

Embryonic Regulation of the Mouse Hematopoietic Niche

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Hematopoietic stem cells (HSCs) can differentiate into several types of hematopoietic cells (HCs) (such as erythrocytes, megakaryocytes, lymphocytes, neutrophils, or macrophages) and also undergo self-renewal to sustain hematopoiesis throughout an organism's lifetime. HSCs are currently used clinically as transplantation therapy in regenerative medicine and are typically obtained from healthy donors or cord blood. However, problems remain in HSC transplantation, such as shortage of cells, donor risks, rejection, and graft-versus-host disease (GVHD). Thus, increased understanding of HSC regulation should enable us to improve HSC therapy and develop novel regenerative medicine techniques. HSC regulation is governed by two types of activity: intrinsic regulation, programmed primarily by cell autonomous gene expression, and extrinsic factors, which originate from so-called "niche cells" surrounding HSCs. Here, we focus on the latter and discuss HSC regulation with special emphasis on the role played by niche cells.

KEYWORDS: embryonic hematopoiesis, hematopoietic stem cell, niche

1. INTRODUCTION

After gastrulation, three germ layers emerge: endoderm, mesoderm, and ectoderm. Among them, mesoderm is the ancestor of hematopoietic cells (HCs), and both extraembryonic and intraembryonic mesoderm contribute to HC generation. In the mouse, the site of blood cell production changes as embryos develop over an approximately 20-day gestation period (Figure 1(a)). Hematopoiesis begins in the yolk sac (YS), producing mainly primitive erythroid cells at 7.5 days postcoitum (dpc). Shortly thereafter, definitive myeloid progenitor cells appear in the YS (around 8.25 dpc), which then seed the fetal liver (FL). This process, termed primitive hematopoiesis, diminishes at 12.5 dpc, when definitive hematopoiesis, which sustains the adult blood system through HSCs, begins in the FL. Although there is controversy over where HSCs are generated—in the extraembryonic mesoderm at the YS or in the intraembryonic para-aortic-splanchnopleural mesoderm (P-Sp)/aorta-gonad-mesonephros (AGM) region—recent studies suggest that both regions contain HSCs capable of reconstituting adult bone marrow hematopoiesis (Figures 1(a) and 1(b)). In addition, HSCs are generated in the placenta (PL), likely independent of the YS and P-Sp/AGM regions. Thereafter, these HSCs are thought to circulate and colonize the FL, where HSC expansion occurs. HSCs then temporarily reside in fetal spleen and finally shift to fetal bone marrow (BM) [1] (Figures 1(a) and 1(b)).

2. THE YOLK SAC

The YS is a bilaminar membranous sac encasing the embryo proper. It is here the “blood islands,” the first site of hematopoiesis. The YS is composed of two distinct layers of cells, the visceral endoderm which is predominantly an absorptive epithelium transporting nutrients to the embryo and the extraembryonic mesoderm which gives rise to the endothelium of the YS and the blood cells that fill these early vessels [2]. Developing mesoderm cells can be programmed either by the visceral endoderm or by soluble factors produced by the visceral endoderm such as Indian hedgehog (Ihh) to become hematopoietic or vascular cells [3, 4] (Figure 2). The extraembryonic mesoderm gives rise to the first hematopoietic cells in the developing conceptus. These include the primitive erythroid cells, macrophages, and megakaryocytes and appear shortly after gastrulation [5]. The primitive erythroid (EryP) cells are larger than their adult-type definitive counterparts (EryD) and express a unique set of embryonic globin genes [6]. EryP dominate the mid-gestation circulation of the mouse embryo and number in the several millions; however, by birth, they will be greatly reduced in frequency, diluted by the vast numbers of EryD generated in the FL [7, 8].

The niche supporting early hematopoietic development in the YS is poorly defined. However, recently some players in this field have been identified. YS endothelial cells and the visceral endoderm express the soluble growth factor angiopoietin 1, whereas early EryP cells express the angiopoietin receptor Tie-2. Interestingly, the EryP cells themselves express soluble growth factors [9]. TGF β 1 expression was detected in the visceral endoderm and the EryP, suggesting that the developing hematopoietic cells may be generating a unique niche themselves. The hypoxic nature of the developing YS is also supportive of hematopoiesis [9, 10]. As the YS develops, a broad range of hematopoietic progenitors are generated. These include definitive erythroid progenitors, myeloid progenitors, and lymphoid progenitors [5, 11, 12]. The migration of hematopoietic progenitors throughout the developing embryo has been investigated capitalizing on knockout mouse strains lacking a heartbeat and subsequent blood flow. These mice lacked the sodium/calcium exchanger Ncx1, failed to develop a heartbeat, and subsequently hematopoietic progenitors remained lodged at their sites of origin, namely, the YS [13] and the placenta [14]. Whether the progenitors isolated in the Ncx-1 knockout placenta are truly HSC is yet to be resolved [14]. Shortly before mid-gestation in the mouse embryo, long-term reconstituting HSCs appear in the YS [15]. These cells show significant differences compared to the HSCs generated within the embryo; however, they are capable of giving rise to the hematopoietic system of newborn mice [15, 16]. The origin of HSC has proven a contentious issue; however, the development of genetic models to monitor HSC sites of production has

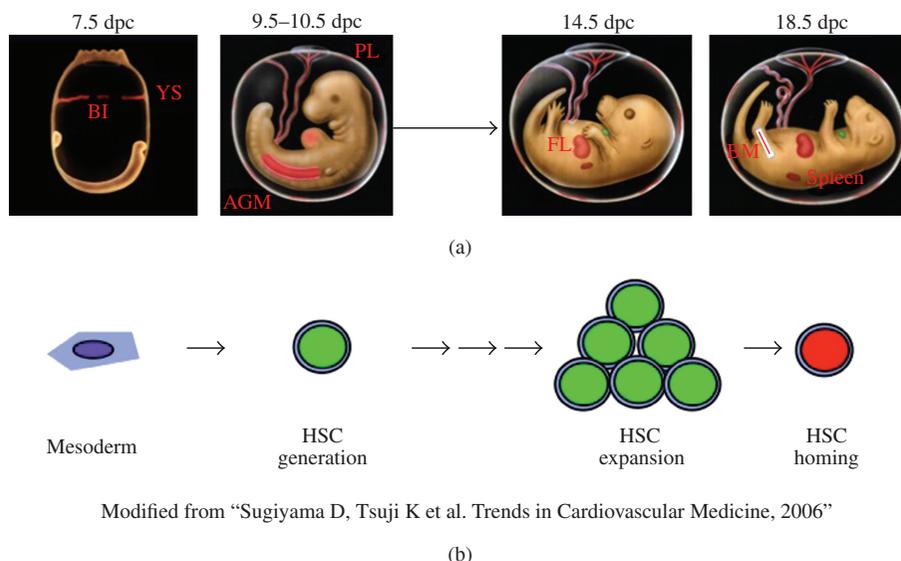


FIGURE 1: Schema of hematopoietic development during mouse embryogenesis. (a) The site of blood cell production changes as embryos develop over an approximately 20-day gestation period in mice. Abbreviations: dpc (day post coitum), YS (yolk sac), BI (blood island), AGM (aorta-gonad-mesonephros), PL (placenta), FL (fetal liver), BM (bone marrow), HSC (hematopoietic stem cell). (b) Corresponding to the time course shown in (a), HSCs emerge in the YS, AGM region, and PL from early to mid-gestation (7.0–11.0 dpc). HSCs proliferate in the FL at mid-gestation and then home to fetal spleen temporarily and to fetal BM at late gestation.

supported the YS as, at least one, source of HSC during embryogenesis. Genetic “tagging and tracing” of early hematopoietic progenitors expressing the transcriptional regulator *Runx1* in the YS suggests that this may indeed be a site of HSC production during embryogenesis [17].

3. THE AGM REGION

After YS hematopoiesis, HSC activity is detected in the p-Sp/AGM region, where cell aggregates, termed HC clusters or intra-aortic/arterial clusters (IACs), are observed. HSCs in the AGM region express *c-Kit/CD117* (the receptor for stem cell factor), *CD31/PECAM-1* (platelet/endothelial cell adhesion molecule 1), and *CD34* (a transmembrane glycoprotein). Presumptive pre-HSCs expressing *c-Kit* and capable of reconstituting neonatal recipients are detected in the p-Sp/AGM region at 9.5 dpc, whereas functional *c-Kit*-expressing HSCs capable of reconstituting adult recipients are first identified in the AGM region at 10.5 dpc. Special microenvironments likely enable pre-HSCs from the p-Sp/AGM region at 9.5 dpc to acquire functional HSC activity by 10.5 dpc. In the AGM region, IACs are frequently observed attached to floors and walls of large arteries, suggesting that they are generated from EC layers (Figure 3(a)). Mouse IACs have been characterized morphologically and are primarily located in the dorsal aorta, the omphalomesenteric artery, and the umbilical artery. Intrinsic regulation of AGM HSCs has been well characterized. The transmembrane receptor encoded by *Notch1* is essential to generate HSCs from ECs [18], and the ubiquitin ligase Mind bomb-1 (encoded by *Mib1*), which regulates Notch ligands, is important for intraembryonic hematopoiesis not only in aortic ECs but in subaortic patches [19]. *Runx1* appears to be required for formation of intra-aortic HC clusters from ECs [20]. Several reports suggest that extrinsic regulation of AGM HSCs is also important. Thrombopoietin (TPO)/*Mpl* (TPO receptor) signaling plays a role in transition from hemogenic ECs to hematopoietic progenitor cells (HPCs)/HSCs via *Runx1* activation and in production of HPCs/HSCs via *Meis1* and *Homeobox (Hox) b4* activation from 10.0 dpc to 10.5 dpc in AGM region [21]. Interleukin-1 (IL-1) reportedly functions as an important homeostatic regulator at

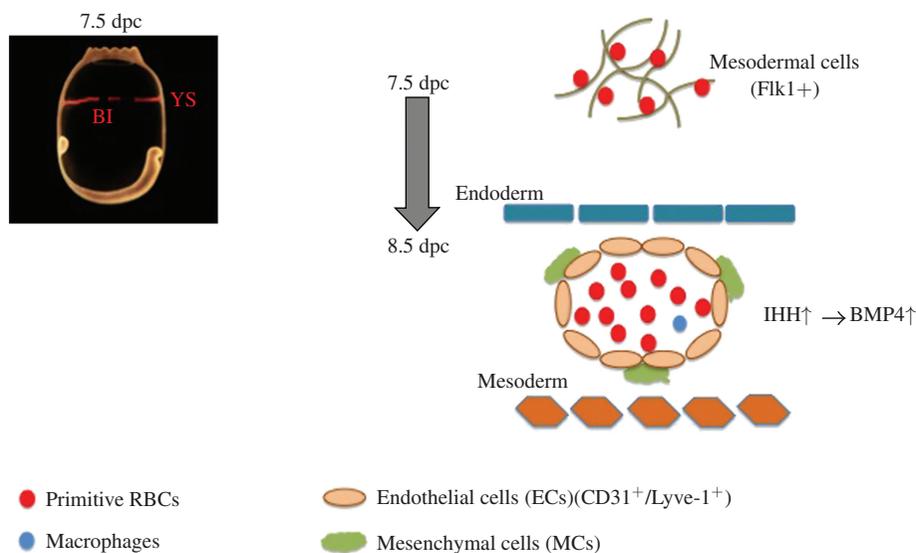


FIGURE 2: Hematopoietic environment in the YS. Mesodermal cells, the ancestors of hematopoietic cells, differentiate into both hematopoietic cells within blood islands and endothelial cells (ECs) outside of that region. Blood vessels, consisting of endothelial cells and containing primitive erythrocytes and macrophages, are located among cells originating from endodermal and mesodermal cells.

earliest stages of HSC development, limiting differentiation of some HSCs along the myeloid lineage [22] (Figure 3(b)). However, it remains unclear which factors regulate AGM HSCs. Since the AGM region consists of ECs, gonad cells, mesonephros cells, and unclassified MCs, those cells likely comprise a niche for HSC generation. *c-Kit*⁻/*CD34*⁻ MCs of the AGM region reportedly express hedgehog receptor/transducer genes, namely, *Ptch1*, *Ptch2*, and *Smo*, as well as genes encoding their downstream effectors, *Gli1*, *Gli2*, and *Gli3*, which act to increase the number of HSCs at 10.5 dpc. In addition, ECs (*CD31*⁺/*CD34*⁺/*Kit*⁻) of the AGM region at 10.5 dpc likely function in HSC regulation through stem cell factor (SCF) secretion [23]. Taken together, the niche is comprised of several factors governing HSC generation.

4. THE PLACENTA

The PL is a highly vascularized structure that functions not only in gas exchange and fetal nutrition but in hematopoiesis at 8.5–13.5 dpc [24]. Similar to the AGM region, *c-Kit*-positive HC clusters in the PL are observed in the vitelline and umbilical arteries of the placenta (Figure 4(a)). A significant proportion of HPCs, including highly proliferative potential colony forming cells (HPP-CFCs), are detected in the mouse placenta [25]. HSCs (*c-Kit*⁺/*CD34*⁺) by 11.5 dpc, and the number increase until 12.5 dpc, resulting in a 15-fold increase in HSC activity compared with that of the AGM region [26, 27]. These findings indicate that mouse PL is a likely site for HSC generation and expansion at mid-gestation. Formation of HC clusters in both the AGM region and PL is impaired in mutant mice deficient in genes encoding specific transcription factors, namely, *Runx1*, *Evi-1*, and *c-Myb*, suggesting the importance of intrinsic regulation [7, 20, 28, 29]. Relevant to extrinsic regulation of PL HSCs, *IL-3* not only is a target of *Runx1* in adult HCs, but also regulates HSC proliferation in the PL [30]. We previously demonstrated regulation of HC clusters in the PL by surrounding niche cells [23]. To do so, we used laser-capture microdissection and flow cytometry to capture niche cells. Among several cytokine genes, only *SCF* was expressed in niche cells, particularly in ECs. SCF protein expression was confirmed by ELISA and immunohistochemistry. Administration of blocking antibodies to *c-Kit*, the SCF receptor, clearly demonstrated that SCF/*c-Kit* signaling is pivotal for

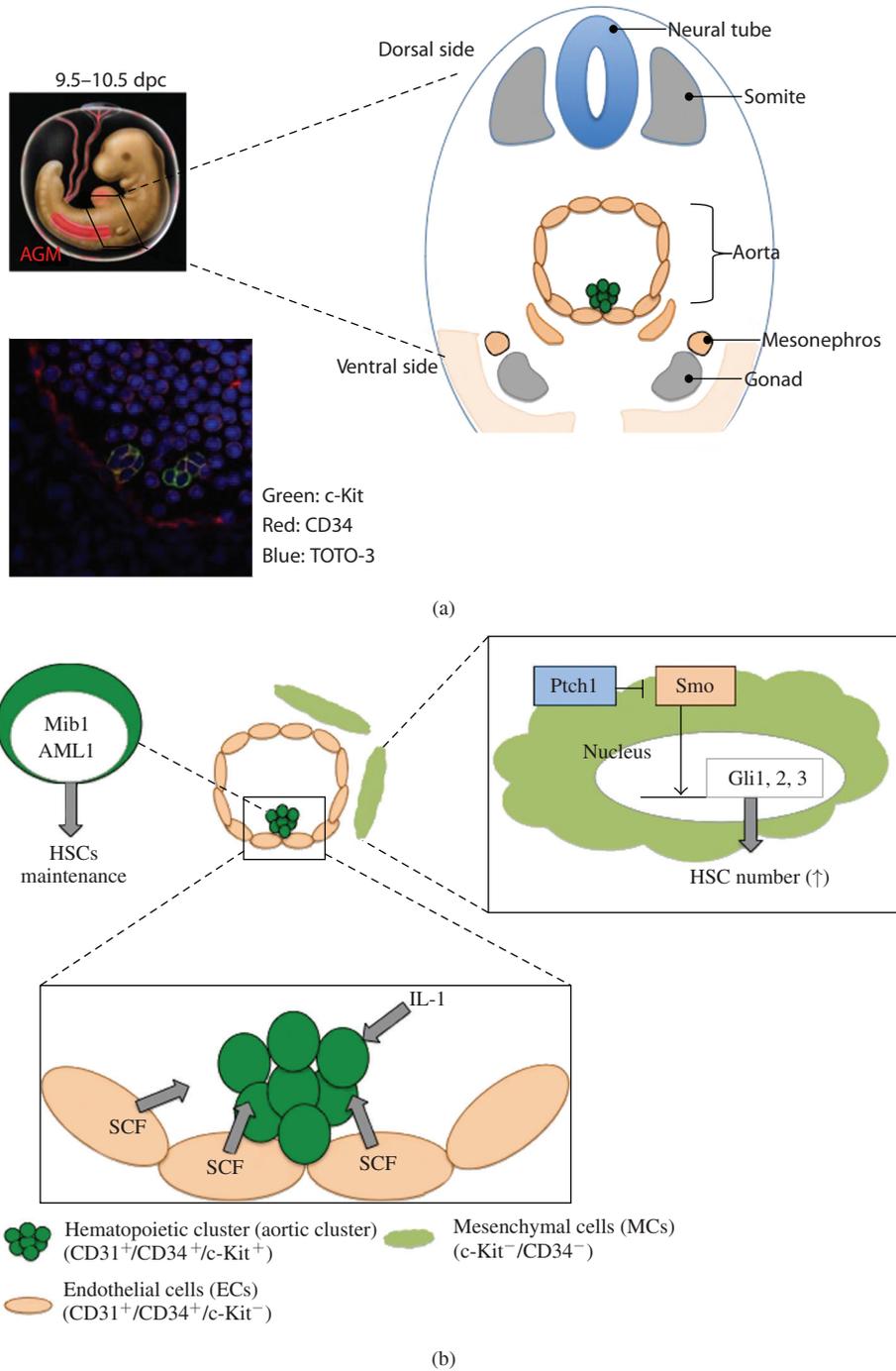


FIGURE 3: Hematopoietic environment in the AGM region. (a) (upper) Model of HC clusters, which are attached to the endothelial wall mainly on the ventral side. (lower) Confocal images of an HC cluster expressing CD34/c-Kit in the aortic region at 10.5 dpc. AGM region tissue sections were prepared from ICR mouse embryos at 10.5 dpc, stained with antibodies, and observed under confocal microscopy. CD34 (red), c-Kit (green), and TOTO-3 (blue). (b) Model of intrinsic and extrinsic regulation at the AGM region. (upper) Transcription factors (such as Runx1) maintain HSC development as intrinsic regulators. Signal transducers (such as Ptch and Smo) and transcription factor (such as Gli1, 2, 3) in mesenchymal cells maintain HSC number and development. (lower) Cytokines (SCF and IL-1) maintain HC clusters and HSC development as extrinsic regulators.

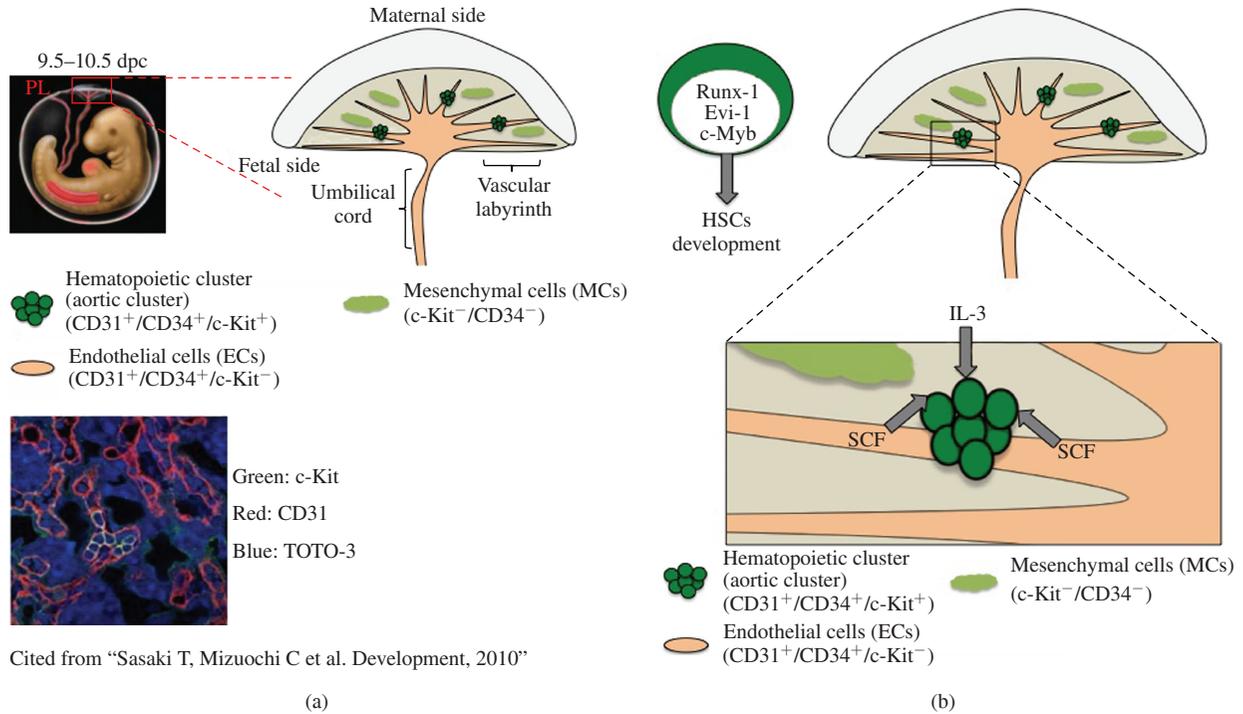


FIGURE 4: Hematopoietic environment in the placenta. (a) (upper) Model of HC clusters in the placenta. HC clusters are attached to the endothelial wall of capillary vessels, the so-called vascular labyrinth region, from 10.5 dpc to 12.5 dpc. (lower) Confocal images of an HC cluster expressing CD31/c-Kit in the placenta at 10.5 dpc. Sections of placenta were prepared from ICR mouse embryos at 10.5 dpc, stained with indicated antibodies and observed under confocal microscopy. CD31 (red), Kit (green), and TOTO-3 (blue). (b) Model of intrinsic and extrinsic regulation in the placenta. (upper) Transcription factors (such as Runx1, Evi-1, c-Myb) maintain HSC development intrinsically. (lower) Cytokines (such as SCF and IL-3) maintain HSCs as extrinsic regulators.

HSC regulation in the PL [23] (Figure 4(b)). Based on gene expression analysis, niche cells do not express *IL-3* [23], although future studies are required to clarify its function *in vivo*.

5. FETAL LIVER

Hematopoiesis occurs in fetal liver (FL) by mid-gestation [31–33]. After HSCs are generated in the YS, AGM region, and PL, they migrate into FL, where HSCs are maintained and the number of HSCs dramatically increases between 12 and 16 dpc, during which time differentiation of mature HCs, particularly erythrocytes, occurs [32–34]. Thus FL is an organ appropriate for investigation of HSC expansion and erythropoiesis *in vitro*. FL HSCs express the markers c-Kit, stem cell antigen-1 (Sca-1), and protein tyrosine phosphatase, receptor type, C (CD45, also known as common leukocyte antigen). Circulating HPCs and HSCs expressing c-Kit have been reported to colonize FL [35]. Both morphological observation and experiments *in vitro* suggest that FL itself does not produce HSCs or HPCs but may be colonized by HCs of extrinsic origin after 9.5 dpc [32, 33, 36, 37]. Intrinsic regulation of hematopoiesis in FL has also been reported. Factors that function in that activity include PU.1, an ETS family transcription factor, which functions in maintenance (differentiation) or expansion of FL HSCs [38]. Class I Hoxb4 and Hoxb3 also positively regulate HSC and HPC proliferation by accelerating the cell cycle [39]. Cited2 (a CBP/p300-interacting transactivator with a glutamic acid [E] and aspartic acid [D]-rich tail 2) maintains FL HSCs and

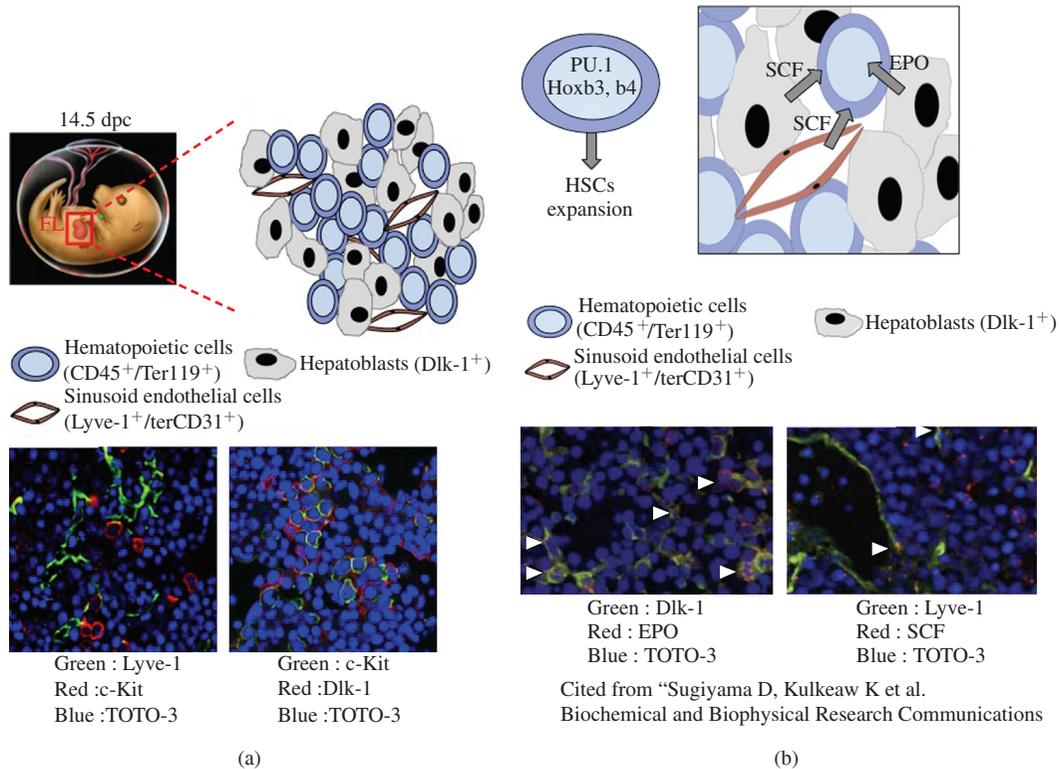


FIGURE 5: Hematopoietic environment in fetal liver. (a) (upper) Structure of fetal liver, which primarily consists of hematopoietic cells, sinusoid endothelial cells, and hepatoblasts. (lower) Confocal images of FL expressing Dlk-1/c-Kit (Right). FL sections were prepared from ICR mouse embryos at 12.5 dpc, stained with indicated antibodies, and observed by confocal microscopy. c-Kit (green), Dlk-1 (red), and TOTO-3 (blue). (b) Intrinsic and extrinsic regulation in FL. (upper) Transcription factors (specifically, PU.1, Hoxb3, and Hoxb4) and cytokines (such as SCF and EPO) play important roles in HSC expansion and differentiation in FL. (lower) Liver sections were prepared from ICR mouse embryos at 12.5 dpc and stained with Dlk-1 (green), EPO (red) and TOTO-3 (blue) (Left), and Dlk-1 (green), SCF (red), and TOTO-3 (blue) (Right).

HPCs [40]. Relevant to extrinsic regulation of FL hematopoiesis, FL consists of hepatoblasts (hepatocyte precursors), ECs, hematopoietic cells [41, 42], and other cells such as MCs (Figure 5(a)). Among them, we recently demonstrated that hepatoblasts expressing Dlk-1 function in HSC differentiation, particularly erythropoiesis, through secretion of erythropoietin (EPO), a cytokine regulating erythroid differentiation and SCF [43] (Figure 5(b)). In addition, hepatoblasts (SCF⁺DLK⁺) function at 15.5 dpc in FL to increase the number of HSCs likely through production of several cytokines, angiopoietin-like 3 (Angptl3), insulin-like growth factor-2 (IGF2), SCF, and thrombopoietin (TPO) [44]. Taken together, hepatoblasts comprise a niche for HSC regulation through cytokine secretion. However, it remains unclear how HSCs maintain self-renewal capacity under intense exposure to cytokines, which stimulate HSC differentiation. Also, in addition to cytokines, the extracellular matrix (ECM), including the interstitial matrix and basement membrane, interacts with cell surface receptors to regulate hematopoiesis extrinsically in FL. Integrins, which function as receptors for ECM, are important effectors of cell adhesion, differentiation, and migration. Particularly in FL hematopoiesis, beta-1 integrin (fibronectin receptor, CD29) is reportedly crucial for HSC colonization [45–48]. Interaction of integrin heterodimers with ECMs could function as a homing mechanism and enable HSCs and hematopoietic progenitor cells (HPCs) to reside in FL [49–51]. ECM proteins are produced in various cell types and interact with cytokines, enabling cells to interpret cytokine signaling in a particular context [52].

6. FETAL SPLEEN AND BONE MARROW

After the number of HSCs increases in the FL, those cells home to fetal-spleen, where HSCs differentiate at 13.5 to 14.5 dpc [53]. Fetal spleen-derived stromal cells enable HSCs to differentiate primarily into macrophages (F4/80⁺Mac1⁺) rather than lymphocytes. After transient hematopoiesis in fetal spleen, HSCs gradually home to BM after 16.5 dpc, where they remain during their entire lifetime. Although fetal BM is formed by 15.5 dpc, HSCs capable of self-renewal and which are positive for the signaling lymphocyte activation molecule (SLAM) family marker CD150 and negative for CD48 and CD41 cannot be detected in this tissue until 17.5 dpc, suggesting that other mechanisms govern the ability of HSCs to home to fetal BM during this period. Expression of genes encoding HSC migration factors, such as Cxcr4 (a chemokine receptor), Col4a1 (related to type IV collagen alpha1 synthesis), Mmp2, Timp2 (related to degradation and regulation of development), is significantly upregulated in HSCs during embryonic development [54].

7. CONCLUDING REMARKS

During embryogenesis, extrinsic regulation by niche cells is pivotal for HSC generation, expansion, differentiation, and maintenance. Increased knowledge of niche cell regulation should facilitate development of novel methods of *in vitro* generation, expansion, and differentiation for HSCs. Furthermore, if artificial niche components that replicate internal environment *in vivo* could maintain the status of stem cell, for example, cell cycle and quiescence, migration of both normal HSCs and leukemic stem cells might be directed as planned. The study of niche regulation of hematopoiesis should impact novel stem cell engineering, which could be applied for clinical therapies in the future.

ABBREVIATIONS

dpc: Days post coitum
 YS: Yolk sac
 BI: Blood island
 AGM: Aorta-gonad-mesonephros
 PL: Placenta
 FL: Fetal liver
 BM: Bone marrow
 HSC: Hematopoietic stem cell
 HPC: Hematopoietic progenitor cell
 HC: Hematopoietic cell.

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