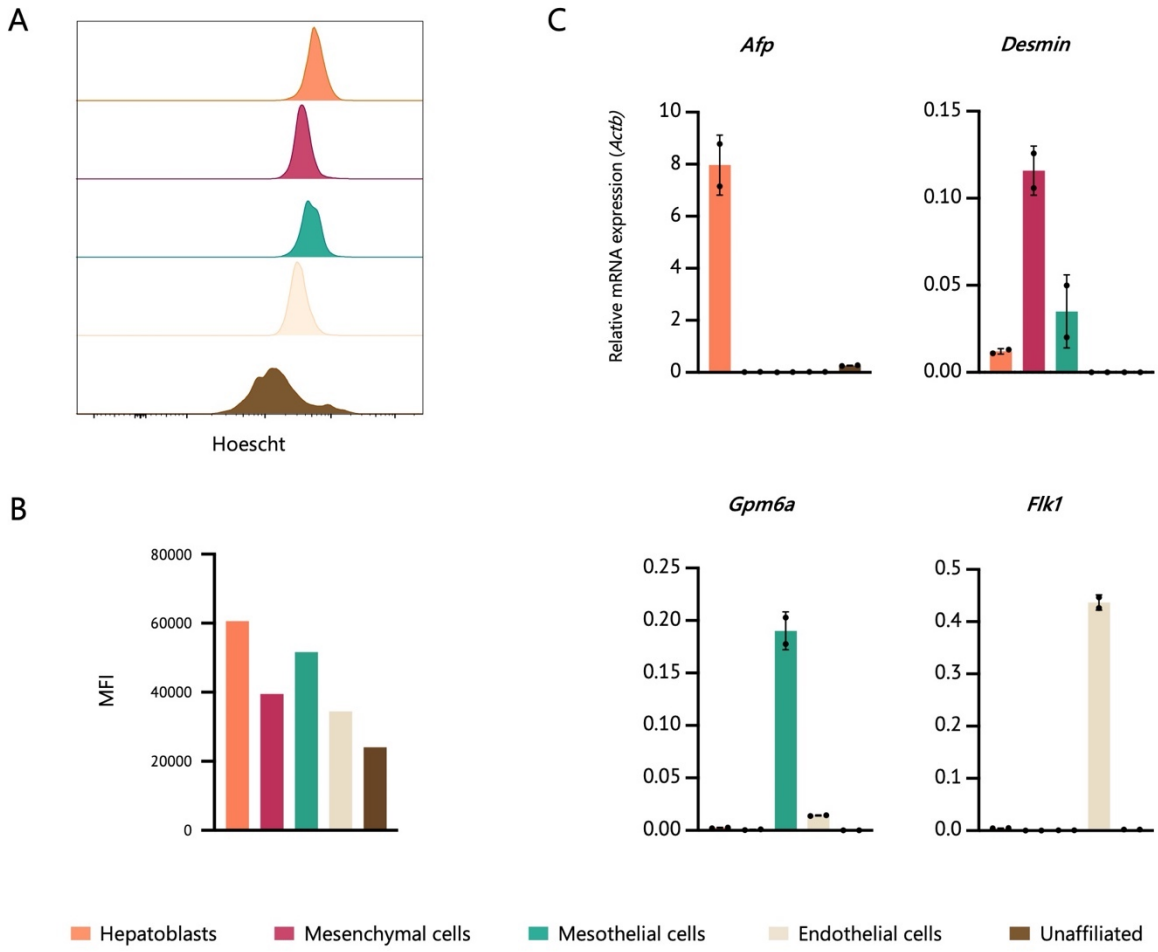

Figure 3 | Hepatoblasts, mesenchymal, mesothelial, and endothelial cells are the major stromal populations of the E14.5 FL

A The majority of the Unaffiliated population presents low Hoescht incorporation, indicative of low viability; **B** Mean Fluorescence Intensity (MFI) of Hoescht staining points out hepatoblasts and mesothelial cells as the most proliferating cell populations and reinforces the lack of DNA content of the Unaffiliated subset; **C** The Unaffiliated populations does not express characteristic transcripts of hepatic (*Afp*), mesenchymal (*Desmin*), mesothelial (*Gpm6a*), or endothelial (*Flk1*) lineage. Unpublish data from Cumano's group.

RESULTS

Characterization of non-hematopoietic cellular subsets of the FL

Gene expression analysis of FACS sorted cellular subsets enabled the characterization of the three major cell populations that were investigated herein (**Figure 4A**). For that and taking advantage of past studies developed in Cumano and Pinto-do-Ó groups', a discriminative panel of structural proteins and cytokines was selected. *Afp*, *Desmin*, and *Gpm6a* discriminate CD54^{+} hepatoblasts, CD140a^{+} perivascular/stellate (further referred to as mesenchymal), and Gp38^{+} mesothelial cells, respectively. Transcript levels of key hematopoietic cytokines identify hepatoblasts as the major producers of *KitL*, *Epo*, and *IL7* whereas *Cxcl12* is mostly expressed in the mesenchymal subset, corroborating previous studies (**Figure 4B**).

On the assumption that physical interactions between stromal and hematopoietic cells might impact hematopoiesis, understanding where these populations locate within the tissue is required. 3D tissue imaging has become a valuable tool in achieving so, as it allows a deeper analysis of tissue complexity. Hepatoblasts can be found distributed throughout the developing FL parenchyma based on Hnf4α immunostaining, a crucial hepatic transcript factor for liver development (**Delaforest** *et al.*, 2019). Additionally, CD146 expression labels vascular structures and allows the identification of both endothelial and perivascular cells. Lhx2, a liver-specific perisinusoidal stellate cell marker, expression can be found near the vasculature and in the sub-mesothelial layer, which goes in agreement with the described locations of stellate cells (**Figure 4C – F**) (**Lua & Asahina**, 2016).

A deeper comprehension of each FL population is key to understanding how the organ functions as a whole and why it has such a crucial role in embryonic hematopoiesis. Nonetheless, one should not devalue the interactions that occur between each cellular subset, as it might be a fundamental aspect to translate the niche environment from *in vivo* to *in vitro*.

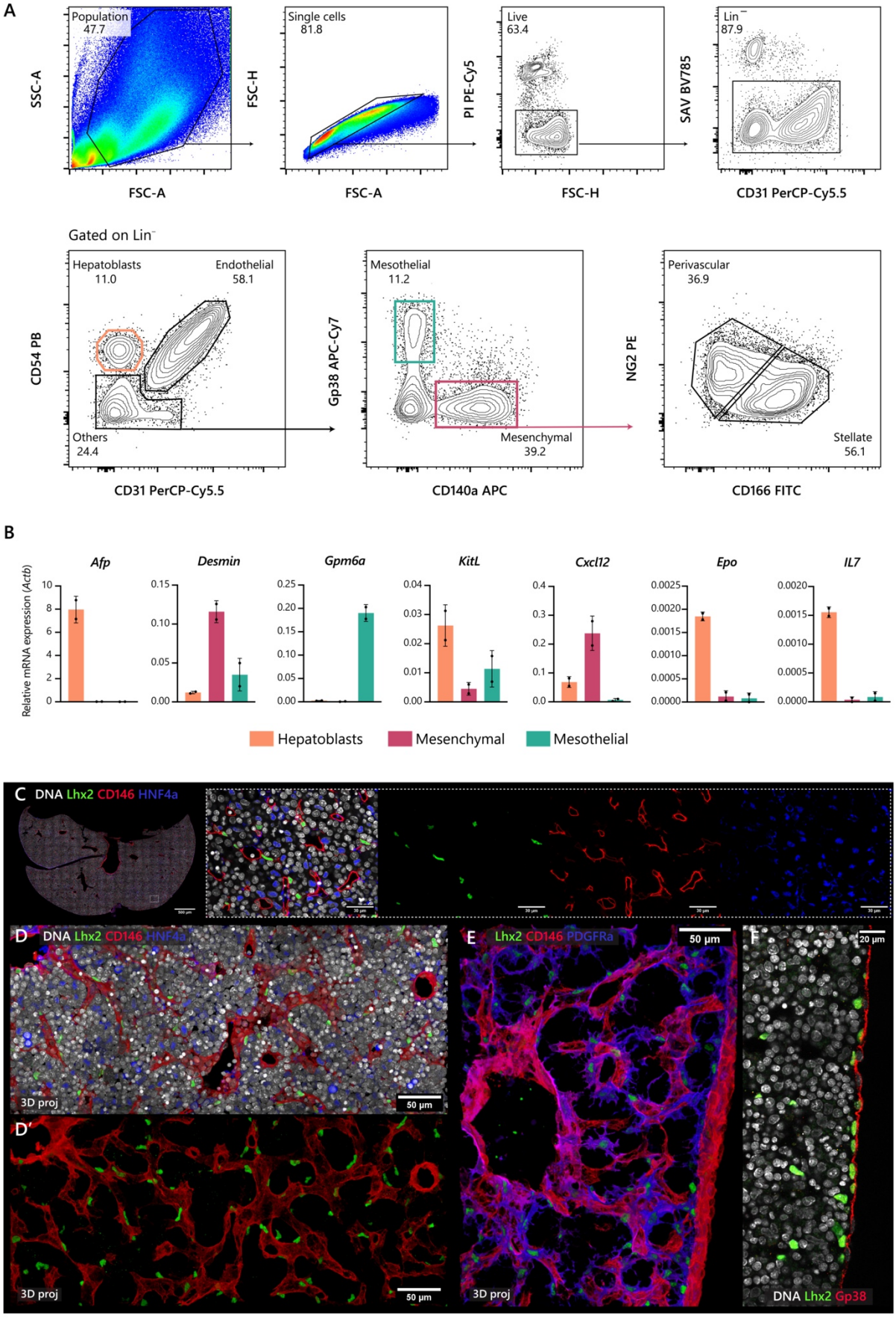

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Figure 4 | Characterization of the major non-hematopoietic FL populations at E14.5

A Flow cytometry profile of Ter119$^-$ CD45$^-$ c-Kit$^-$ CD71$^-$ cells in the FL. CD140a+ cells can be further distinguished based on NG2 and CD166 expression in perivascular and stellate cells, respectively; **B** Gene expression analysis discriminates three major stromal populations present in the FL at this stage of development– hepatoblasts, mesenchymal, and mesothelial cells; **C – D** HNF4α localizes the nuclei of hepatoblasts, that are evenly distributed across the tissue. Stellate cells typically localize near the vasculature, as it can be seen by the close association of Lhx2- and CD146-expressing cells; **E – F** Hepatic stellate cells can also be found in the sub-mesothelial layer, sitting right under the Gp38-expressing mesothelium.

Ex vivo approaches for mimicking the FL niche

2D culture approach

In order to truly understand how stromal and hematopoietic cells behave in culture, this work is rooted in the premise that all of the observed results throughout culture are due to their cellular composition and activity. Culture media and plate coating are therefore seen only as requirements to maintain cells alive, but not as inducers of cellular behavior nor as a source of cytokine supplementation. To assess if cells benefit from co-culture interactions, a combination of sorted hepatoblasts, mesenchymal and mesothelial cells were seeded on a collagen-coated plate, respecting physiological ratios (i.e., the relative ratios obtained from sorting the different populations), alongside individual cultures of the same cellular subsets. Cell populations were sorted with a purity greater than 90% (**Supplementary Figure 1**).

Overall, 2D culture decreases the levels of transcripts for structural proteins and cytokines (**Figure 5A**). However, combining three stromal populations still mantains *Desmin* and *Cxcl12* transcripts that define mesenchymal cells. Additionally, *Gpm6a* expression is also maintained in mesothelial cells in the same co-culture combination. Cultures were analyzed at different time points to further confirm the observed transcript level maintenance. Surprisingly, *Desmin*, *Cxcl12*, and *Gpm6a* are not maintained throughout culture, but rather increase between the 2nd and 8th day of culture (**Figure 5B**). All the other transcripts appear to be substantially lower even after only 2 days in culture (*data not shown*). Altogether, this is suggestive of mesenchymal and mesothelial activity, but impaired hepatic maintenance. Nevertheless, even though hepatoblasts lose their characteristic *Afp* gene expression, *IL7* production is still maintained, as will be further discussed in this section. To evaluate if any other combination of the different stromal populations would result in better culture conditions, distinct co-cultures of only two populations were established, similarly respecting physiological ratios. Transcript levels were maintained irrespective of the stromal combination (**Supplementary Figure 2**).

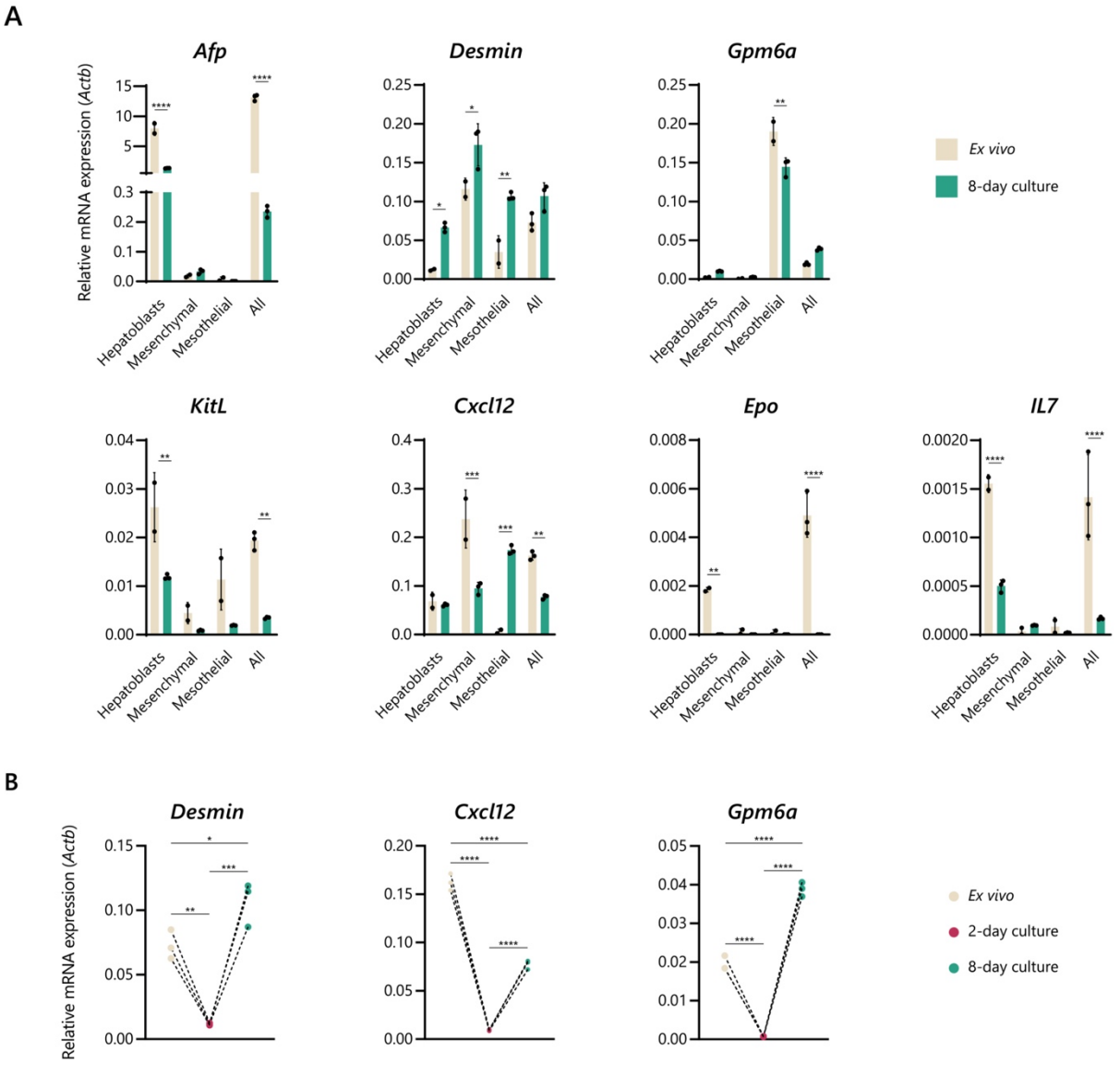

Figure 5 | Transcript levels of structural proteins and cytokines of different culture conditions, in distinct timepoints

A Combination of stromal populations seem to be beneficial in cell maintenance over 8 days of culture when compared to individually cultured populations; **B** *Desmin, Cxcl12,* and *Gpm6a* transcript levels are not simply maintained, but are rather recovered throughout culture. Statistical significance was assessed using Sodak's multiple comparison test (A) or one-way ANOVA followed by Tukey's multiple comparison test (B). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Data are represented as mean ± SD.

To test the hematopoietic supportive ability of these cultures, LSKs were seeded on previously established stromal cultures (stromal cells were cultured 2 days alone), collected 6 days after seeding (gating strategy and purity assessment of sorted LSKs can be found in **Supplementary Figure 3**), and hematopoietic activity was compared to OP9 control cultures. Since many hematopoietic cells visually seemed to still adhere to the well after aspiration, two collection methods were compared to ensure minimal cell loss – aspiration of all the non-adherent cells and scratching off all cellular content of each well, as a way to also collect adherent cells. A significant

number of macrophages was lost using the aspiration method, logically because most macrophages are adherent cells (**Supplementary Figure 4**). To ensure that no hematopoietic subsets were being dismissed, analysis was further performed on scratched samples. Hematopoietic support was confirmed by the identification of lineage-committed cells of lymphoid and myeloid lineage in every tested condition except the one without hepatoblasts, proving the essential role of this cell population in hematopoiesis (**Figure 6**, and FACS analysis plots in **Supplementary Figure 5**). Compared to the seeded cell number (1 000 cells), LSK maintenance was very scarce, and no expansion could be detected regardless of the supportive stromal culture combination. However, a high number of progenitors and mature cells were recovered from cultures containing hepatoblasts. The presence of B cells attests to the expression of IL7 in culture, as this cytokine is necessary for B cell lineage commitment (**Dias** *et al.*, 2005). No T cells or erythrocytes could be found, which goes in agreement with the dependence of cytokine-supplemented conditions for these cells to develop in culture (*data not shown*).

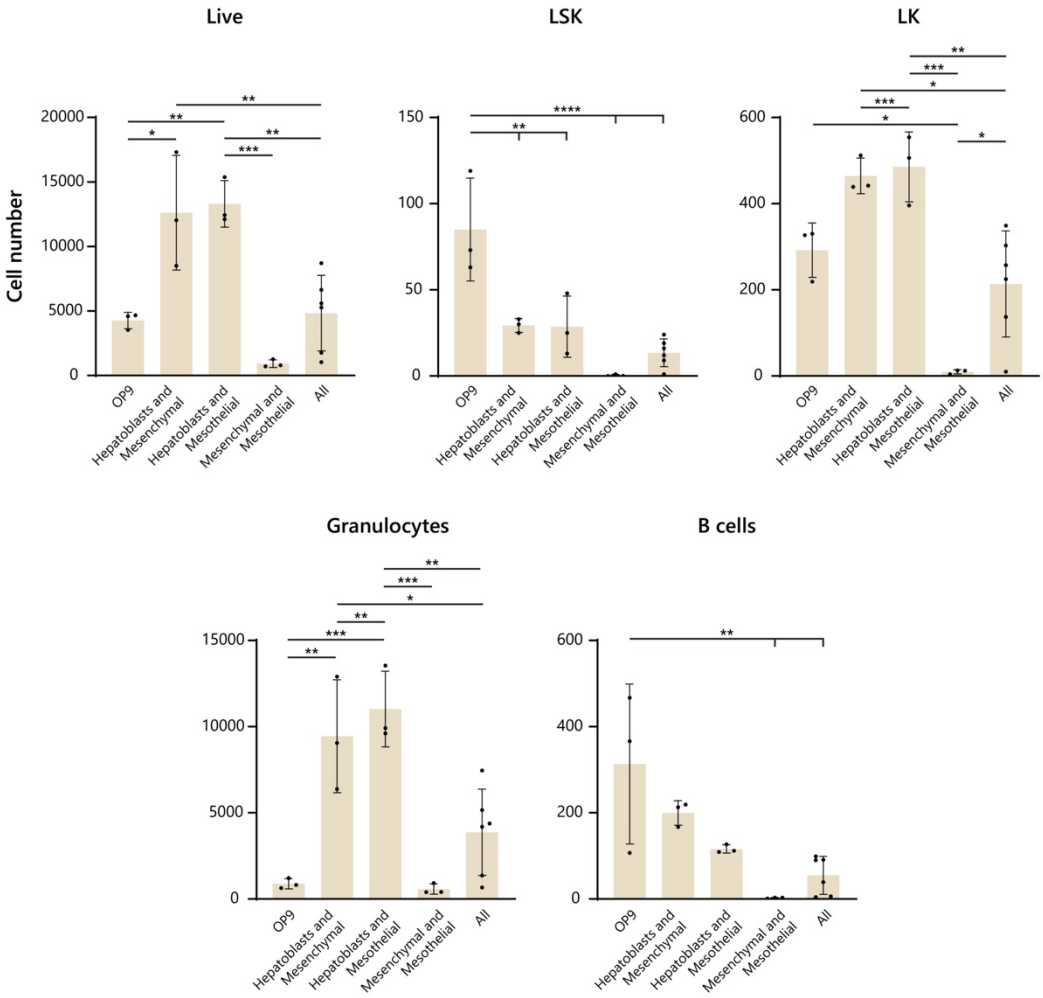

Figure 6 | Recovered hematopoietic cells numbers after 6 days of culture with different supportive stromal layers; a greater number of hematopoietic cells was recovered in combinations containing hepatoblasts
Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparison test. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Data are represented as mean ± SD.

The supportive stromal co-culture system seemed to impact the different hematopoietic lineages as a substantially lower number of cells were recovered from cultures with individual stromal populations as a supportive layer (**Supplementary Figure 6A**). However, no significant differences could be found between the different culture conditions at the transcript level (**Supplementary Figure 6B**). The supportive hematopoietic ability of the established co-cultures remains, therefore, puzzling as no evident link could be found between the greater number of recovered hematopoietic cells from cultures of stromal combinations and their gene expression profile. These results suggest that either hematopoietic differentiation relies greatly on cell-cell contact interactions with different stromal populations or that more relevant cytokines are being disregarded in this analysis.

As growing evidence suggests that crosstalk between the hepatic and hematopoietic systems is a key factor in embryonic hematopoiesis, it was hypothesized that hematopoietic cells might influence the stromal subsets in culture. To confirm such, stromal cultures that were seeded with hematopoietic cells were collected for analysis. No significant expression level differences were observed in any of the tested transcripts, except for *IL7* which increased in the presence of LSKs (**Figure 7**). This suggests that hematopoietic activity does not impact the established stromal cultures.

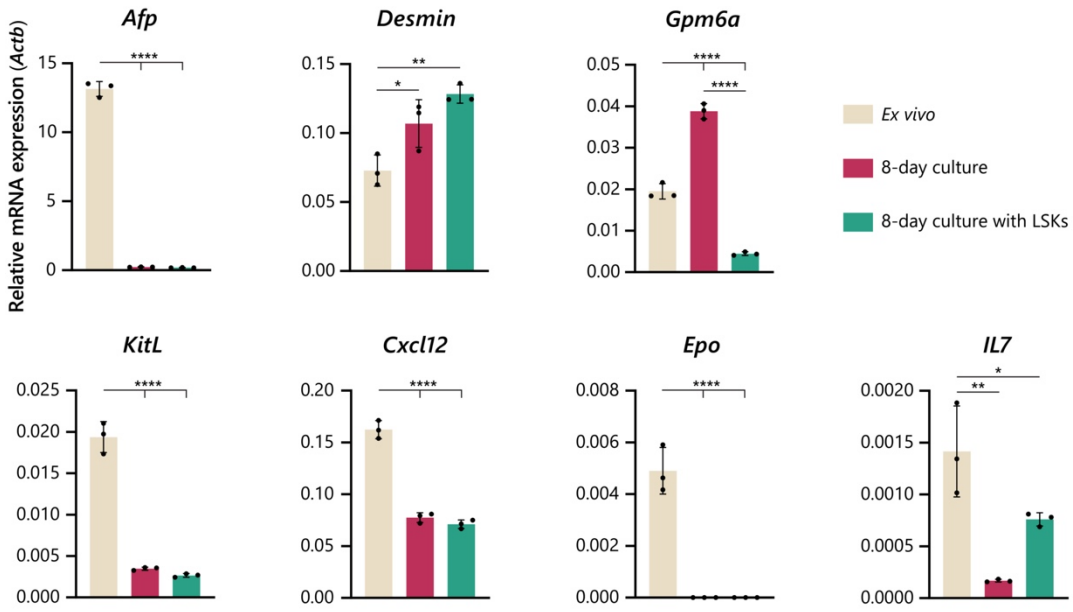

Figure 7 | Gene expression analysis of stromal cells (of the 3 populations combined) in the presence of hematopoietic cells; the obtained results suggest that hematopoietic activity does not impact the established stromal cultures

Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparison test. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Data are represented as mean ± SD.

Microfluidic approach

To retain their phenotype, stem cells require signals from their environment that can arise from soluble factors, cell-cell contact, and interactions with the ECM. In addition to these signaling pathways, cell structure and function may be determined by mechanical cues. It has been proven that biomechanical forces promote embryonic hematopoiesis (**Adamo & García-Cardeña**, 2011).

Taking advantage of Emulate Inc.'s established microfluidic system, it was possible to examine the effects of perfusion on stromal and hematopoietic cell cultures. FL stromal populations were seeded on collagen-coated channels, creating epithelial-like and endothelial-like channels, separated by a porous membrane. On one side of the membrane, freshly sorted hepatoblasts, mesenchymal, and mesothelial cells (epithelial-like channel) were seeded respecting physiological ratios, and on the other side exclusively endothelial cells (endothelial-like channel) (**Figure 8A**). After being left overnight in static conditions (to promote cell adhesion to the membrane), cells were submitted to constant perfusion during culture time. Similar to what was previously known from work developed in Cumano's group, endothelial cells did not grow under these conditions, even in the presence of flow, which was hypothesized as an important physical cue since it mimics blood flow. Eight days after its seeding, stromal cells appeared confluent and attached to the membrane, indicating that they could also serve as a supportive layer for this distinct culture system (**Figure 8A**). However, in comparison to static plate cultures, no significant improvements could be seen at the transcript level, which indicates that a microfluidic approach might not be decisive in stromal maintenance (**Figure 8B**). Looking from a hematopoietic perspective, the differentiation pattern seems to be modified since LSK input was comparable to the one from 2D cultures (**Supplementary Figure 7A**). A significant decrease in recovered B cell numbers might suggest that important soluble factors might be removed due to the constant flow to which these cultures are subjected. This result is corroborated by the OP9 control, as a significant decrease in recovered cell numbers can be seen when compared to the static culture conditions (**Supplementary Figure 7B**).

These results give rise to two different hypotheses – either cell-cell contact interaction is reduced due to the limited space available, or soluble factor signaling is being lost as a result of the constant perfusion. As both stromal and hematopoietic cells do not appear to benefit from this system, no further optimization was performed for this approach.

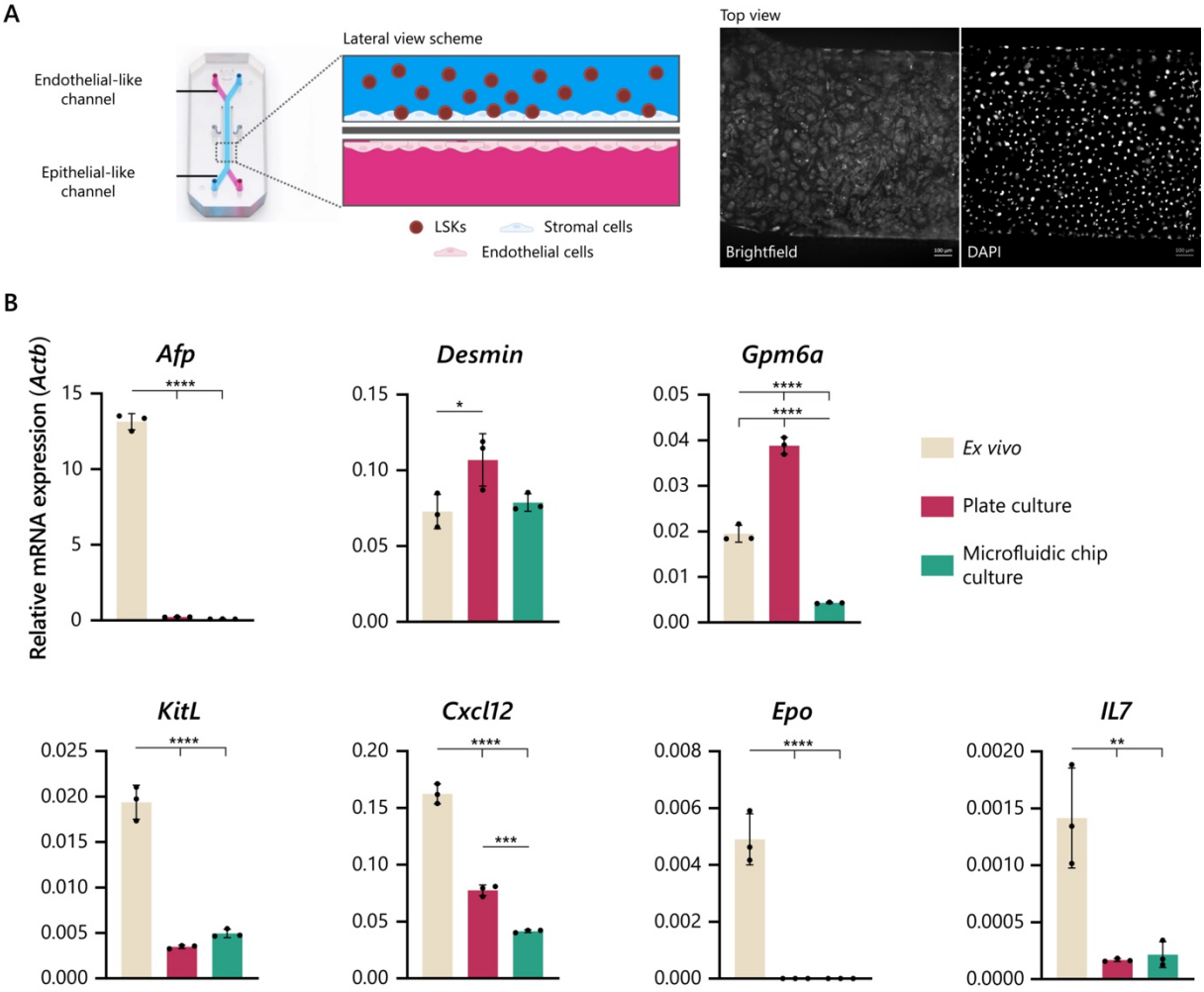

Figure 8 | Microfluidic approach for the *in vitro* maintenance of stromal cells

A Schematic representation of the PDMS microfluidic chip, containing two fluidically independent channels. A combination of stromal cells was seeded on the top channel, creating an epithelial-like structure, and endothelial cells were present in the bottom, endothelial-like channel. The channels are separated by a membrane that allows molecular crosstalk between them. After stromal cell attachment to the membrane, LSKs were seeded on the top channel and cultured for 6 days. At the end of culture time, the stromal layer was confluent; **B** No significant differences could be found in transcript levels from static and perfused cultures. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparison test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Data are represented as mean ± SD.

3D culture approach

As an important role in cell-cell interactions is being demonstrated, it was posed that the establishment of 3D cultures would enhance such interactions. As a starting point, spheroids arose as a simple yet informative approach to studying how would stromal cells behave in a more intricate environment as compared to a monolayer. Spheroids can be defined as cell aggregates

that self-assemble in an environment that prevents attachment to a flat surface. This process is possible since membrane and ECM proteins contribute to the formation of a cohesive structure.

With that in mind, numerous methods of spheroid formulation were tested, the most promising being the hanging drop (HD) and low attachment (LA) plate methods. First, to test if the E14.5 FL populations were capable of self-assemble into a spheroid shape, cell suspensions were depleted of hematopoietic cells and distributed in drops of 20 μL of media in the lid of a Petri dish, which remained inverted for 8 days. By microscopic inspection, spheroids could be detected albeit with substantial debris surrounding the structure. In an attempt to reduce undesirable culture components, an additional depletion for $CD31^+$ endothelial cells was performed (as they were proven to not remain viable in previous culture attempts). Cleaner cultures were attained, however, the HD method proved to be hardly reproducible as the formed spheroid exhibited heterogeneous diameters, cell number input did not correlate with spheroid size, and multiple spheroids were formed per drop (*data not shown*). In a more refined attempt, freshly sorted hepatoblasts, mesenchymal, and mesothelial cells were seeded on LA plates that prevented cell attachment and therefore promoted aggregation. Resorting to sorting as opposed to depletion resulted in an additional reduction of visible debris as only the desired populations are selected to be seeded. The LA plate method proved to be reproducible and efficient in generating homogeneously sized spheroids, which correlated with cell input (**Supplementary Figure 8**). Importantly, only one spheroid (occasionally two) was obtained in each well, increasing the probability of all cell populations being included in the structure. Previous studies done on Cumano's group proved that hepatoblasts could form spheroids, but all the other cellular subsets had not been tested yet. The most compact structures were attained only in the presence of hepatoblasts, as both mesenchymal and mesothelial aggregates resulted in irregular-shaped spheroids (*data not shown*).

To assess if cells within the spheroid remained viable throughout culture, staining with PI was performed after 8 days of culture (**Figure 9B**). No dead cells could be found at the core of the spheroid, indicating that they are still viable at this stage. PI incorporation proved to be an efficient method to asses viability as its staining could penetrate the whole structure of the spheroid (*data not shown*). Transcript level analysis reveals that hepatoblasts are the most benefiting cell population, as levels of *Afp*, *KitL*, and *IL7* are higher than in 2D plate culture at the same culture time point (**Figure 9A**). No significant differences could be found between the assessed methods, nor between different days of culture (*data not shown*). To make sure that all cell populations were present in the aggregates, immunofluorescence staining was performed using discriminative markers for the different populations. E-cadherin-labeled hepatoblasts appear to form an epithelial layer that involves the core of the spheroids, and the $CD140a^+$ cells concentrate in the

middle (**Figure 9C**). Albeit these results are preliminary, two hypotheses can be formulated regarding the observed organization – either the different cell populations have the ability to self-organize or the hepatoblasts are going through an epithelial-to-mesenchymal transition. To exclude the latter, spheroids containing only hepatoblasts need to be subjected to the same analysis.

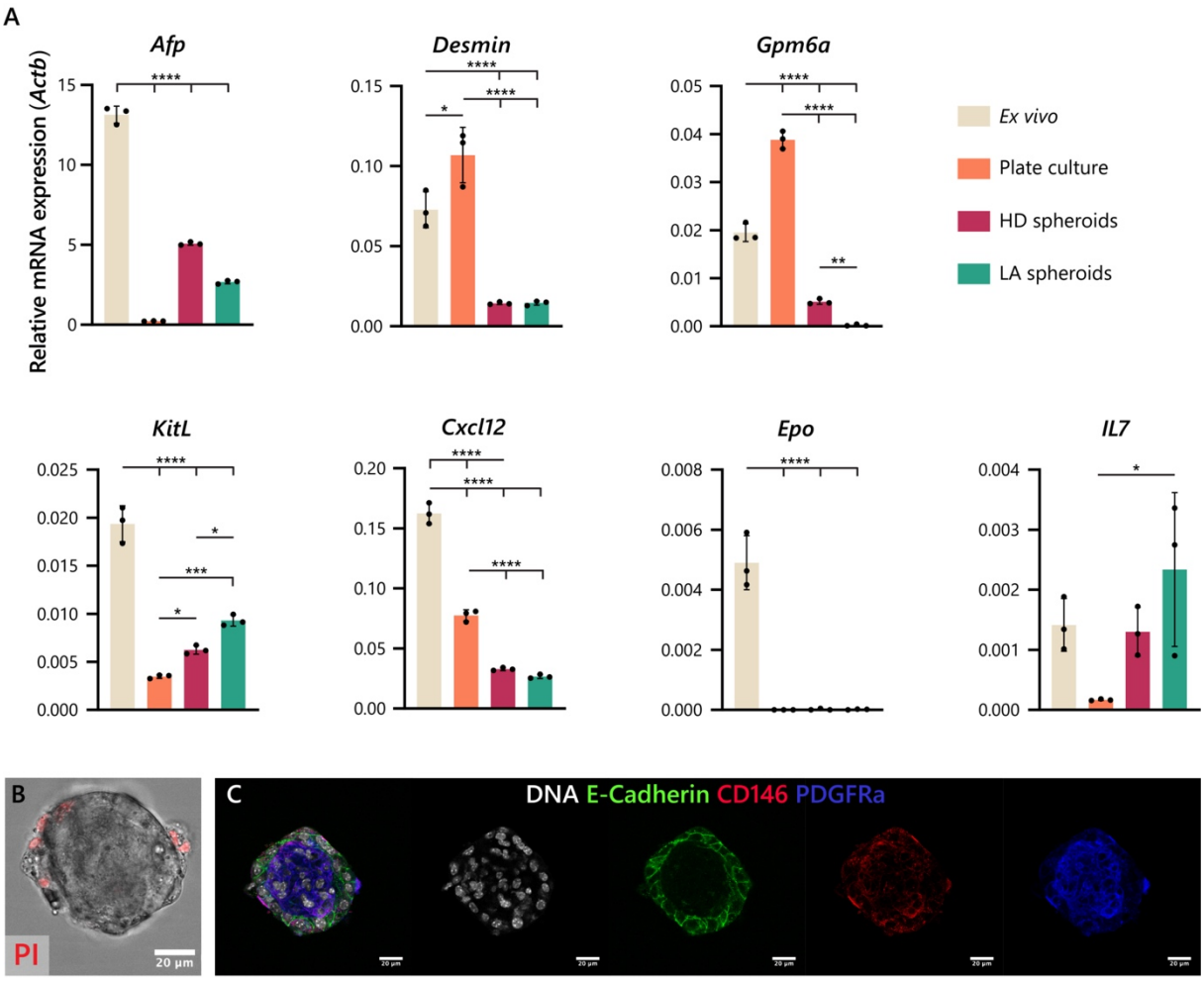

Figure 9 | 3D culture aggregates approach for the *in vitro* maintenance of stromal cells

A Gene expression analysis pointed out an improved maintenance of cultured hepatoblast, as transcript levels of *Afp*, *KitL*, and *IL7* are higher than in plate cultures; **B** Spheroids remained viable at least for 8 days after being formed, as no PI incorporation could be detected; **C** Immunostaining of the stromal populations incorporated in the formed spheroids. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparison test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Data are represented as mean ± SD.

DISCUSSION & FUTURE WORK

Throughout this work, FL cells were isolated and characterized based on their surface protein expression and transcriptomic profile. Three major populations served as a common ground for all the established culture approaches – hepatoblasts, mesenchymal, and mesothelial cells. From the beginning, a striking gap in this work is the absence of endothelial cells in culture, which might be seen as an undervaluation of their contribution to the FL niche environment. Indeed, endothelial cells were identified as one of the major sources of SCF, a cytokine known to be genetically required for HSC maintenance *in vivo* (**Lee** *et al.*, 2021). Additionally, growing evidence suggests that HSCs closely associate with sinusoidal vasculature (**Khan** *et al.*, 2016), reinforcing the idea that this cellular subset might play an important role in hematopoiesis. However, to date, few protocols have been developed to culture primary FL endothelial cells. Neo *et al* (**Neo** *et al.*, 2018) described the use of IL3 and thrombopoietin as supplements required for endothelial culture maintenance. Keeping in mind the starting premise of this work, and since endothelial cells did not remain viable under the drawn culture conditions, it was decided to proceed without this cell population.

The most direct way to prove that FL stromal cells are *bona fide* supportive cells for HSC expansion *ex vivo* is to establish a co-culture assay that does so. However, cells in culture, displaced from their native microenvironment and lacking their receptors' counterparts, change their identity. Providing a system that reflects as closely as possible their *in vivo* conditions might improve culture maintenance. Eight days of culture without any source of supplementation might be too long a period for hepatoblasts to retain their characteristic phenotype, considering that even though most of the published studies keep cultures for a significant period of time, they were cytokine supplemented.

Concerning the hematopoietic influence of these stromal cultures, it was evident that hepatoblasts exert an indispensable role in supporting co-cultured LSKs, as almost all hematopoietic activity was lost in the absence of these cell population. This data goes in

agreement with the reported role of SCF^+DLK^+ hepatic progenitors in supporting >20-fold expansion of HSCs, assayed by co-culture followed by long-term competitive BM transplantation experiments (**Chou & Lodish**, 2010). This cell population expresses several known stem cell supportive cytokines, including TPO, SCF, and Cxcl12. $Nestin^+NG2^+$ periportal cells have also been suggested to be an important niche component, as reaggregate organ culture assays containing this cell population were sufficient to maintain HSCs in culture, whereas in its absence long-term repopulation activity could not be detected (**Khan** *et al.*, 2016). However, from the data of this work, it appears that mesenchymal cells do not support hematopoiesis in the absence of hepatoblasts (either alone or combined with the mesothelial subset). This might suggest that the reported HSC maintenance was due to the influence of the multiple cellular counterparts present in the used FL cell mixture rather than on a single population. Albeit not clarified in the present work, the combination of different stromal populations seems to have an effect on the properties of stromal cultures, especially regarding their hematopoietic supportive ability. A broader analysis of gene expression transcripts is likely to provide a deeper understanding in future studies of the impact of culture in the different cellular subsets, either hepatic or hematopoietic, namely *Scf*, *Flt3L*, *TPO*, and *IGF2* (**Soares-da-Silva** *et al.*, 2020).

Further efforts must be devoted to demonstrating *ex vivo* expansion of HSCs by FL cells, which requires the development of culture conditions that improve the viability of stromal cells yet still allow the maintenance and expansion of HSCs. In the course of this work, and since there are no reports on primary FL stromal cells cultures under these conditions, some fundamental questions arose – Do dead cells affect cultures in anyway? Is confluency an important factor for cell maintenance?, among others. Future work will help to shed a light on these simple yet relevant questions. Additionally, one could question whether E14.5 might be a time when stromal populations are already committed into a more mature hepatic phenotype, reducing their supportive influence on hematopoietic subsets. As dramatic expansion occurs between E12 and E16 in the FL, the expansion switch might be triggered by the stromal environment HSCs encounter at the time of their arrival to the FL. To test this hypothesis, these experiments must be repeated on earlier stages of embryonic development.

Not only molecular but also physical cues encompass the niche's environment characteristics. Cell-cell contact, tissue shear stress, and blood flow might be among some of the aspects that regulate cell activity in the FL. Herein, using a microfluidic device, constant perfusion was imposed on stromal cultures. The continuous supply of nutrients and oxygen together with waste removal through perfused culture media has been proven to enhance albumin production of fetal human hepatocytes as compared to static culture conditions (**Leclerc** *et al.*, 2004). However, this system proved to be inefficient in improving cell maintenance of the hepatic populations in culture when compared to traditional plate culture. Either due to space limitations that reduced cell-cell

interactions or due to the loss of molecular cues caused by constant perfusion, transcript levels of key cytokines and structural proteins were reduced compared to the 2D culture system, which translated into the hampered hematopoietic supportive ability of the stromal layer.

As 3D cellular arrangements increment cell interactions, spheroid aggregates were studied as an approach to culture FL stromal cells. It has been reported that such interaction is highly effective in terms of cellular activity, which seems to be consistent with the present data. Hepatoblasts were the only cell population capable of forming a compact structure when cultured alone, while mesenchymal and mesothelial cells did not display the same ability as they could only form loose clusters. Once again, hepatoblasts prove to be a pivotal stromal population, this time in terms of structural support, as this cellular subset is a major producer of ECM components. As both tested methods for spheroid formation resulted in similar outcomes in terms of gene expression, the LA plate method was chosen as the most promising culture approach as it enables overcoming some technical difficulties that were found throughout the process. Since a significant number of spheroids is required for analysis, to be reproducible and easy to handle are also important characteristics to settle on in a methodology. If this approach continues proving to be beneficial for cell maintenance in culture, a more scalable methodology needs to be employed. As a pattern of organization seemed to be found in the formed spheroids, it is worth pursuing if this process occurs as a self-organization event or as an induced epithelial to mesenchymal differentiation.

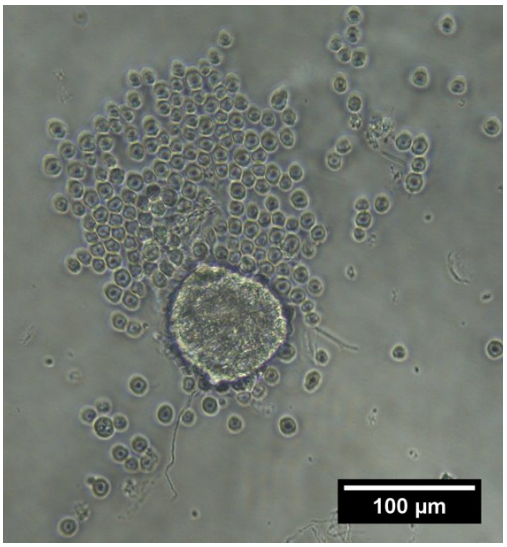

Figure 10 | Co-culture of LSKs with previously formed stromal spheroids

As hepatoblasts showed to be an essential counterpart in supporting hematopoietic activity, and since they appear to be best preserved in this culture approach, it would be interesting to unravel how hematopoietic cells interact with this structure (**Figure 10**). Even though LSKs were cultured both with formed spheroids and as a part of them, optimization efforts were not sufficient to recover hematopoietic cells for analysis in a timely manner. In order to fully understand the effects of aggregates in hematopoietic support, future work must be devoted to do so.

In vitro organotypic cultures should also be considered as a future perspective, similar to the ones employed by Khan *et al* (**Khan** *et al.*, 2016), first described by Medvinsky's group (**Sheridan** *et al.*, 2009). This is widely used for AGM cultures and has the advantage of allowing a gas-liquid interface, as the compact mass of cells is cultured in a membrane on the top of the medium.

The FL niche has been assessed in cell culture systems before, either in mice and human by established cells lines and primary cultures with isolated DLK^+ SCF^+ cells **(Chou** *et al.*, 2013; **Chou & Lodish**, 2010) and reaggregate organ cultures with Nestin-GFP cells (**Khan** *et al.*, 2016). This study was a preliminary assessment of the stromal cells behavior when cultured on a minimal supplementation setup. Better culture systems need to be developed to improve cell viability of the studied cell populations, especially hepatoblasts, as even though they serve as support for hematopoietic development, no evidence of expansion could be seen in any culture approach.

Taking all together, the established methods proved to be beneficial for some cell populations detrimentally to others. A system that results in equal benefits for all of the stromal counterparts, maintaining them with similar *in vivo* properties, would be the ideal hematopoietic supportive system. As such, future work must focus on unraveling the key components that drive not only hematopoiesis, but also stromal cell maintenance in culture.

CONCLUSION

Deeper insights into the FL's composition throughout embryonic development allow the identification of cell populations closely related to hematopoiesis. Unraveling the interactions of hematopoietic and FL cells and mimicking the environment of this unique organ might shed a light on ways to manipulate HSC expansion *in vitro*, as this process only occurs within a limited timeframe over a lifetime. The culture approaches established herein pointed out the potential of FL stromal cells in supporting hematopoiesis *in vitro*, but also the necessity for further improvements on cell maintenance in culture.

Although the road ahead is still long, devoted efforts have contributed to its shortening. The possibility of HSC expansion *ex vivo,* and the consequential clinical improvements on HSC transplantation, seem to be, step by step, increasingly attainable.

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Supplementary Data

Supplementary Table 1 | List of antibodies for Flow Cytometry

Antibody	Species	Conjugation	Clone	Catalog No.	Company
B220	Rat	Pacific Blue	RA3-6B2	558108	BD Biosciences
CD11b	Rat	AF700	M1/70	557960	BD Biosciences
CD16/32	Rat	FITC	93	1106525	SONY
CD19	Rat	Biotin	1D3	553784	BD Biosciences
		BUV661	1D3	565076	BD Biosciences
CD24	Rat	BUV496	M1/69	612953	BD Biosciences
CD31	Rat	PerCP-Cy5.5	390	1112100	SONY
CD34	Rat	PE	RAM34	551387	BD Biosciences
CD41	Rat	BV510	MWReg30	1269615	SONY
CD45	Rat	Biotin	30-F11	1115515	SONY
	Mouse	FITC	104	553772	BD Biosciences
CD45.2	Mouse	APC	104	558702	BD Biosciences
CD48	Hamster	Biotin	HM48-1	103409	BioLegend
		APC	HM48-1	1117060	SONY
CD54	Rat	Pacific Blue	YN1/1.7.4	116116	BioLegend
CD71	Rat	Biotin	RI7217	1169015	SONY
		BV421	RI7217	113813	BioLegend
CD117 (c-Kit)	Rat	APC-Cy7	2B8	1129130	SONY
		Biotin	2B8	1129020	SONY
		BV605	2B8	1275600	SONY
CD135 (FLT3)	Rat	PE-CF594	A2F10.1	562537	BD Biosciences
CD140a	Rat	APC	APA5	1279540	SONY

CD150	Rat	PE-Cy7	TC15-12F12.2	1179570	SONY
CD166	Rat	FITC	eBioALC48	11-1661-82	ThermoFisher
F4/80	Rat	PerCP-Cy5.5	BM8	1215640	SONY
Gp38	Hamster	APC-Cy7	8.1.1	1237090	SONY
Gr-1	Rat	Biotin	RB6-8C5	1142020	SONY
		BUV395	RB6-8C5	563849	BD Biosciences
NG2	Rat	PE	1E6.4	130-100-468	Milteny Biotec
NK1.1	Mouse	APC-Cy7	PK136	1143620	SONY
TCRβ	Hamster	BUV737	H57-597	612821	BD Biosciences
Ter119	Rat	Biotin	TER119	MA5-17819	ThermoFisher
		PE-Cy5	TER119	116209	BioLegend
Sca-1	Rat	BV510	D7	1140645	SONY
		BV711	D7	563992	BD Biosciences
Streptavidin	Mouse	BV785	–	2626245	SONY

Supplementary Table 2 | Antibodies used for MACS depletion of specific cellular subsets in spheroid formation

Antibody	**Species**	**Conjugation**	**Clone**	**Catalog No.**	**Company**
CD31	Rat	Biotin	MEC13.3	1112520	SONY
CD45	Rat		30-F11	1115515	SONY
CD54	Rat		YN1/1.7.4	13-0541-82	ThermoFisher
CD71	Rat		RI7217	1169015	SONY
CD117 (c-Kit)	Rat		2B8	1129020	SONY
CD140a	Rat		APA5	1279550	SONY
Gp38	Hamster		eBio8.1.1	13-5381-82	ThermoFisher
Ter119	Rat		Ter119	116203	ThermoFisher

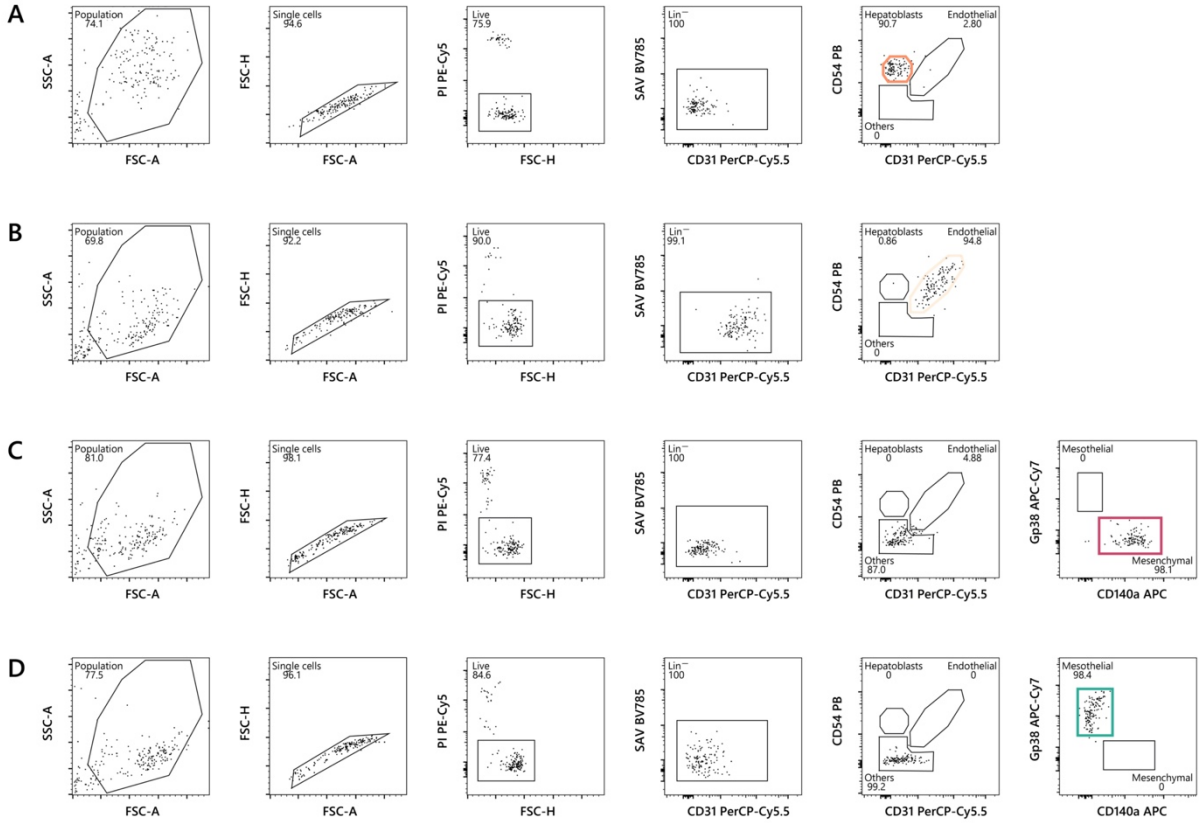
Supplementary Table 3 | Antibodies used for immunofluorescence imaging

Primary Antibody	Species	Catalog No.	Company
CD146	Sheep	AF6106	R&D
E-cadherin	Rabbit	3195S	Cell Signaling
Gp38	Hamster	NB600-1015	Novus Biologicals
Hnf4α	Mouse	MA1-199	ThermoFisher
Lhx2	Rabbit	ABE1402	Millipore
PDGFRα	Goat	AF1062	R&D

Secondary Antibody	Species	Catalog No.	Company
Ch anti-mouse 647	Chicken	A21463	Life Technologies
Dk anti-goat 647	Donkey	A21099	Life Technologies
DK anti-rabbit 488	Donkey	A21206	Life Technologies
DK anti-sheep 568	Donkey	A21463	Life Technologies
Goat anti-hamster 647	Goat	106-605-142	Jackson ImmunoResearch

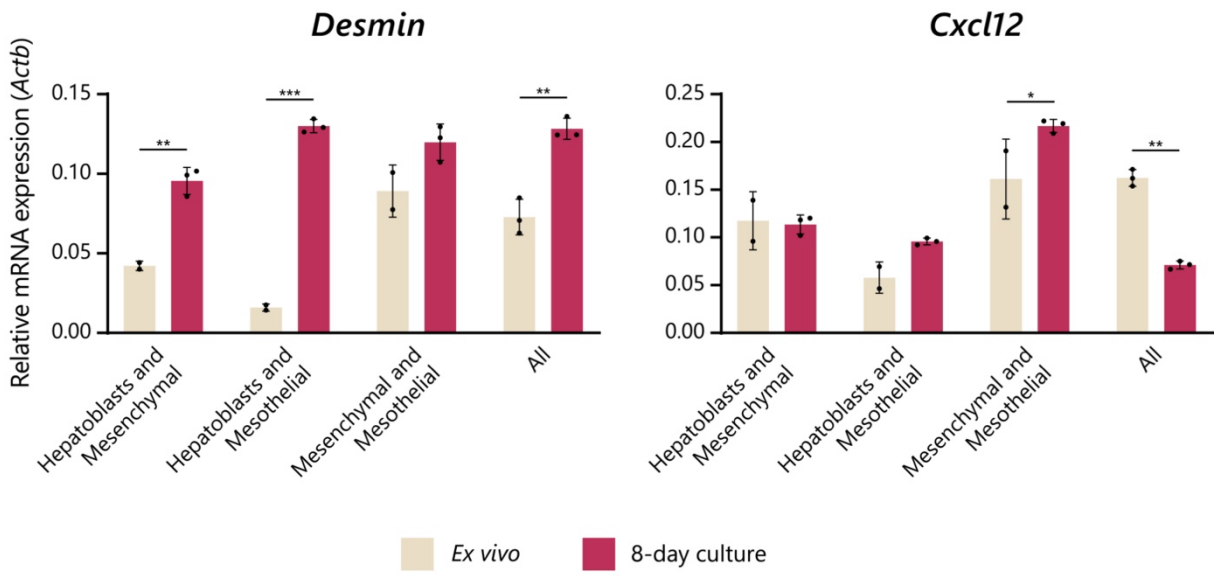
Supplementary Table 4 | Primers' sequences used for amplification of mouse transcripts for qPCR

Name		Primer sequence
Actb	FW	GCTTCTTTGCAGCTCCTTCGT
	RV	ATCGTCATCCATGGCGAACT
Afp	FW	CTTCCCTCATCCTCCTGCTAC
	RV	ACAAACTGGGTAAAGGTGATGG
Cxcl12	FW	TGCATCAGTGACGGTAAACCA
	RV	TTCTTCAGCCGTGCAACAATC
Desmin	FW	GTGGATGCAGCCACTCTAGC
	RV	TTAGCCGCGATGGTCTCATAC
Epo	FW	ACAAAGCCATCAGTGGTCTACG
	RV	TCTGGAGGCGACATCAATTCC
Gpm6a	FW	TGCTTCGAGTGCTGCATTAAA
	RV	TTGCCAACTCAAAGTAGGTCTG
IL7	FW	TTCCTCCACTGATCCTTGTTCT
	RV	AGCAGCTTCCTTTGTATCATCAC
KitL	FW	GAATCTCCGAAGAGGCCAGAA
	RV	GCTGCAACAGGGGGTAACAT



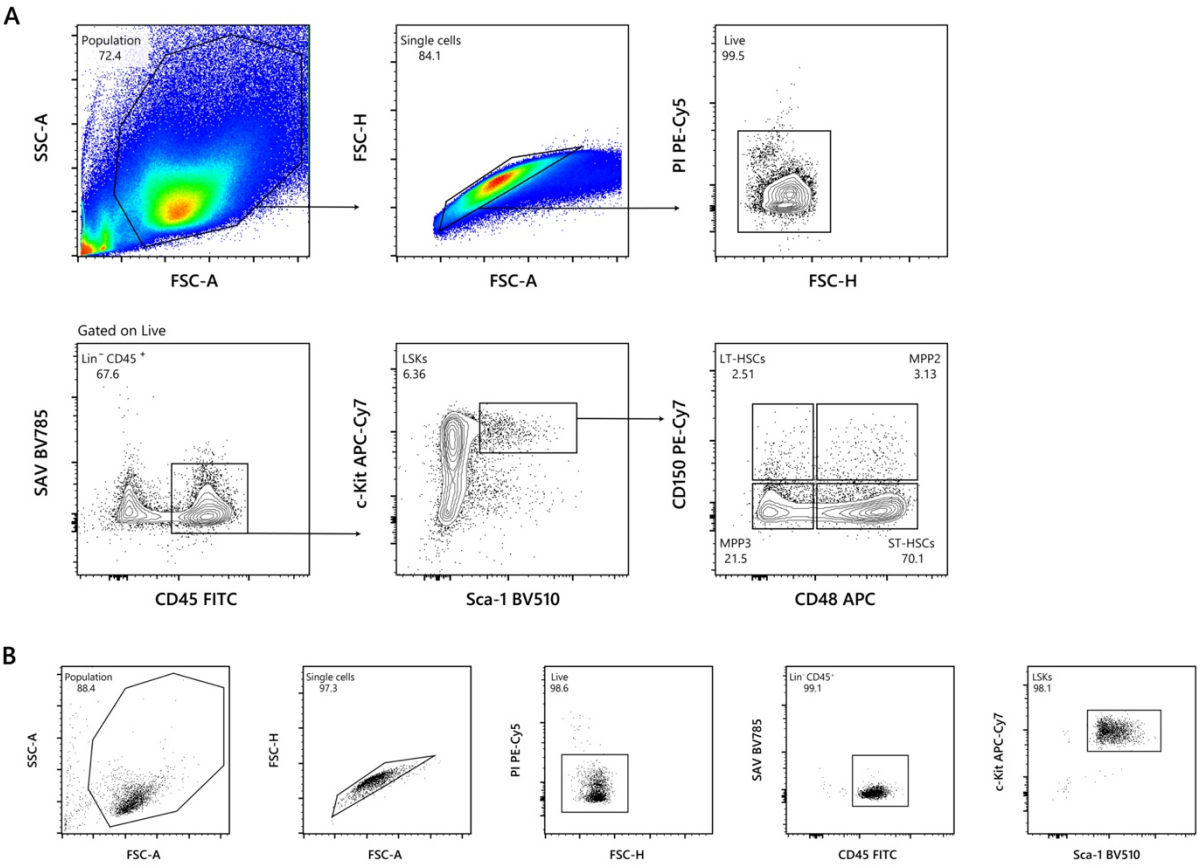
Supplementary Figure 1 | Representative FACS plots of sample's purity assessment

A Sorted hepatoblasts; **B** Sorted endothelial cells; **C** Sorted mesenchymal cells; **D** Sorted mesothelial cells.



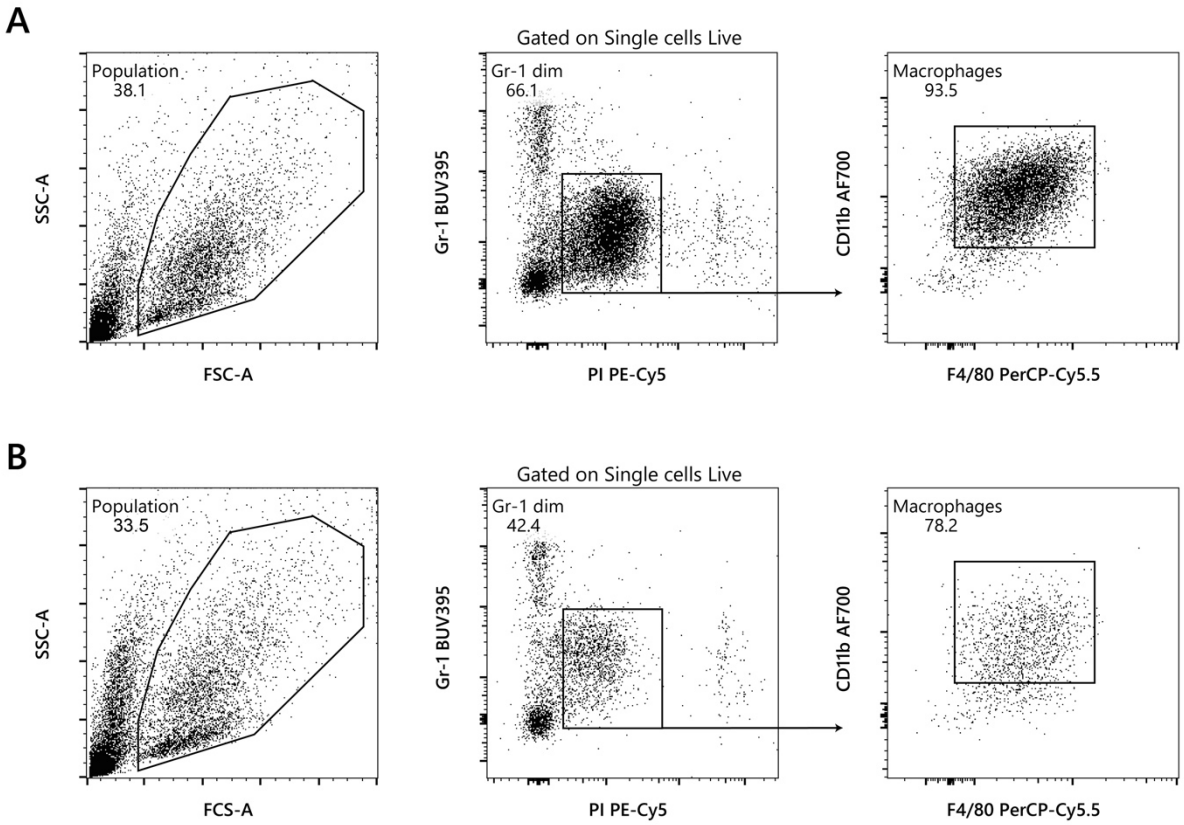
Supplementary Figure 2 | Distinct culture combinations do not result in transcript expression differences after 8 days of culture

Statistical significance was assessed using Sodak's multiple comparison test (A) or one-way ANOVA followed by Tukey's multiple comparison test (B). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Data are represented as mean ± SD.



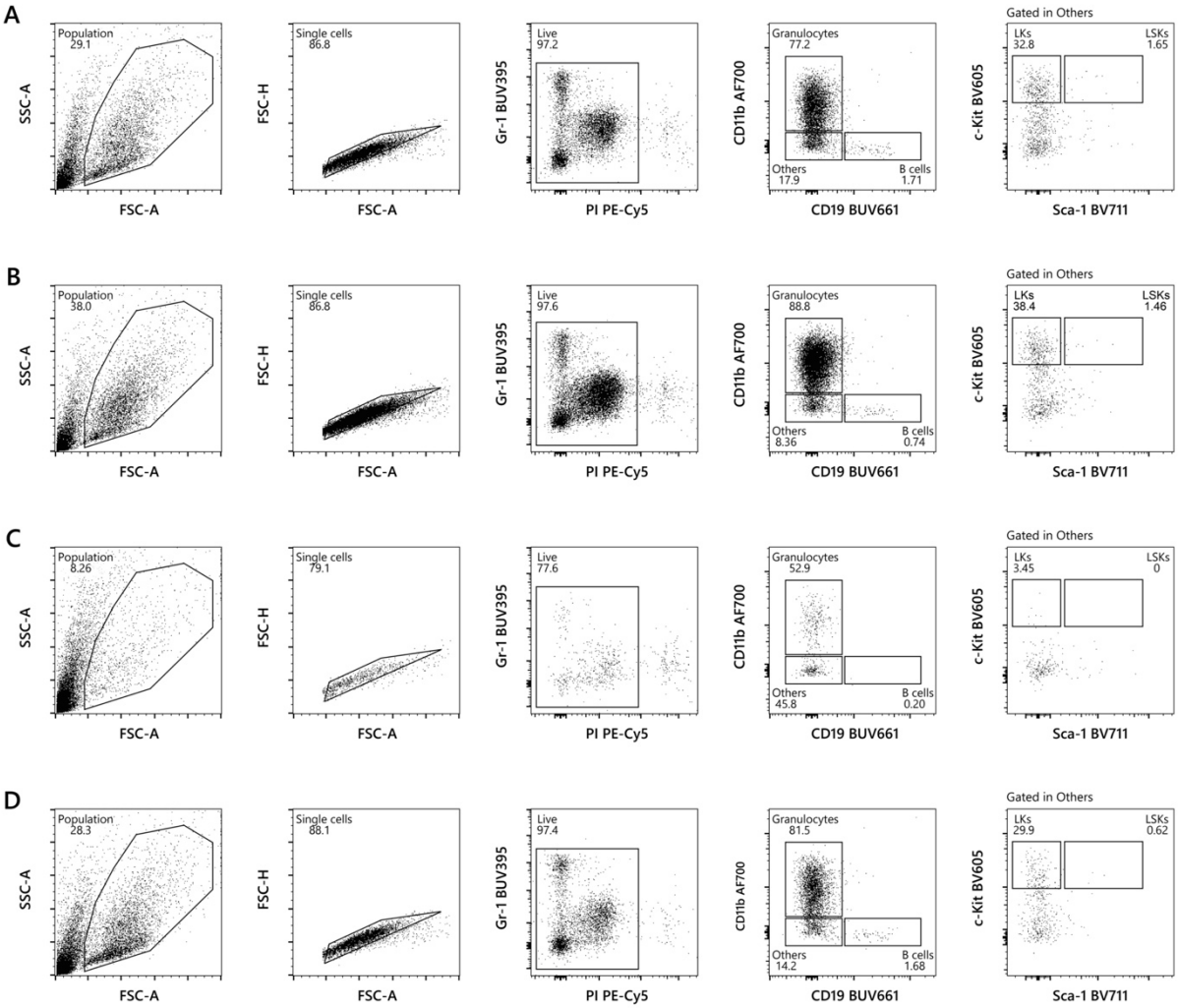
Supplementary Figure 3 | Representative FACS plots of LSK samples

A Gating strategy for LSK isolation; **B** Representative purity assessment.

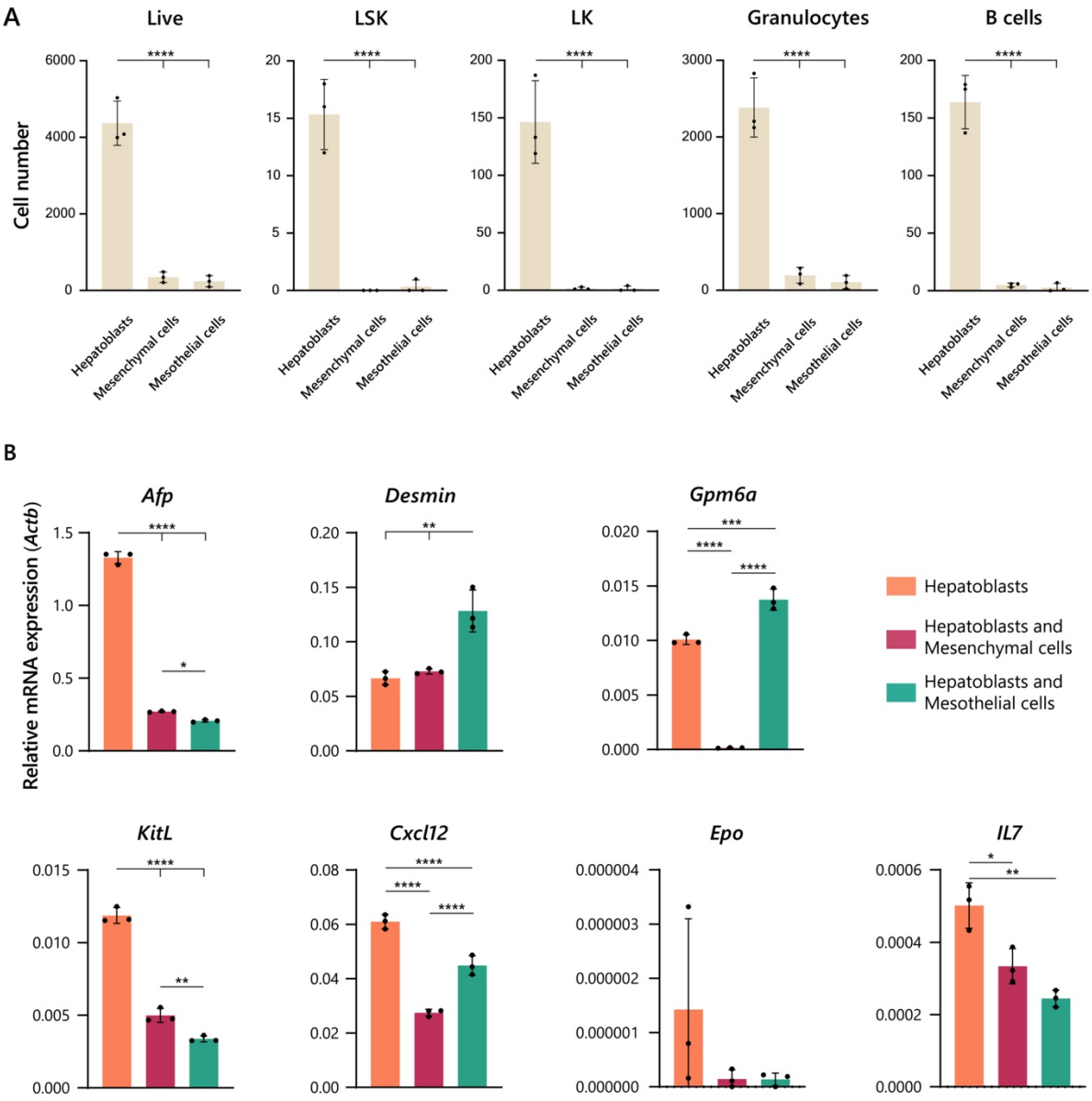


Supplementary Figure 4 | Cell loss as a result of different collection methods

The scratching method **(A)** resulted in greater cell recovery, as no macrophages were being lost as observed using the aspiration method **(B)**.

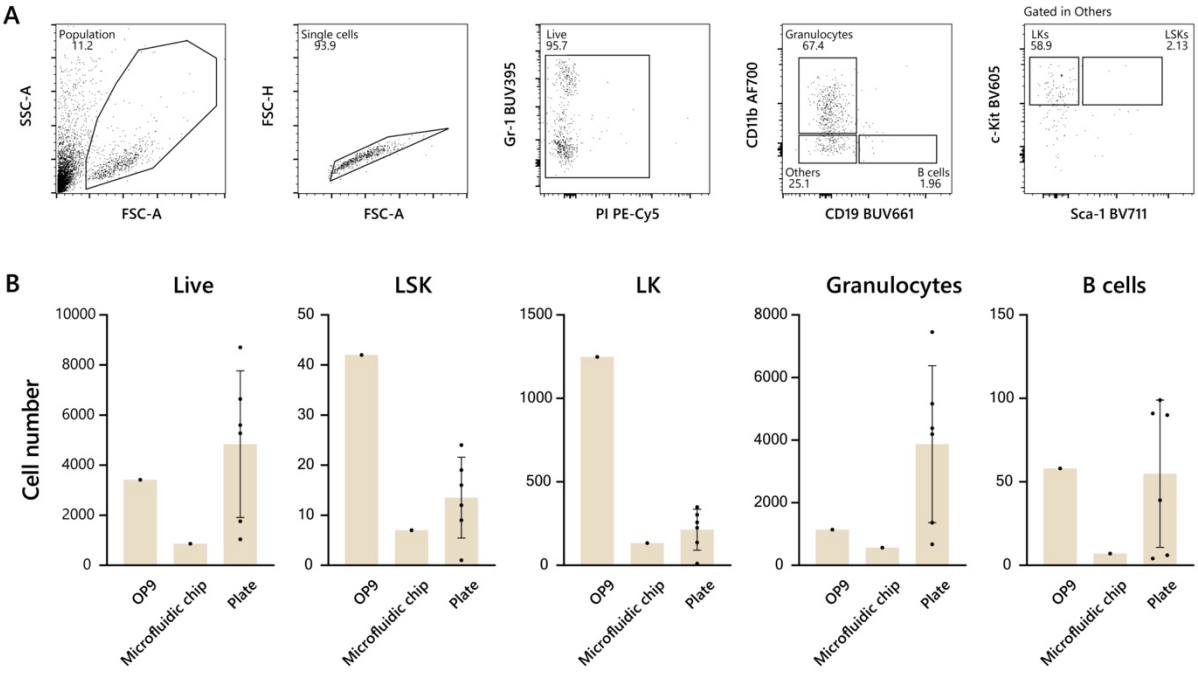


Supplementary Figure 5 | FACS analysis of the recovered hematopoietic populations after 6 days of culture with **A** Hepatoblasts and mesenchymal cells; **B** Hepatoblasts and mesothelial cells; **C** Mesenchymal and mesothelial cells; **D** All

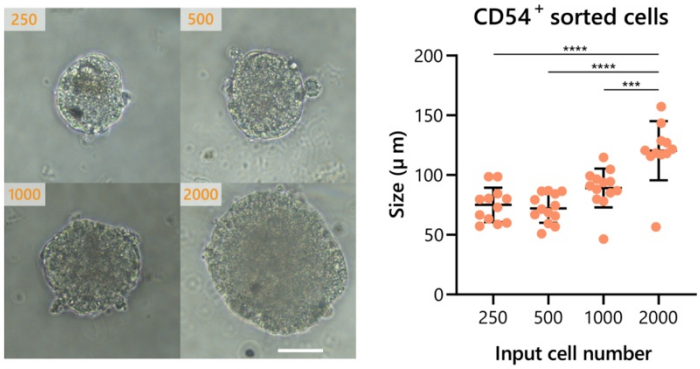


Supplementary Figure 6 | Correlation between recovered cell numbers and transcript levels

A A much smaller cell number was recovered in co-cultures of LSKs with individual populations as compared to cultures with combinations of populations; **B** Looking at the transcript level, no direct link can be found between the increased hematopoietic supportive ability of cultures with combinations containing hepatoblasts as compared to hepatoblasts alone. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparison test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Data are represented as mean ± SD.



Supplementary Figure 7 | Recovered hematopoietic cells after 6 days of culture under perfusion revealed a much smaller frequency of hematopoietic cells, as compared to static conditions



Supplementary Figure 8 | Cell input correlates with spheroid size, demonstrated in a preliminary attempt of spheroid formation, composed of hepatoblasts and using a LA plate

Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparison test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Data are represented as mean ± SD.