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Hematopoietic Stem Cell Niche in Foetal Liver and Production of Pluripotent Stem Cell-Derived Liver Organoids

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Abstract

There is a considerable demand but limited supply for hematopoietic stem cells (HSCs) in clinics. To meet clinical needs of HSCs, new efforts focus on *de novo* HSCs generation from pluripotent stem cells (PSCs). Although previous attempts have yielded precursors and progenitors of HSCs, the production of fully functional HSCs has largely been unsuccessful. The failure of PSC-derived HSCs to mature to foetal liver stage is not surprising, as most methods are trying to generate hemogenic endothelium resembling that found in the aorta-gonad-mesonephros (AGM) region, highlighting the importance of understanding human foetal liver niche and developing protocols to mimic this environment. This paper investigates the diverse cellular interactions within the fetal liver niche that contribute to HSC maturation and explores the potential for generating human fetal liver organoids that can recreate these supportive environments *in vitro*. Such organoids could provide a groundbreaking model for studying HSC maturation and potentially offer a scalable solution for the *ex vivo* production of functional HSCs, paving the way for advances in both regenerative medicine and hematopoietic stem cell transplantation.

Keywords

Hematopoietic Stem Cell (HSC), HSC Maturation, Fetal Liver Niche, Fetal Liver Organoid

1. Introduction & Aims

Multipotent HSCs that give rise to all mature blood cell lineages possess unique self-renewal capacity, perpetuating the HSC pool throughout life. This ensures sustained lifelong blood, immune cell production and the ability to respond to

changing demands upon injury or infection. Adult HSCs engrafted in the bone marrow (BM), where the HSC pool is sustained through a balance between self-renewal and differentiation, are mostly quiescent at homeostatic state and rarely divide only to replenish the HSC pool or to maintain a suitable level of differentiated blood cells. Due to these properties, HSC transplantation can hierarchically reconstitute the entire hematopoietic system in a recipient.

HSC-based therapy has enormous potential and promise for treating untreatable diseases. Examples include but are not limited to gene therapy for severe combined immunodeficiency caused by deficiency in adenosine deaminase (ADA-SCID), HIV cure based on CCR5 mutant, and use of TCR- or CAR-engineered HSC for cancer immunotherapy. Nevertheless, the expansion and ease of access to these therapeutic approaches are dependent on having an unlimited supply of transplantable HSC that can be easily manipulated in culture. Traditionally, there are two main types of HSC transplantation: allogenic or autologous. HSCs can be harvested from the patient's or donor's BM by aspiration or mobilized peripheral blood (PB) via apheresis, or from umbilical cord blood (CB) at birth. Allogenic HSC transplantation is highly effective in treating hereditary blood disorders and malignant hematologic diseases if a suitable human leukocyte antigen (HLA) matched donor can be found. Poor HLA-matching between donor and recipient can lead to engraftment failure and/or graft versus host disease (GVHD). Because of the limited availability of HLA-matched donors and limited applications of autologous transplantation, we need new ways for generating and increasing the supply of transplantable HSCs.

Pluripotent stem cells (PSCs) are a potential source of HSC. Human PSCs can be derived from blastocysts (human embryonic stem cells, hESC) or can be reprogrammed from fibroblasts (induced PSCs, iPSCs). The generation of HSCs along with blood and immune cells from iPSCs *in vitro* would offer considerable promise for regenerative or cancer therapies and disease modelling because this method would avoid GVHD and other allograft-related issues. However, efficient derivation of robustly engraftable iPSC-derived HSC with multilineage potential *in vitro* remains challenging due to inability to achieve functional maturation which occurs in foetal liver (FL) during development. In human embryogenesis, definitive (transplantable into the adults) HSCs first appear in the intra-aortic hematopoietic clusters (IAHCs). These clusters form through a process known as endothelial-to-hematopoietic transition (EHT), in which blood vessel-lining cells (endothelial cells) transform into blood-forming HSCs. This process occurs in a specific region called the aorta-gonad-mesonephros (AGM) between 4 - 6 weeks of gestation (Carnegie Stages CS13-17). After their formation, these immature HSCs migrate to the fetal liver, where they mature and expand, forming a pool of HSCs capable of supporting lifelong blood production and robust bone marrow engraftment. However, at this early stage, HSCs are functionally immature and barely engraftable [1].

Several groups have successfully specified definitive hemogenic endothelium

and progenitors from PSCs, mimicking the early steps leading to AGM haematopoiesis, but failed to generate long-term repopulating HSCs [2]-[4]. These protocols have focused on mimicking the AGM-like environment—inducing HSC emergence, not the FL-like environment—inducing HSC functional maturation. Accordingly, it is highly unrealistic to achieve the goal of engraftable HSC generation *ex vivo* unless recreating the FL-like niche induces HSC functional maturation. To recapitulate this process in a 3D FL-emulating organoid, it is essential to understand the environmental cues provided in foetal liver at cellular and molecular levels.

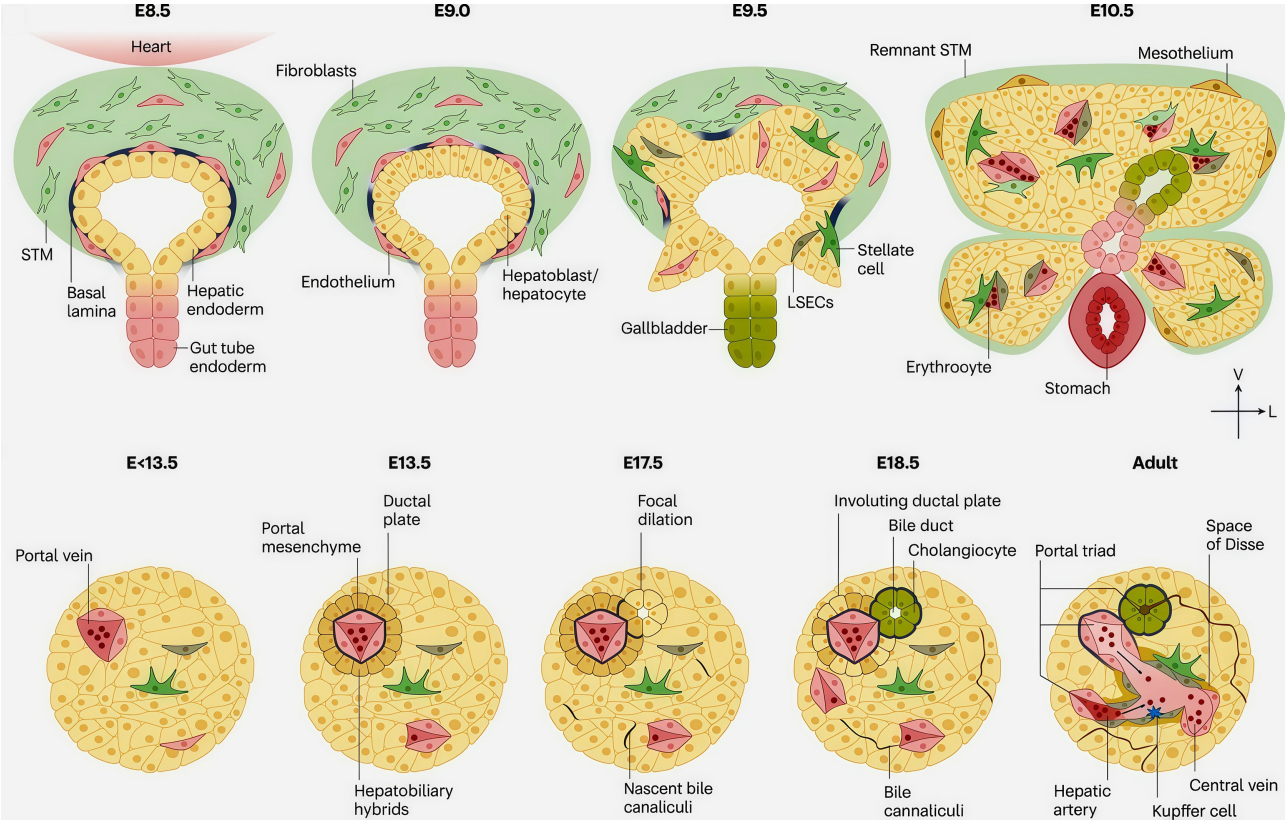
This dissertation will explore and characterize how the populations in foetal liver niche support a maturing and expanding HSC pool. Additionally, the current ways of generating liver organoids to emulate/reconstitute an HSC-supportive human foetal liver niche in a dish.

2. Liver Organogenesis (Hepatogenesis)

Early liver development starts with formation and patterning of the endoderm. Nodal, a member of the TGFβ family, is responsible for mammalian endoderm specification, promoting endoderm specification at high levels and mesoderm specification at low levels [5]. The gradient created by this signal triggers the expression of high levels of EOMES and low levels of T, which are essential transcription factors. These factors work in synergy with NODAL-SMAD2/3 signal transduction to stimulate the production of endodermal patterning genes, including CER1, SOX17, and FOXA2 [6].

During gastrulation, the endoderm layer experiences a sequence of developmental changes that lead to the establishment of the gut tube. This structure is organized along the anterior-posterior (front-to-back) axis into the foregut, midgut, and hindgut regions in mammals [7]. The spatial organization is determined by the graded distribution of Wnts, bone morphogenetic proteins (BMPs), and fibroblast growth factors (FGFs), which are present in low amounts at the anterior end and increase towards the posterior end. This gradient orchestrates the developmental destinies of the anterior foregut, posterior foregut, and the combined midgut-hindgut areas [8]. The posterior foregut endoderm will eventually commit to the hepatic lineage along with pancreas and gallbladder development.

Liver development starts with the coordinated action of BMPs and FGFs, emanating from the septum transversum mesenchyme (STM) and the cardiac mesoderm (**Figure 1**). Endothelial cells are also crucial in defining liver development [9]. As the hepatic endoderm matures, it changes from a columnar shape to a multi-layered (pseudostratified) tissue, eventually forming an early liver structure known as a diverticulum [10]. This process is accompanied by the breakdown of the surrounding basal lamina, enabling the precursor cells, *i.e.* hepatoblasts, to penetrate the septum transversum mesenchyme and create the primitive liver, or liver bud. The transcription factor HHEX is pivotal in the developmental transition from a columnar to a multi-layered epithelial structure, while TBX3 and



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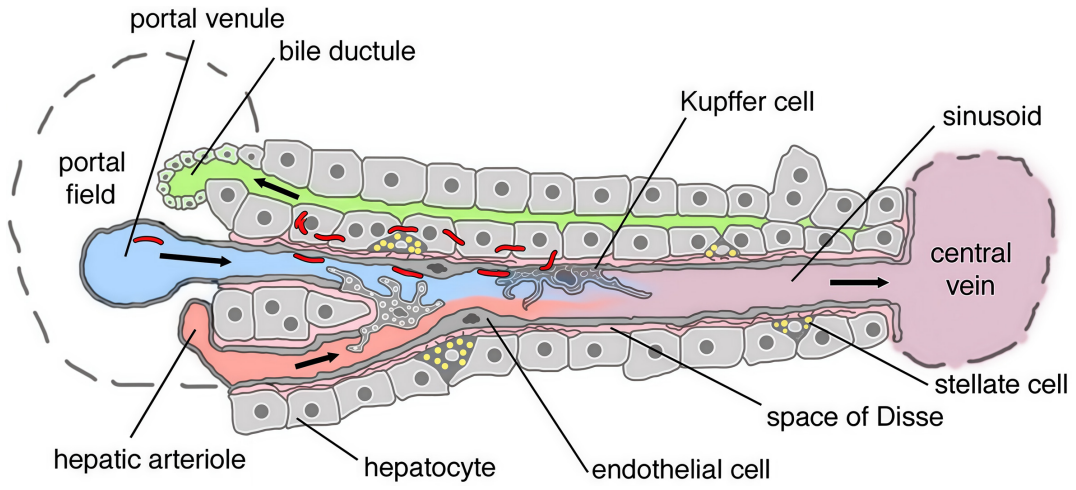


(b)

Figure 1. Basic mammalian liver structure. (a) Developmental stages of liver formation (E8.5 to adult) [12]. This panel illustrates the stepwise development of the liver in mice from embryonic day 8.5 (E8.5) to adulthood. Key processes such as the formation of hepatic endoderm, differentiation into hepatoblasts and endothelial cells, and the development of bile ducts and vasculature are shown. The transitions between these stages highlight the importance of cellular interactions, particularly between hepatoblasts, endothelial cells, and stellate cells, which establish the liver's functional architecture. These developmental steps are critical for understanding how the fetal liver environment supports hematopoietic stem cell (HSC) maturation. (b) Adult liver structure [13]. This panel shows the structural organization of the adult liver, focusing on the liver's vasculature and cellular compartments, including hepatocytes, Kupffer cells, and liver sinusoidal endothelial cells (LSECs). Blood flows from the hepatic artery and portal vein through the sinusoids, supporting nutrient exchange and immune functions. This mature architecture is essential for maintaining lifelong liver function and HSC support, linking back to the developmental processes described in the text.

PROX1 stimulate proliferation and migration of hepatoblasts [10]. HNF4a is essential for developing the liver's bud structure and plays a significant role in the liver bud's growth and maturation [11]. Hepatoblast are bipotent and have the capacity to differentiate into either a hepatocyte or a cholangiocyte, in response to TGFβ, Wnt, and Notch as well as BMP and FGF [8]. Along with non-parenchymal cell types, hepatocytes and cholangiocytes together, contribute to the final structure of the adult liver.

3. HSC Maturation and Expansion in Foetal Liver

The foetal liver is the primary hematopoietic organ, which can be described as a "melting pot" for multiple waves of haematopoiesis, and the main site for HSC expansion and maturation (**Figure 2(a)**). The liver rudiment in human emerges at around CS10 (week 3) and becomes colonized by first "primitive" wave blood progenitors and yolk sac-derived myeloerythroid progenitors (YSMP) from the second "transient definitive" hematopoietic wave [14] [15]. Between CS14-CS16 (4 - 6 weeks), a third, HSC-forming, definitive haematopoiesis wave, that generates nascent HSCs, arises in the AGM region. The molecular-defined HSCs colonize the foetal liver only at CS17 (6 weeks), after migration through yolk sac and placenta [15]. In liver, HSCs experience developmental maturation, limited expansion and lineage differentiation. They begin to produce progeny with multiple lineages already in the first trimester foetal liver and move to BM staying quiescence for postnatal life during the second trimester (**Figure 2(a)**).

Mouse haematopoiesis is broadly comparable with that of human, with the process occurring over much shorter periods (**Figure 2(a)**). HSC activity cannot be detected in the murine foetal liver until the embryonic day of development 10.5 (E10.5) [16]. After E12.5, foetal liver becomes main site where HSCs undergo expansion and differentiation, reaching a maximum amount of ~1000 by E15.5 - 16.5 [17]. This parallels human fetal development, where the fetal liver serves as the critical niche for HSC maturation, supporting lifelong hematopoiesis.

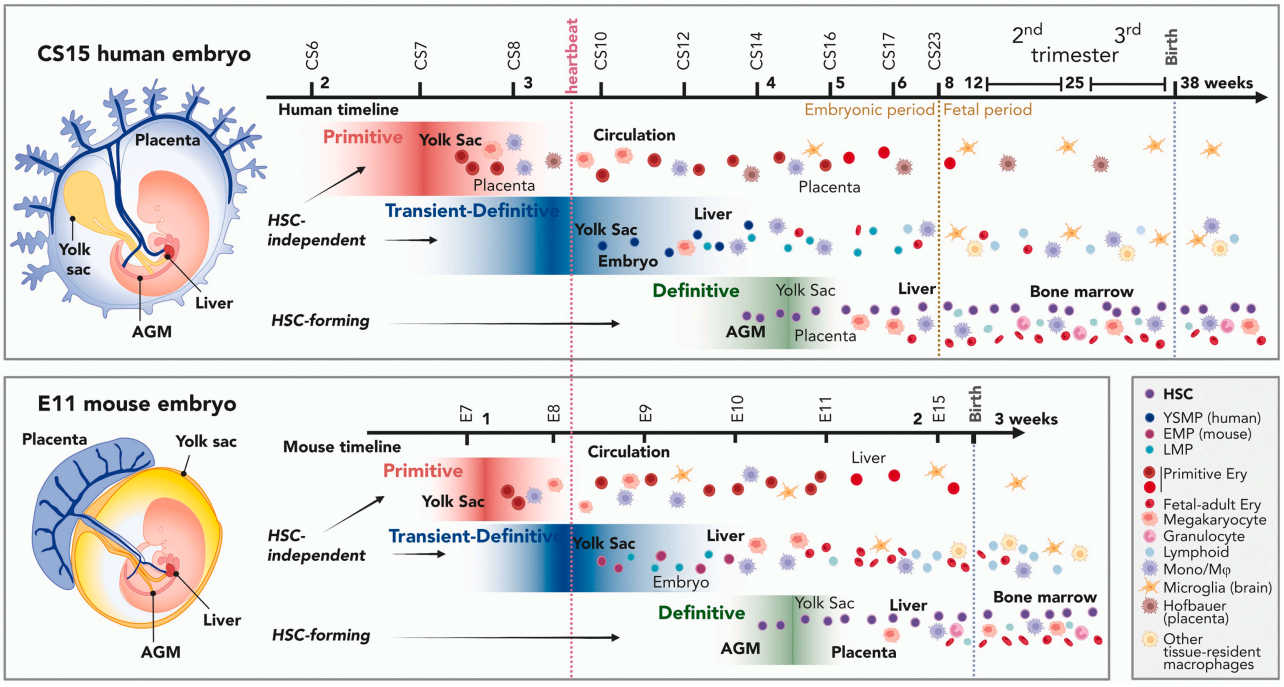
In addition to human and mouse, zebrafish (*Danio rerio*) is a popular model to study HSCs because of embryo transparency, ease of genetic manipulations, conserved hematopoietic pathways and rapid development. In contrast to human and mice, zebrafish definitive HSCs colonize the first niche, caudal haematopoietic tissue (CHT), where expansion and differentiation to multilineages take place, functionally equivalent to mammalian foetal liver in haematopoiesis, and this is followed by migration to the kidney marrow (**Figure 2(b)**). **Table 1** compares the anatomical sites of hematopoiesis across different species, including humans, mice, and zebrafish. Many studies explore the interactions between HSCs and their niches in CHT of zebrafish. Upon arrival of HSCs within the CHT, the vascular niche is remodelled by CXCR1 to promote HSPC engraftments [18]. Once HSCs lodged in the CHT, a stem cell pocket was formed by a small group of endothelial cells surrounding a single HSC, in a process referred to as "Endothelial Cuddling" [19]. This arrangement ensures that endothelial and stromal cells

remain in direct contact with HSCs, promoting HSC proliferation. Strikingly, prolonged HSCs in the CHT with lycorine treatment, as a promoter of HSPC-niche interactions, can ultimately lead to an expanded stem cell pool into adulthood [19]. This research provides evidence of the dynamic nature of niche interactions upon stem cell colonization. Besides, NACA (nascent polypeptide-associated complex alpha subunit) deficient stromal cell progenitors within the CHT niche compromise to maturation, resulting in a niche failed in supporting HSPCs maintenance, expansion and differentiation in zebrafish [20]. Caudal hematopoietic embryonic stromal tissue (CHEST) cells isolated from CHT produce hematopoietic-supportive cytokines and exhibit properties of perivascular endothelial cells that support HSPCs expansion and differentiation *in vitro* [21]. Additionally, *ism*1 (*isthmin* 1), a secreted protein with human orthologous, is required to form and modulate HSPCs and their progeny during zebrafish haematopoiesis [22] (p. 1)]. These findings allow comparison of hematopoietic-supportive niches in zebrafish and mammals, contributing to a deeper comprehension of the evolution of the vertebrate hematopoietic system.

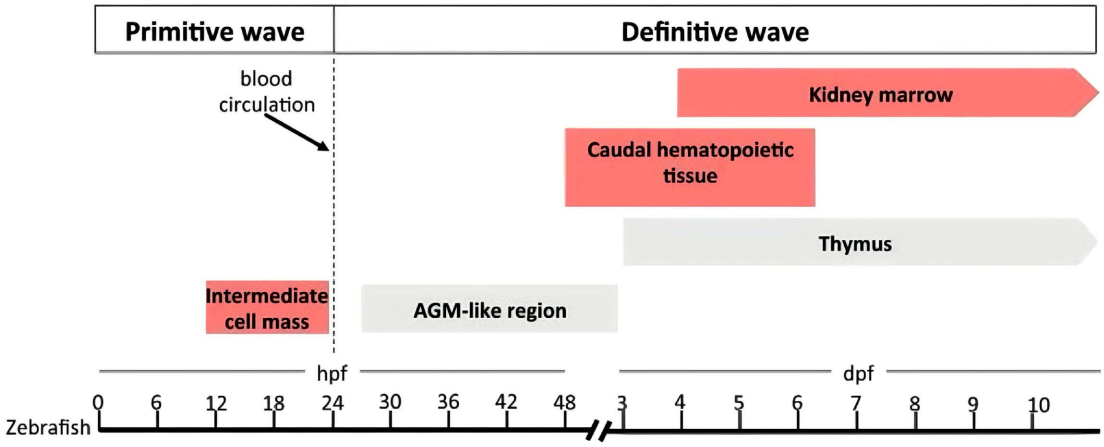
Table 1. Anatomical site of haematopoiesis in Human, Mouse and Zebrafish.

Species	Definitive HSC emerge	Definitive HSC expansion	Postnatal hematopoiesis
Human	AGM	FL	BM
Mouse	AGM	FL	BM
Zebrafish	VDA/AGM	CHT	KM/Thymus

However, it not feasible to directly translate the mouse and zebrafish findings to human contexts or to assess functional maturation in mice and zebrafish, due to different timing of developmental milestones and anatomy of extraembryonic structures. The function and development of these structures can vary significantly among different species. For instance, humans have a gestation period of approximately 280 days, in stark contrast to mice, which have a much shorter gestational span of about 20 days. While zebrafish do not have a gestation period in the same way that mammals like humans and mice do, as they are oviparous, meaning they lay eggs that hatch approximately 2 to 3 days post-fertilization (dpf) at standard temperatures. The compressed mice/zebrafish developmental timeline results in a great overlap between hematopoietic populations and developmental events. The human placenta is villous, characterized by tree-like chorionic villi structures, and completely envelopes the embryo and amniotic membrane and the human yolk sac is a small, balloon-like structure that is inside the amnion. In contrast, the mouse placenta exhibits labyrinthine architecture, which means it has a complex structure with an intermingled network of maternal and foetal blood vessels, and the yolk sac is the predominant structure surrounding the embryo and amnion. Zebrafish not only lack a placenta and other extraembryonic structures such



(a)



(b)

Figure 2. Haematopoiesis map during human, mouse and zebrafish development. (a) Cited from [23]. Mammalian haematopoiesis occurs in three temporal waves in multiple anatomical niches during embryonic development, that give rise both differentiated blood cells required for embryonic development and undifferentiated HSCs for life-long haematopoiesis. Primitive wave of haematopoiesis is derived from yolk-sac at CS7 or E7, in human or mouse respectively, producing transient hematopoietic cells to satisfy the urgent needs of the developing embryo. The second (transient-definitive) haematopoiesis wave also initiates in the yolk sac and probably embryo at around CS8-9 or E8, in human or mouse respectively. In human, the second wave is marked by the emergence of Yolk Sac-derived Macrophage Progenitors (YSMPs) which primed for myeloid specification. In contrast, the equivalent progenitors in mouse yolk sac (EMPs) exhibit a tendency towards erythromyeloid differentiation. Before the definitive haematopoiesis, "primitive" and "transient definitive" hematopoietic waves generates extra-embryonic derived blood progenitors populating the embryo, but those will not contribute to most adult multilineage haematopoiesis. Definitive haematopoiesis generate nascent HSCs in AGM region during CS14-17 in human, and E10.5-birth in mice. (b) Cited from [24]. Between 12 - 24 hpf, primitive haematopoiesis occur in intermediate cell mass. Between 26 and 48 hpf, definitive haematopoiesis arises in the ventral wall of the dorsal aorta in AGM region. Definitive HSCs and progenitors then enter the circulation and colonize the caudal hematopoietic tissue, followed by movement to the kidney and thymus. YSMPs, Yolk Sac-derived Macrophage Progenitors; EMPs, erythromyeloid progenitors; hpf, hours post fertilization.

as the amniotic sac and allantois, but their yolk sac also does not participate in haematopoiesis.

Single-cell RNA sequencing studies of human HLF+ HSCs over time revealed changes in gene expression during their residency in the liver, indicating the maturation of HSCs [15]. During HSCs functional maturation, transcriptional programs are enhanced and surface phenotypes change, pinpointing distinct biological processes. They show increased expression of the self-renewal regulator MLLT3 and its targets, including HLF, HEMGN, and MSI2, while suppressing fetal genes such as LIN28B and IGFBP2. Additionally, they acquire specific maturity markers, including CD133 (Prominin 1) and HLA-DR. These markers allow researchers to track HSC maturation in real-time during iPSC-derived HSPC culture experiments.

Recent studies using mouse models and confetti-based lineage tracing challenge the existing belief that the foetal liver primarily functions as a site for extensive HSC expansion, arguing that most cell divisions undertaken by FL-HSCs in mice do not result in an expanded pool of cells destined to contribute to the adult HSC pool. Instead, FL-HSCs that significantly proliferate during E12.5 - 14.5 in foetal liver are biased to differentiation rather self-renewal [25]. Hence, HSC with lifelong haematopoiesis potential only modestly expands in the foetal liver, implying that the increase in transplantable HSCs seen in the mouse foetal liver may be due to maturation rather than proliferation. This revised understanding of the fetal liver's role provides new insights into the mechanisms of HSC development and could inform strategies for optimizing HSC expansion *in vitro*.

3.1. Stromal Cells

Understanding the spatial location and cell types of the FL-HSC niche has long remained a significant focus of scientific research. Haematopoiesis already begins in the foetal liver before the hepatic vascular system has fully developed or matured. As a result, HSCs and hematopoietic cells within in the liver parenchyma can directly interact with various types of cells. A research group led by Paul Frenette found that mouse FL-HSCs are closely associated with Nestin+NG2+ periportal stromal cells which create a niche that supports HSC expansion [26]. This relationship scales with the fractal branching patterns of portal vessels, which are tributaries of the umbilical vein. Following the closure of the umbilical inlet after birth, a transition in portal vessels occurs, coinciding with the loss of Nestin+NG2+ cells and the migration of HSCs away from these vessels. The study suggests that the expansion of HSCs is synchronized with the growth of Nestin+NG2+ cells and portal vessels, supporting a niche-driven expansion model, rather than the concept of an HSC expansion driven by increased expression of HSC niche genes (SCF, Angplt2, IGF2) which remain at similar levels from E12 to E14.5.

To identify the stromal cells and factors that support HSCs and expansion of HSCs, stromal cells from the foetal liver were extracted and tested for their ability

to support HSC. AFT024, a single stromal cell line derived from foetal liver in mouse, has been reported the capacity to sustain long-term repopulating mouse stem cells for 4 to 7 weeks *in vitro* [27]. Later, a study found that AFT024 stromal cell line can support survival and maintenance of human hematopoietic progenitors *in vitro* in an undifferentiated state with engraftment and multilineage differentiation potential, superior to human stromal cells [28]. This undifferentiated/more primitive state of human hematopoietic stem and progenitor cells (HSPCs) maintained by AFT024 cell line is achieved through specific histone modifications, suggesting a mechanism by which the microenvironment can influence stem cell characteristics and potentially improve their therapeutic utility by preserving their stemness [29].

Besides, Lodish team identified a novel stromal population in mice: $CD3^+Ter119^-$ cells, isolated from E15 foetal liver, which have HSC-supportive ability with a significant expansion of long-term HSCs (LT-HSCs) in culture, distinguishing them from other $CD3^+$ cell populations [30]. These FL $CD3^+$ cell express high levels of insulin-like growth factor 2 (IGF-2) identified as a growth factor for HSCs. All mouse FL and adult BM HSCs express IGF-2 receptors, and the study demonstrated that IGF-2, when supplemented with other growth factors, significantly enhances the quantity and function of LT-HSCs. They subsequently identified angiopoietin-like proteins specifically expressed in these cells, particularly Angiopoietin-like 2 (Angptl2) and Angiopoietin-like 3 (Angptl3), as potent stimulators of *in vitro* HSC expansion [31]. However, later investigations using RT-PCR showed that this FL population did not express CD3 protein complex, and suggested CD3+ surface phenotype of these cells to constitute an artifact [32]. These cells are actually SCF+DLK+ hepatic progenitors, expressing a range of factors crucial for HSC expansion not only including Angptl and IGF2, but also stem cell factor (SCF/KitL), thrombopoietin (TPO) and CXCL12. These key cytokines and growth factors produced by fetal liver cells that support HSC culture are summarized in **Table 2**. Therefore, SCF+DLK+ cells are likely the primary stromal cells in the foetal liver supporting HSC expansion, maintenance and homing. Based on these findings, a serum-free culture system for both FL and BM-HSCs was developed, containing low but saturating levels of just IGF-2, Angptl3, SCF and TPO, shown > 20-fold expansion.

Stellate cells (Ito cells), described as fat-sorting cells by Toshio Ito, are a subtype of stromal cells that are *Desmin*-positive and express alpha-smooth muscle actin (α-SMA) [33] [34]. They are located in the perisinusoidal space of the liver, also known as space of Disse, and found in close contact with, particularly intercalated between, hepatocytes [35]. Hepatic stellate cells in mice express a variety of cytokines and extracellular matrix mRNAs which likely contribute to a supportive niche for liver haematopoiesis, such as SCF/KitL, colony-stimulating factor (CSF), erythropoietin (EPO), TPO, oncostatin M (OSM), FMS-like tyrosine kinase 3 ligand (Flt3l), and remarkably high levels of insulin-like growth factor (Igf2) [36] [37]. By genetic dissection of the developing liver niche in mice, perisinusoidal

hepatic stellate and endothelial cells were identified as the primary source of SCF, a key factor for HSC niche maintenance, and almost all HSCs were depleted when SCF was removed from both hepatic stellate and endothelial cells [38]. Most HSCs were found to be located close to the sinusoidal vasculature. SCF from endothelial or hepatic stellate cells synergistically promote HSC maintenance in the foetal liver with only modest depletion of HSC was observed when SCF was deleted from either endothelial or hepatic stellate cells. The redundancy of these cells as sources SCF could confer extra robustness to the system, potentially providing an evolutional advantage.

3.2. Endothelial Cells

As HSCs are derived from endothelium, a range of endothelial-cell specific antigens are expressed on developing HSCs. The expression of endothelial cell-selective adhesion molecule (ESAM), a lifelong marker of HSCs in mouse and human, on HSCs and endothelial cells is crucial for definitive haematopoiesis, particularly adult-type erythropoiesis, in the foetal liver [39] [40]. Despite ESAM-deficient HSCs in foetal liver retained their long-term ability to regenerate and give rise to erythrocytes, their ability to produce adult-type globins was significantly impaired [40]. Therefore, interactions mediated by ESAM may be indispensable for the acquisition of authentic differentiation potential in developing HSCs.

The majority of HSCs are in direct contact with endothelial cells in mouse foetal liver [41], as well as in the zebrafish CHT [19]. Notably, the study found that two subtypes of endothelial cells, arterial endothelial cells (AECs) and sinusoidal endothelial cells (SECs), along with other foetal liver cell types contribute unique signalling molecules to the niche of HSCs, suggesting their unique roles in supporting HSCs [41]. This contact might be mediated by E-selectin and VCAM1 [42] [43]. In addition, the endothelial protein C receptor, encoded by PROCR gene and expressed by murine and human FL-HSCs, stands out as one of the most reliable and conserved surface markers for the isolation and identification of FL-HSCs [44] [45]. This activates signalling through protease-activated receptor 1, which suppresses apoptosis and sustains the self-renewal activity of HSCs [45] [46].

Apart from endothelial factors expressed on HSCs, endothelial cells also express genes crucial for promoting HSC expansion and maturation. Endothelial cell-derived WNT5A, involvement in the non-canonical Wnt pathway, from the human foetal liver vascular niche has been demonstrated to serve a pivotal function in supporting maturation and expansion of foetal liver multilineage HSPCs in a cell autonomous manner [47]. The addition, soluble WNT5A can indeed improve the maturation and proliferation of HSPCs in stromal-free conditions. However, this setup does not recapitulate quantitatively the robust generation of phenotypic HSPCs or multilineage colony-forming progenitors achieved through co-culturing with foetal liver endothelial cells, indicating that further studies are needed to uncover additional niche factors involved in foetal liver haematopoiesis, potentially

working alongside WNT5A, to fully understand and replicate the supportive environment provided by the foetal liver endothelium.

3.3. Hepatoblasts

Interaction between HSCs and hepatoblasts was observed in the human foetal liver. Yong et al. identified a novel cell population in the human foetal liver, with low CD34 and CD133 expression [48]. Isolated $CD34^{lo}CD133^{lo}$ foetal liver cells at 15 - 23 weeks of gestation, shown to express hepatic and mesenchymal markers including AFP, CK18, CK19 and EpCAM, and can differentiate into hepatocytes both *in vivo* and *ex vivo* [49]. These $CD34^{lo}CD133^{lo}$ cells express essential growth factors (e.g., SCF, IGF2, CXCL12 and Angiopoietin-like proteins) known to support the expansion of human HSCs. In co-culture experiments, they not only support and expand autologous and allogenic HSCs derived from cord blood but also maintain their self-renewing and repopulating ability after 7 days co-culture, highlighting their pivotal role in establishing a conducive microenvironment for HSC proliferation [48].

Most studies of the niche compartments crucial for foetal haematopoiesis were focused primarily on specific subsets of cells, often analysing individual stages, and neglecting the initial phase of haematopoiesis. A recent study explored the intricate dynamics of haematopoiesis in the foetal liver, defined by an environment that is surprisingly characterized by low levels of cytokines [50]. At E12.5 mice, hematopoietic progenitors from the yolk sac (YS) and intraembryonic sources converge to the foetal liver sub-mesothelial region characterized by the presence of CXCL12-producing stellate cells, hepatoblasts and mesothelial cells that produce KITL. YS and intraembryonic-derived progenitors have been shown to make direct contact with hepatoblasts but not with stellate or endothelial cells. During this stage, the sub-mesothelial region is notably low in oxygen, a condition that could be responsible for the detected peak levels of EPO in hepatoblasts. At E14.5, mesenchymal cells, primarily perivascular cells, show an increase in CXCL12 expression, leading to an increase in CXCR4 levels in intraembryonic hematopoietic progenitors which then move away from the sub-mesothelial area. Meanwhile, YS-derived progenitors, which lack CXCR4 expression, remain in the sub-mesothelial region. Later in development, by E18.5, hepatic cells, predominantly cholangiocytes and hepatocytes, show a reduction in the production of KITL, IL-7, CXCL12 and EPO. This occurs simultaneously with the initiation of haematopoiesis in BM. Throughout development, foetal liver is strikingly a low cytokine niche compared to BM, revolutionizing our understanding of the FL HSC niche properties.

Novel single cell RNA-sequencing (scRNA-seq) studies in human foetal liver have highlighted the heterogeneous cell composition of this organ throughout human development, highlighting a predominant haematopoietic component in the foetal liver (**Figure 3**) [51]. Nonetheless, despite the growing body of knowledge, the specific physical and molecular interaction of each cell type to HSCs within

this dynamic organ needs further investigation [15]. Understanding these processes is crucial for harnessing the regenerative potential of HSCs and could pave the way for novel therapeutic strategies in the treatment of haematological diseases.

Table 2. Summary of growth factors/cytokines produced from foetal liver *in vivo* that may be crucial for HSC culture.

Cytokine/growth factor	Expressing cell	Reference
Angptl	Nestin+ cells	[26]
	SCF+Dlk1+ hepatoblasts	[32]
SCF/KitL	Stellate cells	[37]
	Nestin+ cells	[26]
	Dlk1+ hepatoblasts	[32] [52]
	$CD34^{lo}CD133^{lo}$ cells	[48] [49]
TPO	Stellate cells	[37]
	SCF+Dlk1+ hepatoblasts	[32]
FGF-1	Dlk1+ hepatoblasts	[32]
IGF-2	SCF+Dlk1+ hepatoblasts	[32] [52]
	Nestin+ cells	[26]
	Stellate cells	[37]
	$CD34^{lo}CD133^{lo}$ cells	[48] [49]
CSF	Stellate cells	[37]
OSM	Stellate cells	[37]
EPO	Stellate cells	[37]
	SCF+DLK1+ hepatoblasts	[32] [52]
Flt3l	Stellate cells	[37]
CXCL12	$CD34^{lo}CD133^{lo}$ cells	[48] [49]
	SCF+Dlk1+ hepatoblasts	[32] [52]

3.4. Metabolic Factors and Others

In addition to various cellular elements of foetal liver niche, other metabolic factors and small molecules may also play an important role in HSC expansion and maturation in the foetal liver. Even with the elevated production of proteins during HSC expansion, FL-HSCs are resilient to the accumulation of unfolded/misfolded protein, endoplasmic reticulum (ER) stress, because of the protective role of bile acids which traditionally known for fat digestion and nutrients absorption. Maternal and foetal bile acids serving as chemical chaperones are essential in mitigating ER protein stress in expanding HSCs [55]. Reducing bile acids level *in vivo* results in elevated ER-stress levels, and the accumulation of protein aggregates, as well as a substantial decrease in the number of FL-HSCs. Interestingly, bile acids treatment *in vitro* maintained engraftment ability of

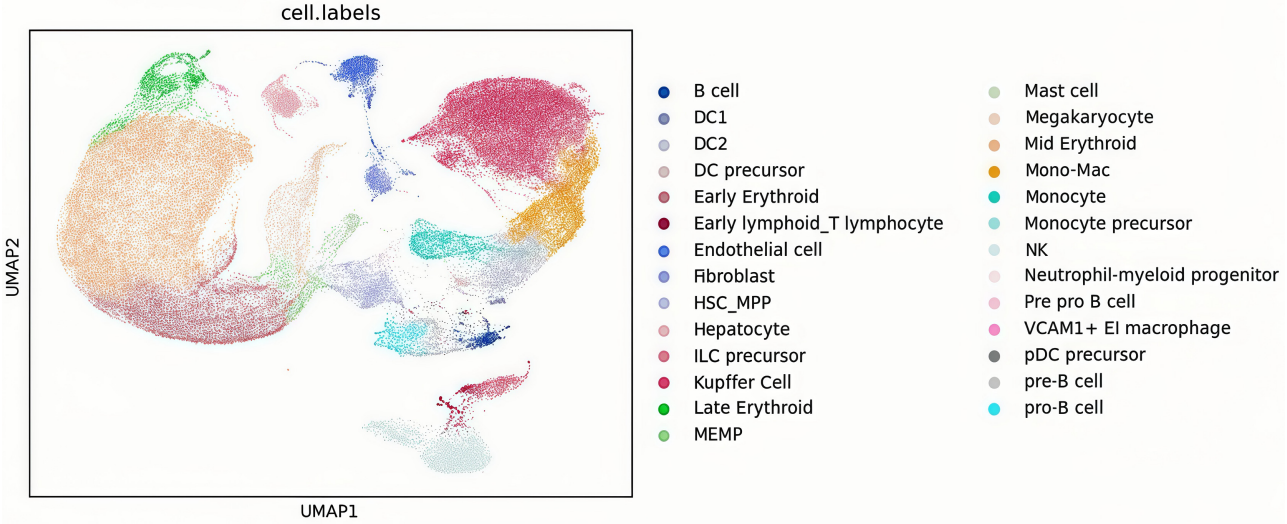


Figure 3. UMAP Analysis of Human Fetal Liver Cells at 7 to 17 Weeks Post-Conception. UMAP analysis of human fetal liver cells at 7 to 17 weeks post-conception, identifying 27 major cell states via 10x Genomics Chromium Single Cell 3' RNA-sequencing. Cells were sequenced on an Illumina NovaSeq 6000 with an average depth of 50,000 reads per cell. Data were processed with the Cell Ranger pipeline and analyzed using the Seurat package in R. The black-circled population represents HSCs and multipotent progenitors (HSC_MPPs). Neutrophils, basophils, and eosinophils were not detected [51], consistent with granulocyte development during the fetal bone marrow stage [53]. Early samples favored erythroid lineages, while lymphoid and myeloid lineages emerged later, in line with previous findings [54]. Data from https://developmentcellatlas.ncl.ac.uk//datasets/hca_liver/.

cultured FL HSCs, indicating a promising application of bile acids for *in vitro* expansion of HSC.

Metabolism plays an essential role in maintaining normal functions of HSCs. Genes linked to mitochondrial oxidative phosphorylation gradually supressed in human FL-HSCs during maturation in foetal liver, transiting to a more homeostatic state [15]. However, the molecular mechanisms by which mitochondrial dynamics are regulated to orchestrate metabolism and HSCs stemness are not yet fully understood. Zhang *et al.* found that Zeb1, an EMT inducer, is essential for maintaining long-term stemness in both adult BM- and FL-HSCs in mice [56]. They found that a subset of functional LT-HSCs, Zeb1+Lin−Sca-1+c-Kit+ cells, inhibit mitochondrial fusion, specifically by suppressing the action of a fusion protein Mitofusin-2, therefore sustaining a low mitochondrial metabolic state and an immature mitochondrial machinery with reduced mitochondrial ROS levels, membrane potential, mass and oxidative phosphorylation activities. These attributes together benefit the long-term self-renewal and multilineage commitment of HSCs.

4. Liver Organoids

Understanding the function of embryo/foetal liver niche components in culture for their effects in HSC maturation and ultimately recapitulate the HSC functional maturation in a dish is important to interpret transcriptomic and phenotypic data. One way to model this *in vitro* for functional testing would be the generation of foetal liver organoids. An organoid is a 3D structure derived from stem cells that

can recapitulate its *in vivo* counterparts through a process of self-organization. Nowadays, scientists can generate liver organoids using various cell sources, but some design principles have to be considered (Table 3): 1) Space design: composition of starting cell population (derivation from homogenous or heterogenous) along with their shape and size (single cell or cell aggregate). 2) Biological environmental control: include factors/cytokines (BMP, FGF, RA), liver specific ECM (collagen, glycosaminoglycans, laminin, fibronectin, and scaffolds/molds), FL-specific cell types (stellate cells, LSECs, Kupffer cells, cholangiocytes); specification signals from niche (STM, cardiac mesoderm, tissue boundary [57]-[59]). 3) Synthetic niche factors: gene circuits engineering [60], match the physiological stiffness by mechano-modulatory 3D culture system [61], hexagonal bioprinted constructs [62] or needle-array system for scalable FL-like tissue [63].

Table 3. Design principles of human liver organoid.

Space design	Starting cell population	Homogenous	[64]
		Heterogenous	[65]-[67]
	Size & Shape	Single cells in ECM	
		Multicellular spheroid/aggregate	
Biological factors	Soluble factors/cytokines	BMPs	[8]
		FGFs	
		Retinoic acid	[68]
		Liver X receptors/retinoid X receptors (LXR/RXR)	[69]
		Platelet-derived growth factor (PDGF)	
		Transforming growth factor beta (TGFβ)	[70]
		Insulin-like growth factor 1 (IGF1)	
	Liver specific ECM	Collagen	[71] [72]
		Glycosaminoglycans	
		Laminin	
		Fibronectin	
		Scaffolds/mould	
	FL-specific cell types	Stellate cells	[73]
		LSECs	
		Kupffer cells	
		Cholangiocytes	
	Specification signals from niche	Hepatic nervous system	[57]-[59]
		STM	
		Cardiac mesoderm	
		Tissue boundary	
Synthetic factors	Gene circuits		[60]
	Stiffness modulation		[61]
	Hexagonal bioprinted constructs		[62]
	Needle-array system		[63]
	3D perfusable chip system		[74]

4.1. Generation of Human Liver Organoids from Pluripotent Stem Cells

Hepatic cell lineages derived from hPSCs offers a valuable cell source that could revolutionize our knowledge of how embryonic cells achieve functional maturity. Advancements in human stem cell research enable several groups reported successful generation of hPSCs-derived liver organoid (**Figure 4**).

Early studies by Michalopoulos and their team on various adult rat liver cells led to the development of tissue structures that mimicked liver architecture, marking the first documentation of liver organoids [75]. It wasn't until 2013 that studies demonstrated the ability to maintain liver organoids with self-renewing properties, showing phenotypic and genetic stability, derived from a progenitor population in adult mouse liver [76]. In the same year, Takebe and colleages initiated the first creation of 3D liver tissue cultures that emulated the developing liver bud during early embryogenesis, pioneered in using hiPSC-derived hepatic endoderm cells [77]. These cells were cultivated with human umbilical vein endothelial cells and human BM mesenchymal stem cells to form liver bud organoids. This innovative approach successfully replicated the initial phase of liver development, producing a vascularized liver bud organoid that matured into functional liver tissue upon transplantation into mice. This system has been refined by 2017, in which they utilized hiPSCs to generate hepatic endoderm, endothelial, and STM cells, combined at a ratio of 10:7:2, producing a scalable human liver bud entirely from hiPSCs (**Figure 4(a)**) [65]. Upon seeding the triple progenitors onto the omniwell plate, endothelialized liver buds were self-organized. In 2019, Pettinato et al. outlined a comparable approach, where hPSCs were co-cultured with human adipose microvascular endothelial cells (HAMECs) at a ratio of 3:1, leading to their differentiation into hepatocytes (**Figure 4(a)**) [67]. This protocol generates liver organoids composed of 89% Albumin+ cells and 15% CD31+ cells, demonstrating a higher yield of differentiation and substantial enhancements in various hepatic functions. Interestingly, HAMECs self-organized within the organoids in rosette-like structures. Another approach involved forming 3D aggregates of hPSCs-derived hepatoblast-like cells, which were then cultured together with human foetal liver mesenchymal cells (hFLMCs) at a 2:1 ratio (**Figure 4(a)**) [66]. By day 14 of differentiation, the expression of albumin, a marker of mature hepatocytes, was predominantly higher along the periphery of the organoids, while PDGFR-β+ hFLMCs were mainly located at the centre. Moreover, co-culture with hFLMCs was shown to enhance hepatic differentiation in the hEHOs, as indicated by increased levels of hepatic function markers, including albumin secretion, CYP3A4 activity, and urea production.

Besides beginning with mixed cell types to generate human liver organoids, other research has shown methods for developing complex human liver organoids from homogenous cell populations. Ouchi et al. from Takebe group successfully developed a reproducible way to create hPSCs-derived multicellular liver organoids consisting of hepatocytes, stellate cells, Kupffer-like cells, and other liver-

specific cell types, exhibiting transcriptomic similarities to human liver tissues *in vivo* (**Figure 4(b)**) [64]. Initially, hPSCs were differentiated into foregut spheroids through definitive endoderm specification. These spheroids were then embedded in Matrigel and incubated with retinoic acid (RA), a compound known to contribute to the specification of both parenchymal and non-parenchymal cells.

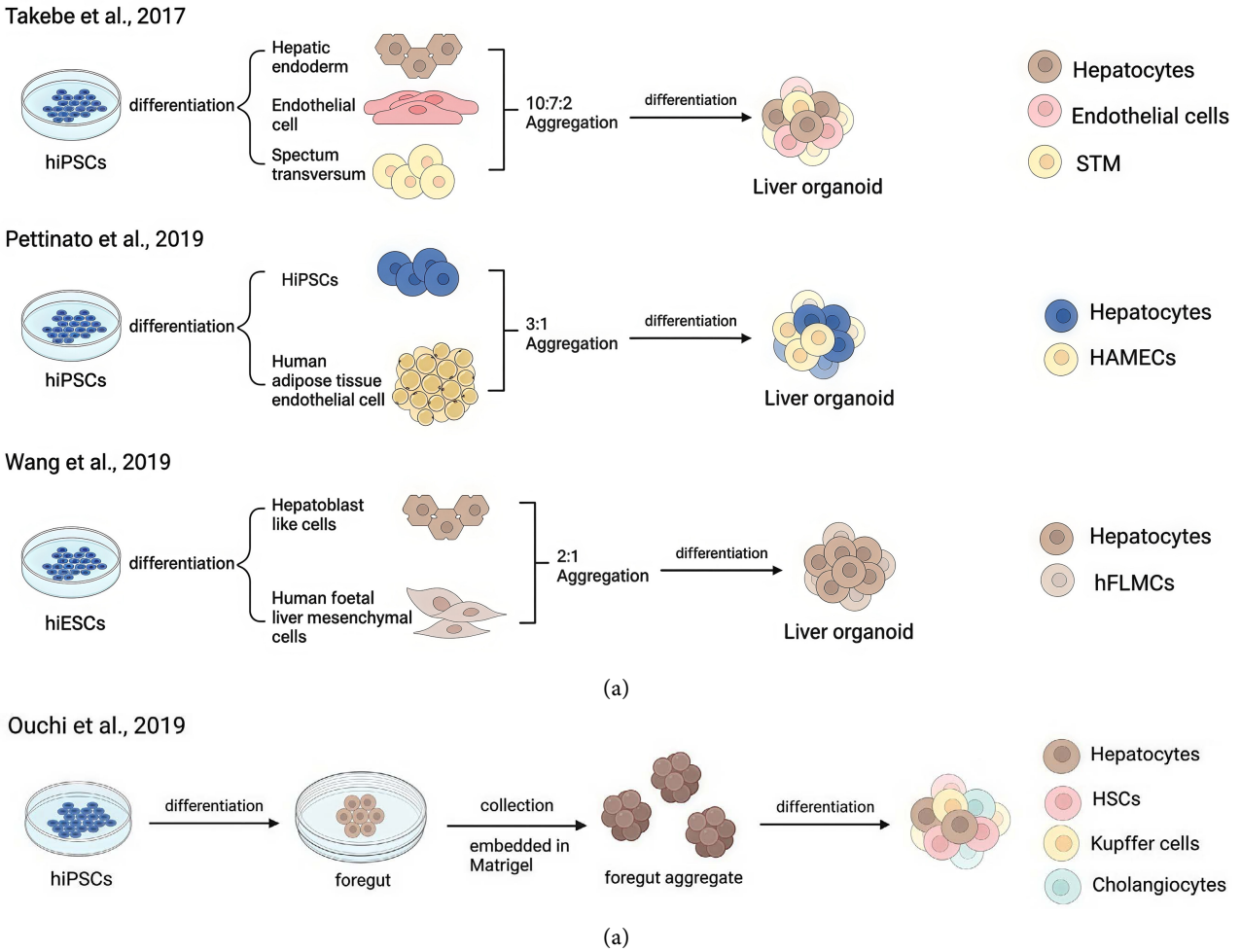


Figure 4. A summary picture of strategies for the generation of human liver organoids from PSCs. (a) Starting from heterogenous cell population such as, differentiated hepatic cell lineages or isolated human cells that support the differentiation and maturation of liver organoid. (b) Starting from homogenous cell population. This figure was created from BioRender and adapted from [78].

4.2. Biological and Non-Biological Environmental Control

4.2.1. Biological Factors

A range of soluble factors are employed to recapitulate the early liver development that starts from iPSCs-induced definitive endoderm, followed by the specification of the posterior foregut, and then the specification of hepatocytes. [12] showed a pseudo-temporal map depicting the liver development in mice from E7.5 to E10.5. The study elucidated crucial interactions between cells, mediated by paracrine and juxtracrine signalling, such as TGFβs, IGF1, and PDGF, which play pivotal roles in the early establishment of the sinusoidal structure.

Apart from soluble elements and paracrine cues, direct interactions through cell-to-ECM contacts play a crucial role in promoting the self-organization of liver organoids. A study focused on enhancing the functionality of liver organoids through the use of a 3D liver-derived extracellular matrix hydrogel (LEMgel) [71]. This system was developed through an optimized decellularization method from sheep liver, preserves essential components including glycosaminoglycans, laminin, collagen, and fibronectin. These components are crucial for maintaining the structural and functional integrity of the liver organoids. Additionally, decellularized liver scaffolds preserved intact vascular structure and bile duct network are utilized as channels for cell seeding, blood flow, and bile flow for generation of transplantable human liver graft [72].

By definition, cells that reside in their native niche, are inherently exposed to appropriate cues which enable their proper development and function. Interactions between anterior and posterior gut spheroids in a three-dimensional culture allow for the RA-dependent emergence of organ domains, hepato-biliary-pancreatic (HBP) primordium, without extrinsic factors [57]. The study by Wiilnow *et al.* presents a comprehensive examination of the developmental trajectories and lineage differentiation within the hepato-pancreato-biliary (HPB) system, revealing the existence of a multipotent progenitor niche, situated at the liver and pancreato-biliary boundary, that contributes to the liver, pancreas, and gallbladder [58]. Thus, liver organoids with physiological and homeostatic functions require not only interactions at cellular or tissue levels but also inter-organ crosstalk for accurately simulating human liver development *in vitro*.

4.2.2. Synthetic Factors

Recent advances in fine-tuning liver development *in vitro* involve non-biological elements including synthetic gene circuits, 3D biomaterials/bioprinting, etc. Through engineering and integrated analysis of gene regulatory networks, we can steer the maturation and vascular morphogenesis of liver organoids *in vitro* via included overexpression of specific transcription factors (PROX1 and ATF5) and CRISPR-based activation of endogenous gene (CYP3A4), which reprogrammed tissue gene regulatory networks and improved native liver functions [60]. This approach led to the development of organoids with superior liver identity, featuring hepatocyte, biliary, endothelial and stellate-like cell populations, and exhibited functional hepatic features both *in vitro* and *in vivo*. In addition, research by Yusuke et al. introduces innovative techniques for producing scalable liver bud-like spheroids optimized for 3D printing, leading to the successful biofabrication of liver-like tissue that exhibits self-organization and functional integration upon transplantation [63].

5. Summary and Discussion

The primary challenge limiting patient access to HSC-based therapy is the scarcity of safe, transplantable HSCs that perfectly match the recipient, coupled with our restricted capabilities to modify or increase HSC numbers outside the body.

Overcoming these obstacles could be possible through the efficient production of transplantable HSCs from patient-specific iPSCs. This dissertation dissects the intricate environment of HSC niche within the foetal liver and PSC-derived 3D organoid generation aimed to mimic the foetal liver niche.

While previous research has predominantly honed in on cell type differentiation by replicating early developmental stages, these populations are not suitable for function in an adult tissue niche where they would be transplanted. Hence, future effort should pivot towards comprehending the acquisition of functional HSC maturation in foetal liver post-specification, a big gap towards therapeutic applications, intended as the ability of robust engraftment and hematopoietic reconstitution upon transplantation. Understanding the environmental and molecular cues provided by various foetal liver cell types is essential for studying the physiological and developmental context of the HSC functional maturation process. However, foetal liver niche, particularly in humans, is barely understood. Therefore, an in-depth investigation of the various cell populations in human embryonic and foetal liver is critical. Such research will illuminate the role of the foetal liver niche in guiding HSC maturation and how to emulate these conditions *in vitro* for generating fully functional HSCs.

The protocols described above are shown to effectively generate hepatic cell and precursors of all the main lineages expected to be present in the embryonic and foetal liver, which sustain HSC function and maturation. However, these human liver organoids are missing the other main components present in the foetal liver: HSC with HSC-derived progenitors, and non-definitive yolk sac-derived hematopoietic progenitors. To fully recapitulate human *in vivo* foetal liver, it is essential to integrate iPSCs-derived AGM-like HSPCs and iPSCs-derived hematopoietic progenitors and cells resemble those from yolk sac into the foetal liver organoid protocol. Generating a heterogeneous organoid system containing cells from multiple lineages will be challenging, yet undertaking this effort could enable more accurate simulations of early liver organogenesis and recapitulate in a dish the process of HSC functional maturation in foetal liver.

Although autologous iPSC-based approaches represent the ideal source for patient-tailored regenerative medicine, it is arguably considered not widely available to the major population soon. Making "off-the-shelf" HSC products from hypoimmunogenic PSCs is a more feasible strategy, in which these cells are universally compatible and simpler for mass production, offering a practical option for standard treatments aimed at blood reconstitution. Several research teams are currently pursuing this strategy, but refined protocols for HSC functional maturation are still required [79] [80]. The potential societal and economic benefits of these breakthroughs are substantial, as these treatments are primarily curative, thereby eliminating the expenses and burdens associated with continuous therapy and a diminished quality of life. However, the success of these applications hinges on our capacity to generate safe and effective HSCs from a readily available and limitless source.

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Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

References

[1] Ivanovs, A., Rybtsov, S., Ng, E.S., Stanley, E.G., Elefanty, A.G. and Medvinsky, A. (2017) Human Haematopoietic Stem Cell Development: From the Embryo to the Dish. *Development*, **144**, 2323-2337. https://doi.org/10.1242/dev.134866

[2] Ditadi, A., Sturgeon, C.M., Tober, J., Awong, G., Kennedy, M., Yzaguirre, A.D., *et al.* (2015) Human Definitive Haemogenic Endothelium and Arterial Vascular Endothelium Represent Distinct Lineages. *Nature Cell Biology*, **17**, 580-591. https://doi.org/10.1038/ncb3161

[3] Ng, E.S., Azzola, L., Bruveris, F.F., Calvanese, V., Phipson, B., Vlahos, K., *et al.* (2016) Differentiation of Human Embryonic Stem Cells to *HOXA*$^{+}$ Hemogenic Vasculature That Resembles the Aorta-Gonad-Mesonephros. *Nature Biotechnology*, **34**, 1168-1179. https://doi.org/10.1038/nbt.3702

[4] Nafria, M., Bonifer, C., Stanley, E.G., Ng, E.S. and Elefanty, A.G. (2020) Protocol for the Generation of Definitive Hematopoietic Progenitors from Human Pluripotent Stem Cells. *STAR Protocols*, **1**, Article 100130. https://doi.org/10.1016/j.xpro.2020.100130

[5] Vincent, S.D., Dunn, N.R., Hayashi, S., Norris, D.P. and Robertson, E.J. (2003) Cell Fate Decisions within the Mouse Organizer Are Governed by Graded Nodal Signals. *Genes & Development*, **17**, 1646-1662. https://doi.org/10.1101/gad.1100503

[6] Faial, T., Bernardo, A.S., Mendjan, S., Diamanti, E., Ortmann, D., Gentsch, G.E., *et al.* (2015) Brachyury and SMAD Signalling Collaboratively Orchestrate Distinct Mesoderm and Endoderm Gene Regulatory Networks in Differentiating Human Embryonic Stem Cells. *Development*, **142**, 2121-2135. https://doi.org/10.1242/dev.117838

[7] Tremblay, K.D. and Zaret, K.S. (2005) Distinct Populations of Endoderm Cells Converge to Generate the Embryonic Liver Bud and Ventral Foregut Tissues. *Developmental Biology*, **280**, 87-99. https://doi.org/10.1016/j.ydbio.2005.01.003

[8] Gordillo, M., Evans, T. and Gouon-Evans, V. (2015) Orchestrating Liver Development. *Development*, **142**, 2094-2108. https://doi.org/10.1242/dev.114215

[9] Matsumoto, K., Yoshitomi, H., Rossant, J. and Zaret, K.S. (2001) Liver Organogenesis Promoted by Endothelial Cells Prior to Vascular Function. *Science*, **294**, 559-563. https://doi.org/10.1126/science.1063889

[10] Bort, R., Signore, M., Tremblay, K., Barbera, J.P.M. and Zaret, K.S. (2006) Hex Homeobox Gene Controls the Transition of the Endoderm to a Pseudostratified, Cell Emergent Epithelium for Liver Bud Development. *Developmental Biology*, **290**, 44-56. https://doi.org/10.1016/j.ydbio.2005.11.006

[11] Parviz, F., Matullo, C., Garrison, W.D., Savatski, L., Adamson, J.W., Ning, G., *et al.* (2003) Hepatocyte Nuclear Factor 4α Controls the Development of a Hepatic

Epithelium and Liver Morphogenesis. *Nature Genetics*, **34**, 292-296. https://doi.org/10.1038/ng1175

[12] Lotto, J., Stephan, T.L. and Hoodless, P.A. (2023) Fetal Liver Development and Implications for Liver Disease Pathogenesis. *Nature Reviews Gastroenterology & Hepatology*, **20**, 561-581. https://doi.org/10.1038/s41575-023-00775-2

[13] Frevert, U., Engelmann, S., Zougbédé, S., Stange, J., Ng, B., Matuschewski, K., *et al.* (2005) Intravital Observation of Plasmodium Berghei Sporozoite Infection of the Liver. *PLOS Biology*, **3**, e192. https://doi.org/10.1371/journal.pbio.0030192

[14] Bian, Z., Gong, Y., Huang, T., Lee, C.Z.W., Bian, L., Bai, Z., *et al.* (2020) Deciphering Human Macrophage Development at Single-Cell Resolution. *Nature*, **582**, 571-576. https://doi.org/10.1038/s41586-020-2316-7

[15] Calvanese, V., Capellera-Garcia, S., Ma, F., Fares, I., Liebscher, S., Ng, E.S., *et al.* (2022) Mapping Human Haematopoietic Stem Cells from Haemogenic Endothelium to Birth. *Nature*, **604**, 534-540. https://doi.org/10.1038/s41586-022-04571-x

[16] Ema, H. and Nakauchi, H. (2000) Expansion of Hematopoietic Stem Cells in the Developing Liver of a Mouse Embryo. *Blood*, **95**, 2284-2288. https://doi.org/10.1182/blood.v95.7.2284

[17] Mikkola, H.K.A. and Orkin, S.H. (2006) The Journey of Developing Hematopoietic Stem Cells. *Development*, **133**, 3733-3744. https://doi.org/10.1242/dev.02568

[18] Blaser, B.W., Moore, J.L., Hagedorn, E.J., Li, B., Riquelme, R., Lichtig, A., *et al.* (2017) CXCR1 Remodels the Vascular Niche to Promote Hematopoietic Stem and Progenitor Cell Engraftment. *Journal of Experimental Medicine*, **214**, 1011-1027. https://doi.org/10.1084/jem.20161616

[19] Tamplin, O.J., Durand, E.M., Carr, L.A., Childs, S.J., Hagedorn, E.J., Li, P., *et al.* (2015) Hematopoietic Stem Cell Arrival Triggers Dynamic Remodeling of the Perivascular Niche. *Cell*, **160**, 241-252. https://doi.org/10.1016/j.cell.2014.12.032

[20] Murayama, E., Sarris, M., Redd, M., Le Guyader, D., Vivier, C., Horsley, W., *et al.* (2015) NACA Deficiency Reveals the Crucial Role of Somite-Derived Stromal Cells in Haematopoietic Niche Formation. *Nature Communications*, **6**, Article No. 8375. https://doi.org/10.1038/ncomms9375

[21] Wolf, A., Aggio, J., Campbell, C., Wright, F., Marquez, G., Traver, D., *et al.* (2017) Zebrafish Caudal Haematopoietic Embryonic Stromal Tissue (CHEST) Cells Support Haematopoiesis. *Scientific Reports*, **7**, Article No. 44644. https://doi.org/10.1038/srep44644

[22] Berrun, A., Harris, E. and Stachura, D.L. (2018) *Isthmin* 1 (*ism*1) Is Required for Normal Hematopoiesis in Developing Zebrafish. *PLOS ONE*, **13**, e0196872. https://doi.org/10.1371/journal.pone.0196872

[23] Calvanese, V. and Mikkola, H.K.A. (2023) The Genesis of Human Hematopoietic Stem Cells. *Blood*, **142**, 519-532. https://doi.org/10.1182/blood.2022017934

[24] Kulkeaw, K. and Sugiyama, D. (2012) Zebrafish Erythropoiesis and the Utility of Fish as Models of Anemia. *Stem Cell Research & Therapy*, **3**, Article No. 55. https://doi.org/10.1186/scrt146

[25] Ganuza, M., Hall, T., Myers, J., Nevitt, C., Sánchez-Lanzas, R., Chabot, A., *et al.* (2022) Murine Foetal Liver Supports Limited Detectable Expansion of Life-Long Haematopoietic Progenitors. *Nature Cell Biology*, **24**, 1475-1486. https://doi.org/10.1038/s41556-022-00999-5

[26] Khan, J.A., Mendelson, A., Kunisaki, Y., Birbrair, A., Kou, Y., Arnal-Estapé, A., *et al.* (2016) Fetal Liver Hematopoietic Stem Cell Niches Associate with Portal Vessels. *Science*, **351**, 176-180. https://doi.org/10.1126/science.aad0084

[27] Moore, K.A., Ema, H. and Lemischka, I.R. (1997) *In Vitro* Maintenance of Highly Purified, Transplantable Hematopoietic Stem Cells. *Blood*, **89**, 4337-4347. https://doi.org/10.1182/blood.v89.12.4337

[28] Nolta, J., Thiemann, F., Arakawa-Hoyt, J., Dao, M., Barsky, L., Moore, K., *et al.* (2002) The AFT024 Stromal Cell Line Supports Long-Term *ex vivo* Maintenance of Engrafting Multipotent Human Hematopoietic Progenitors. *Leukemia*, **16**, 352-361. https://doi.org/10.1038/sj.leu.2402371

[29] Chung, Y.S., Choi, B., Kwon, C.H.D., Joh, J.W. and Kim, S.J. (2010) AFT024 Cell Line in Co-Culture System Using High Pore Density Insert (HPDI) Maintains Hematopoietic Stem/Progenitor Cells (HSCs/HPCs) as More Primitive State through Histone Modification. *Transplantation Proceedings*, **42**, 4611-4618. https://doi.org/10.1016/j.transproceed.2010.09.175

[30] Zhang, C.C. and Lodish, H.F. (2004) Insulin-Like Growth Factor 2 Expressed in a Novel Fetal Liver Cell Population Is a Growth Factor for Hematopoietic Stem Cells. *Blood*, **103**, 2513-2521. https://doi.org/10.1182/blood-2003-08-2955

[31] Zhang, C.C., Kaba, M., Ge, G., Xie, K., Tong, W., Hug, C., *et al.* (2006) Angiopoietin-Like Proteins Stimulate *ex vivo* Expansion of Hematopoietic Stem Cells. *Nature Medicine*, **12**, 240-245. https://doi.org/10.1038/nm1342

[32] Chou, S. and Lodish, H.F. (2010) Fetal Liver Hepatic Progenitors Are Supportive Stromal Cells for Hematopoietic Stem Cells. *Proceedings of the National Academy of Sciences*, **107**, 7799-7804. https://doi.org/10.1073/pnas.1003586107

[33] Kiassov, A.P., Van Eyken, P., van Pelt, J.F., Depla, E., Fevery, J., Desmet, V.J., *et al.* (1995) Desmin Expressing Nonhematopoietic Liver Cells during Rat Liver Development: An Immunohistochemical and Morphometric Study. *Differentiation*, **59**, 253-258. https://doi.org/10.1046/j.1432-0436.1995.5940253.x

[34] Ramadori, G., Saile, B., Ramadori, G. and Saile, B. (2002) Mesenchymal Cells in the Liver—One Cell Type or Two? *Liver*, **22**, 283-294. https://doi.org/10.1034/j.1600-0676.2002.01726.x

[35] Ramadori, G. (1992) The Stellate Cell (Ito-Cell, Fat-Storing Cell, Lipocyte, Perisinusoidal Cell) of the Liver. *Virchows Archiv B*, **61**, 147-158. https://doi.org/10.1007/bf02890417

[36] Kubota, H., Yao, H. and Reid, L.M. (2007) Identification and Characterization of Vitamin A-Storing Cells in Fetal Liver: Implications for Functional Importance of Hepatic Stellate Cells in Liver Development and Hematopoiesis. *Stem Cells*, **25**, 2339-2349. https://doi.org/10.1634/stemcells.2006-0316

[37] Tan, K.S., Kulkeaw, K., Nakanishi, Y. and Sugiyama, D. (2017) Expression of Cytokine and Extracellular Matrix mRNAs in Fetal Hepatic Stellate Cells. *Genes to Cells*, **22**, 836-844. https://doi.org/10.1111/gtc.12517

[38] Lee, Y., Leslie, J., Yang, Y. and Ding, L. (2020) Hepatic Stellate and Endothelial Cells Maintain Hematopoietic Stem Cells in the Developing Liver. *Journal of Experimental Medicine*, **218**, e20200882. https://doi.org/10.1084/jem.20200882

[39] Ishibashi, T., Yokota, T., Tanaka, H., Ichii, M., Sudo, T., Satoh, Y., *et al.* (2016) ESAM Is a Novel Human Hematopoietic Stem Cell Marker Associated with a Subset of Human Leukemias. *Experimental Hematology*, **44**, 269-281.E1. https://doi.org/10.1016/j.exphem.2015.12.010

[40] Ueda, T., Yokota, T., Okuzaki, D., Uno, Y., Mashimo, T., Kubota, Y., *et al.* (2019) Endothelial Cell-Selective Adhesion Molecule Contributes to the Development of Definitive Hematopoiesis in the Fetal Liver. *Stem Cell Reports*, **13**, 992-1005. https://doi.org/10.1016/j.stemcr.2019.11.002

[41] Lu, Y., Liu, M., Yang, J., Weissman, S.M., Pan, X., Katz, S.G., *et al.* (2021) Spatial Transcriptome Profiling by MERFISH Reveals Fetal Liver Hematopoietic Stem Cell Niche Architecture. *Cell Discovery*, **7**, Article No. 47. https://doi.org/10.1038/s41421-021-00266-1

[42] Schweitzer, K.M., Dräger, A.M., van der Valk, P., Thijsen, S.F., Zevenbergen, A., Theijsmeijer, A.P., van der Schoot, C.E. and Langenhuijsen, M.M. (1996) Constitutive Expression of E-Selectin and Vascular Cell Adhesion Molecule-1 on Endothelial Cells of Hematopoietic Tissues. *The American Journal of Pathology*, **148**, 165-175.

[43] Wittig, O., Paez-Cortez, J. and Cardier, J.E. (2010) Liver Sinusoidal Endothelial Cells Promote B Lymphopoiesis from Primitive Hematopoietic Cells. *Stem Cells and Development*, **19**, 341-350. https://doi.org/10.1089/scd.2009.0300

[44] Balazs, A.B., Fabian, A.J., Esmon, C.T. and Mulligan, R.C. (2006) Endothelial Protein C Receptor (CD201) Explicitly Identifies Hematopoietic Stem Cells in Murine Bone Marrow. *Blood*, **107**, 2317-2321. https://doi.org/10.1182/blood-2005-06-2249

[45] Iwasaki, H., Arai, F., Kubota, Y., Dahl, M. and Suda, T. (2010) Endothelial Protein C Receptor-Expressing Hematopoietic Stem Cells Reside in the Perisinusoidal Niche in Fetal Liver. *Blood*, **116**, 544-553. https://doi.org/10.1182/blood-2009-08-240903

[46] Subramaniam, A., Talkhoncheh, M.S., Magnusson, M. and Larsson, J. (2018) Endothelial Protein C Receptor (EPCR) Expression Marks Human Fetal Liver Hematopoietic Stem Cells. *Haematologica*, **104**, e47-e50. https://doi.org/10.3324/haematol.2018.198515

[47] Choi, Y.J., Heck, A.M., Hayes, B.J., Lih, D., Rayner, S.G., Hadland, B., *et al.* (2021) WNT5A from the Fetal Liver Vascular Niche Supports Human Fetal Liver Hematopoiesis. *Stem Cell Research & Therapy*, **12**, Article No. 321. https://doi.org/10.1186/s13287-021-02380-z

[48] Yong, K.S.M., Keng, C.T., Tan, S.Q., Loh, E., Chang, K.T., Tan, T.C., *et al.* (2015) Human $CD34^{lo}CD133^{lo}$ Fetal Liver Cells Support the Expansion of Human $CD34^{hi}CD133^{hi}$ Hematopoietic Stem Cells. *Cellular & Molecular Immunology*, **13**, 605-614. https://doi.org/10.1038/cmi.2015.40

[49] Chen, Q., Khoury, M., Limmon, G., Choolani, M., Chan, J.K.Y. and Chen, J. (2013) Human Fetal Hepatic Progenitor Cells Are Distinct From, but Closely Related to, Hematopoietic Stem/Progenitor Cells. *Stem Cells*, **31**, 1160-1169. https://doi.org/10.1002/stem.1359

[50] Peixoto, M.M., Soares-da-Silva, F., Bonnet, V., Ronteix, G., Santos, R.F., Mailhe, M.-P., *et al.* (2023) Spatiotemporal Dynamics of Cytokines Expression Dictate Fetal Liver Hematopoiesis. Preprint. https://doi.org/10.1101/2023.08.24.554612

[51] Popescu, D., Botting, R.A., Stephenson, E., Green, K., Webb, S., Jardine, L., *et al.* (2019) Decoding Human Fetal Liver Haematopoiesis. *Nature*, **574**, 365-371. https://doi.org/10.1038/s41586-019-1652-y

[52] Sugiyama, D., Kulkeaw, K., Mizuochi, C., Horio, Y. and Okayama, S. (2011) Hepatoblasts Comprise a Niche for Fetal Liver Erythropoiesis through Cytokine Production. *Biochemical and Biophysical Research Communications*, **410**, 301-306. https://doi.org/10.1016/j.bbrc.2011.05.137

[53] Ohls, R.K., Li, Y., Abdel-Mageed, A., Buchanan, G., Mandell, L. and Christensen, R.D. (1995) Neutrophil Pool Sizes and Granulocyte Colony-Stimulating Factor Production in Human Mid-Trimester Fetuses. *Pediatric Research*, **37**, 806-811. https://doi.org/10.1203/00006450-199506000-00022

[54] Kashem, S.W., Haniffa, M. and Kaplan, D.H. (2017) Antigen-Presenting Cells in the

Skin. *Annual Review of Immunology*, **35**, 469-499.
https://doi.org/10.1146/annurev-immunol-051116-052215

[55] Sigurdsson, V., Takei, H., Soboleva, S., Radulovic, V., Galeev, R., Siva, K., *et al.* (2016) Bile Acids Protect Expanding Hematopoietic Stem Cells from Unfolded Protein Stress in Fetal Liver. *Cell Stem Cell*, **18**, 522-532.
https://doi.org/10.1016/j.stem.2016.01.002

[56] Zhang, K., Zhao, H., Sheng, Y., Chen, X., Xu, P., Wang, J., *et al.* (2022) Zeb1 Sustains Hematopoietic Stem Cell Functions by Suppressing Mitofusin-2-Mediated Mitochondrial Fusion. *Cell Death & Disease*, **13**, Article No. 735.
https://doi.org/10.1038/s41419-022-05194-w

[57] Koike, H., Iwasawa, K., Ouchi, R., Maezawa, M., Giesbrecht, K., Saiki, N., *et al.* (2019) Modelling Human Hepato-Biliary-Pancreatic Organogenesis from the Foregut-Midgut Boundary. *Nature*, **574**, 112-116. https://doi.org/10.1038/s41586-019-1598-0

[58] Willnow, D., Benary, U., Margineanu, A., Vignola, M.L., Konrath, F., Pongrac, I.M., *et al.* (2021) Quantitative Lineage Analysis Identifies a Hepato-Pancreato-Biliary Progenitor Niche. *Nature*, **597**, 87-91. https://doi.org/10.1038/s41586-021-03844-1

[59] Koike, H., Iwasawa, K., Ouchi, R., Maezawa, M., Kimura, M., Kodaka, A., *et al.* (2021) Engineering Human Hepato-Biliary-Pancreatic Organoids from Pluripotent Stem Cells. *Nature Protocols*, **16**, 919-936. https://doi.org/10.1038/s41596-020-00441-w

[60] Velazquez, J.J., LeGraw, R., Moghadam, F., Tan, Y., Kilbourne, J., Maggiore, J.C., *et al.* (2021) Gene Regulatory Network Analysis and Engineering Directs Development and Vascularization of Multilineage Human Liver Organoids. *Cell Systems*, **12**, 41-55.E11. https://doi.org/10.1016/j.cels.2020.11.002

[61] Sorrentino, G., Rezakhani, S., Yildiz, E., Nuciforo, S., Heim, M.H., Lutolf, M.P., *et al.* (2020) Mechano-Modulatory Synthetic Niches for Liver Organoid Derivation. *Nature Communications*, **11**, Article No. 3416.
https://doi.org/10.1038/s41467-020-17161-0

[62] Kang, H.K., Sarsenova, M., Kim, D., Kim, M.S., Lee, J.Y., Sung, E., *et al.* (2021) Establishing a 3D *in Vitro* Hepatic Model Mimicking Physiologically Relevant to *in vivo* State. *Cells*, **10**, Article 1268. https://doi.org/10.3390/cells10051268

[63] Yanagi, Y., Nakayama, K., Taguchi, T., Enosawa, S., Tamura, T., Yoshimaru, K., *et al.* (2017) *In vivo* and *ex vivo* Methods of Growing a Liver Bud through Tissue Connection. *Scientific Reports*, **7**, Article No. 14085.
https://doi.org/10.1038/s41598-017-14542-2

[64] Ouchi, R., Togo, S., Kimura, M., Shinozawa, T., Koido, M., Koike, H., *et al.* (2019) Modeling Steatohepatitis in Humans with Pluripotent Stem Cell-Derived Organoids. *Cell Metabolism*, **30**, 374-384.E6. https://doi.org/10.1016/j.cmet.2019.05.007

[65] Takebe, T., Sekine, K., Kimura, M., Yoshizawa, E., Ayano, S., Koido, M., *et al.* (2017) Massive and Reproducible Production of Liver Buds Entirely from Human Pluripotent Stem Cells. *Cell Reports*, **21**, 2661-2670.
https://doi.org/10.1016/j.celrep.2017.11.005

[66] Wang, S., Wang, X., Tan, Z., Su, Y., Liu, J., Chang, M., *et al.* (2019) Human Esc-Derived Expandable Hepatic Organoids Enable Therapeutic Liver Repopulation and Pathophysiological Modeling of Alcoholic Liver Injury. *Cell Research*, **29**, 1009-1026.
https://doi.org/10.1038/s41422-019-0242-8

[67] Pettinato, G., Lehoux, S., Ramanathan, R., Salem, M.M., He, L., Muse, O., *et al.* (2019) Generation of Fully Functional Hepatocyte-Like Organoids from Human Induced Pluripotent Stem Cells Mixed with Endothelial Cells. *Scientific Reports*, **9**, Article No. 9820. https://doi.org/10.1038/s41598-019-45514-3

[68] Shinozawa, T., Kimura, M., Cai, Y., Saiki, N., Yoneyama, Y., Ouchi, R., *et al.* (2021) High-Fidelity Drug-Induced Liver Injury Screen Using Human Pluripotent Stem Cell-Derived Organoids. *Gastroenterology*, **160**, 831-846.E10. https://doi.org/10.1053/j.gastro.2020.10.002

[69] Mu, T., Xu, L., Zhong, Y., Liu, X., Zhao, Z., Huang, C., *et al.* (2020) Embryonic Liver Developmental Trajectory Revealed by Single-Cell RNA Sequencing in the Foxa2^{eGFP} Mouse. *Communications Biology*, **3**, Article No. 642. https://doi.org/10.1038/s42003-020-01364-8

[70] Lotto, J., Drissler, S., Cullum, R., Wei, W., Setty, M., Bell, E.M., *et al.* (2020) Single-Cell Transcriptomics Reveals Early Emergence of Liver Parenchymal and Non-Parenchymal Cell Lineages. *Cell*, **183**, 702-716.E14. https://doi.org/10.1016/j.cell.2020.09.012

[71] Saheli, M., Sepantafar, M., Pournasr, B., Farzaneh, Z., Vosough, M., Piryaei, A., *et al.* (2018) Three-Dimensional Liver-Derived Extracellular Matrix Hydrogel Promotes Liver Organoids Function. *Journal of Cellular Biochemistry*, **119**, 4320-4333. https://doi.org/10.1002/jcb.26622

[72] Takeishi, K., Collin de l'Hortet, A., Wang, Y., Handa, K., Guzman-Lepe, J., Matsubara, K., *et al.* (2020) Assembly and Function of a Bioengineered Human Liver for Transplantation Generated Solely from Induced Pluripotent Stem Cells. *Cell Reports*, **31**, Article 107711. https://doi.org/10.1016/j.celrep.2020.107711

[73] Xu, Q. (2021) Human Three-Dimensional Hepatic Models: Cell Type Variety and Corresponding Applications. *Frontiers in Bioengineering and Biotechnology*, **9**, Article 730008. https://doi.org/10.3389/fbioe.2021.730008

[74] Wang, Y., Wang, H., Deng, P., Chen, W., Guo, Y., Tao, T., *et al.* (2018) *In Situ* Differentiation and Generation of Functional Liver Organoids from Human iPSCs in a 3D Perfusable Chip System. *Lab on a Chip*, **18**, 3606-3616. https://doi.org/10.1039/c8lc00869h

[75] Michalopoulos, G.K., Bowen, W.C., Mulè, K. and Stolz, D.B. (2001) Histological Organization in Hepatocyte Organoid Cultures. *The American Journal of Pathology*, **159**, 1877-1887. https://doi.org/10.1016/s0002-9440(10)63034-9

[76] Huch, M., Dorrell, C., Boj, S.F., van Es, J.H., Li, V.S.W., van de Wetering, M., *et al.* (2013) *In Vitro* Expansion of Single Lgr5$^+$ Liver Stem Cells Induced by Wnt-Driven Regeneration. *Nature*, **494**, 247-250. https://doi.org/10.1038/nature11826

[77] Takebe, T., Sekine, K., Enomura, M., Koike, H., Kimura, M., Ogaeri, T., *et al.* (2013) Vascularized and Functional Human Liver from an iPSC-Derived Organ Bud Transplant. *Nature*, **499**, 481-484. https://doi.org/10.1038/nature12271

[78] Cotovio, J.P. and Fernandes, T.G. (2020) Production of Human Pluripotent Stem Cell-Derived Hepatic Cell Lineages and Liver Organoids: Current Status and Potential Applications. *Bioengineering*, **7**, Article 36. https://doi.org/10.3390/bioengineering7020036

[79] Charlesworth, C.T., Hsu, I., Wilkinson, A.C. and Nakauchi, H. (2022) Immunological Barriers to Haematopoietic Stem Cell Gene Therapy. *Nature Reviews Immunology*, **22**, 719-733. https://doi.org/10.1038/s41577-022-00698-0

[80] Chen, Y., Zhou, Y., Zhou, Z., Fang, Y., Ma, L., Zhang, X., *et al.* (2023) Hypoimmunogenic Human Pluripotent Stem Cells Are Valid Cell Sources for Cell Therapeutics with Normal Self-Renewal and Multilineage Differentiation Capacity. *Stem Cell Research & Therapy*, **14**, Article No. 11. https://doi.org/10.1186/s13287-022-03233-z