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Original article

Multiple specialized stem cell niches characterize hematopoiesis in the human fetal liver

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Abstract

Before birth, different organs retain hematopoiesis. The liver is involved in the 6th-8th week of gestation. The hepatic hematopoietic stem cell (HSC) niche begins when HSCs colonize the liver and make contact with sinusoidal endothelial cells. The aim of this study is to characterize the development of each hematopoietic cell lineage in 9 human liver specimens between 10 and 29 weeks of gestation with immunohistochemistry. Hematopoietic activity was coherent with gestational age. Erythropoiesis was the greatest fraction of fetal liver hematopoiesis: myeloid/erythroid ratio was inverted compared to the adult bone marrow. Myelopoiesis was compartmentalized in the periportal zone. Factor-VIII-positive cells were small, with a megakaryoblast-like morphology. Lymphoid precursors were absent. A small quantity of T and B lymphocytes was found. CD34-reactive hematopoietic stem and progenitor cells were found in a minority of cells. The understanding of the hematopoietic process during gestation could in the future contribute to better understand child and adult hematologic conditions.

Keywords

Liver, stem cell niches, hepatic hematopoiesis, human, erythropoiesis, myelopoiesis.

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Introduction

During adult life, the site of hematopoiesis is the bone marrow, which is the only organ with the ability to produce blood cells. Before birth, however, this function is retained by different organs. Primitive hematopoiesis has been located in the yolk sac [1] and in the aorta-gonad-mesonephros region [2], which produce nucleated erythrocytes. As gestation progresses, the liver takes the upper hand in the production of blood cells. In mice, liver hematopoiesis has been reported to start from 7 to 10 days post-conception [3]. As the fetus grows, hepatic hematopoiesis gradually increases, reaches a peak, and then disappears as the bone marrow substitutes the liver as the only hematopoietic organ. In humans, the exact timing of liver hematopoiesis is still unclear. It is generally accepted that the liver becomes a site of hematopoiesis around the 6^{th}-8^{th} week of gestation, and it represents the main hematopoietic organ during gestation. Hepatic hematopoiesis can also occur during adult life. In pathological conditions, the liver is one of the sites where extramedullary hematopoiesis can arise in adulthood, thanks to the ability of the human liver to retain part of its capacity to support hematopoiesis. Liver extramedullary hematopoiesis could be used as a tool to gain more insights on the relationships that exist between hematopoietic cells and liver cells [4, 5].

The production of blood cells in the fetal liver has to be supported by a fully-fledged stem cell niche. The hepatic hematopoietic stem cell (HSC) niche is likely a highly complex one. It has been proposed that this niche is constituted when HSCs migrate from the yolk sac, colonize the liver [6], and make contact with sinusoidal endothelial cells [7]. This migration is promoted and guided by chemokines, such as CXCL10 and CXCL12, produced by sinusoidal endothelial cells [8]. Later on, the developing hepatoblasts, stellate cells and the endothelial cells that line the sinusoids produce a wide number of molecules, including the stem cell factor (CD117), erythropoietin and IL-3, that stimulate, and perhaps maintain, hematopoiesis [8, 9]. The cessation of hematopoiesis occurs as hepatoblasts gradually differentiate into hepatocytes and stop supporting HSCs [10, 11].

The phenomenon of hepatic hematopoiesis during fetal life has previously been studied with multiple techniques. Hematopoiesis in the fetal and embryonic liver has generally been observed during the most part of gestation. Different studies described its localization and morphology regarding all cell lineages that can arise from an HSC [12]. Erythropoiesis has been reported to be predominant with respect to other lineages: it is constantly organized in "erythropoietic foci" that are situated among hepatoblasts or in a perisinusoidal location [12-14]. Great investigation has also been done on myelopoiesis: indeed, it appears to be predominantly located in the stroma that surrounds large blood vessels in the portal tract. This particular localization suggests that stromal cells could specifically support granulocytopoiesis [15]. Megakaryocytopoiesis is also detectable in the fetal liver, developing megakaryocytes being usually isolated among hepatoblasts, appearing smaller than those observable in the bone marrow during adult life [16]. The effective presence of lymphopoiesis is yet to be clarified: the fetal liver has been recognized as a potential site for lymphopoiesis [17, 18], but the identification of lymphoid precursors in the fetal liver has not produced clear results [12].

Substantial attention also needs to be devoted to the changes in the hematopoietic process itself at different times of gestation. Attempts to characterize this evolution produced successful results both on murine and human models, such as the identification of four stages of hepatic hematopoiesis [19, 20]. This approach to the study of hepatic hematopoiesis could be critical to reaching a better understanding of the whole process and might give powerful insights on how it could relate to pathological processes. Thus, here we aim to further expand this knowledge by employing immunohistochemical staining techniques, both to improve the characterization of each stage of hepatic hematopoiesis and to gain more insights on how each hematopoietic cell lineage develops at different times of gestation.

Materials and methods

We obtained a total of 9 liver tissue specimens from human embryos and fetuses with a gestational age that varied between 10 and 29 weeks of gestations.

Each specimen was formalin-fixed routinely processed for histological observation and then stained with hematoxylin-eosin. In order to perform immunohistochemical analysis of every specimen, a total of seven 3μm thick sections were obtained from each paraffin block. Sections that had to be immunostained with Glycophorin A, Myeloperoxidase, Factor-VIII, CD7 and CD34 monoclonal antibodies were dewaxed, rehydrated and pretreated with a 10-minutes heat-induced epitope retrieval in buffer pH 6.00 at 95°C (EnVisionTM FLEX Target Retrieval Solution Low pH – Dako Denmark A/S, Glostrup, Denmark, Code: DM828). The staining process was completed by incubating each section at room temperature for 20 minutes with the respective anti-human monoclonal antibody. The same process was performed with sections that had to be stained with CD79a and Terminal Deoxynucleotidyl Transferase; after being dewaxed and rehydrated, these sections were however pretreated with a 10-minutes heat-induced epitope retrieval in buffer pH 9.00 at 95°C. All staining procedures were performed with the aid of the EnvisionTM FLEX+ (Dako Denmark A/S, Glostrup, Denmark, code: K8002) Detection System and the AutostainerLink 48 instrument according to the manufacturer's instructions.

All sections were then observed with an optical microscope (Leica DM2000 from Leica Microsystems) at multiple magnifications (25, 40, 200, 400 HPF) and images were obtained using a Leica DFC295 Camera and the software Leica Application Suite V4.0 (Switzerland). Hematoxylin-eosin-stained slides were evaluated to determine the hepatic hematopoiesis stage of each specimen, according to a previous work from our group [20]. The stage was then related to the gestational age. Sections stained with monoclonal antibodies were then observed in order to characterize each cell lineage and its localization in the hepatic parenchyma. Glycophorin A staining helped to identify the erythroid lineage, Myeloperoxidase to identify the myeloid lineage, Factor-VIII to identify megakaryocytes and megakaryoblasts, and CD7 and CD79a to identify the T and B lymphoid lineages respectively, Terminal Deoxynucleotidyl Transferase staining was used to identify lymphoid precursors, and CD34 staining to reveal hematopoietic stem/progenitor cells.

Results

Stage of hepatic hematopoiesis

Staining with hematoxylin-eosin permitted to identify the stage of hepatic hematopoiesis in each fetal liver specimen. Out of nine specimens, only two were graded as stage II and were both from embryos at 10 weeks of gestation. The hepatic parenchyma in three specimens showed the characteristics of both stage II and III; one of these specimens had a gestational age of 10 weeks, while the other two had an age of 12 weeks. In these cases, the parenchymal areas graded at stage III were mostly detected in the subcapsular areas, while stage II hematopoiesis was mainly located in deeper areas of the liver. Two specimens were graded as stage III, with a gestational age of 14 and 29 weeks, and two more showed a stage IV hematopoiesis, with a gestational age of 21 and 23 weeks, respectively (**Tab. 1**).

Table 1. Hepatic hematopoiesis in fetal liver specimens.

Weeks	Stage	Glycophorin A	Myeloperoxidase	Factor-VIII	CD7	Cd79a	Terminal Deoxynucleotidyl Transferase	CD34
10	II	++	++	+	+/-	+/-	-	-
10	II	++	++	+	+/-	+/-	-	+/-
10	II/III	++	++	+	+/-	+	-	+/-
12	II/III	+++	++	+	+/-	+/-	-	-
12	II/III	+++	++	+	+/-	+	-	+/-
14	III	+++	++	+	+/-	+/-	-	-
21	IV	++	++	+	+/-	+/-	-	+/-
23	IV	++	++	+	+/-	+/-	-	+/-
29	III	+++	++	+	+/-	+/-	-	+/-

+++: ≥ 75% of the hepatic parenchyma occupied by positive cells; ++: ≥ 50% of the parenchyma occupied by positive cells (for Glycophorin A only) or positive cells concentrated in specific areas; +: 5% to 10% of the parenchyma occupied by positive cells; +/-: 1% to 2% of the parenchyma occupied by positive cells; -: no observable positive cells.

Glycophorin A

Every liver specimen showed strong, but variable, reactivity for Glycophorin A. The strongest immunostaining was found in liver samples with stage III hematopoiesis, where multiple erythropoietic foci were easily identifiable. These foci presented a spherical or ellipsoidal shape and roughly measured up to 64 μm in diameter (**Fig. 1**). Glycophorin A immunostaining decreased in liver specimens at stage II and IV, coherently with the presence of smaller, linear or spherical, erythropoietic foci measuring 26 to 50 μm in diameter.

Myeloperoxidase

Staining with Myeloperoxidase helped to identify the myeloid cell lineage. Overall, myelopoiesis was at least 3 to 4 times lower than erythropoiesis. Myeloid cells were found in every liver specimen, scattered and isolated between hepatocytes or in small clusters of 2-3 cells. Moreover, greater clusters of cells reactive to Myeloperoxidase were observed in the stroma at the periphery of portal tracts (**Fig. 2**). These cells were organized in linear groups of roughly 5 to 9 cells and were relatively large (8-9 μm) (**Fig. 3**). Overall, each blood vessel in the portal tracts could be surrounded by a stroma with 10 or much more Myeloperoxidase-positive cells, depending on its size. A similar pattern of myelopoiesis was also identified in the subcapsular areas of the hepatic parenchyma, as Myeloperoxidase-positive cells were present in small clusters right below the hepatic capsule.

Factor-VIII

All liver specimens also showed cells positive to Factor-VIII, which were found isolated and scattered in the hepatic parenchyma. Factor-VIII-reactive cells were surrounded by developing hepatoblasts and showed variable dimensions. Smaller cells showed the morphological characteristics of megakaryoblasts, retaining small round nuclei filled with condensed chromatin (**Fig. 4**). Mature megakaryocytes were visible as much larger cells with lobulated nuclei and stained positive as well to Factor-VIII (**Fig. 5**). The smaller, immature cells were present in every specimen and mostly in the hepatic parenchyma of

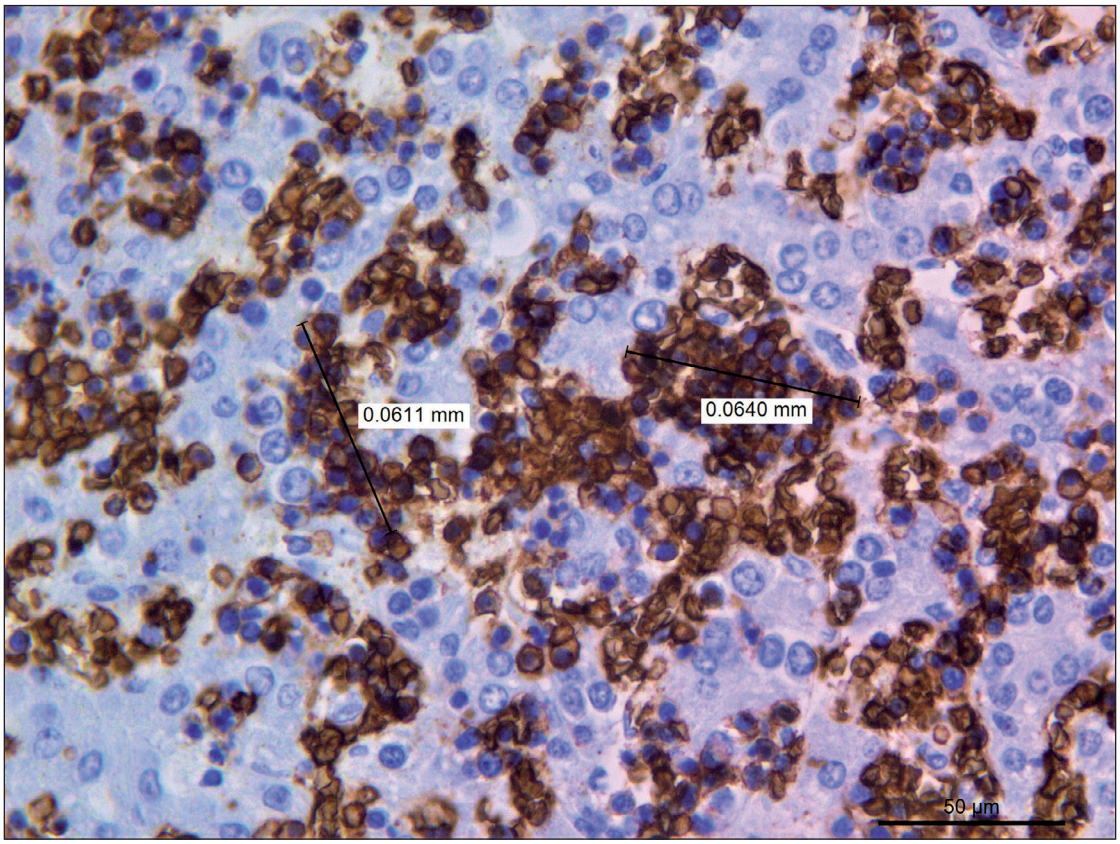

Figure 1. Erythropoietic foci dimensions during stage III hematopoiesis after Glycophorin-A staining (Glycophorin-A; 40x; scale bar 50 μm).

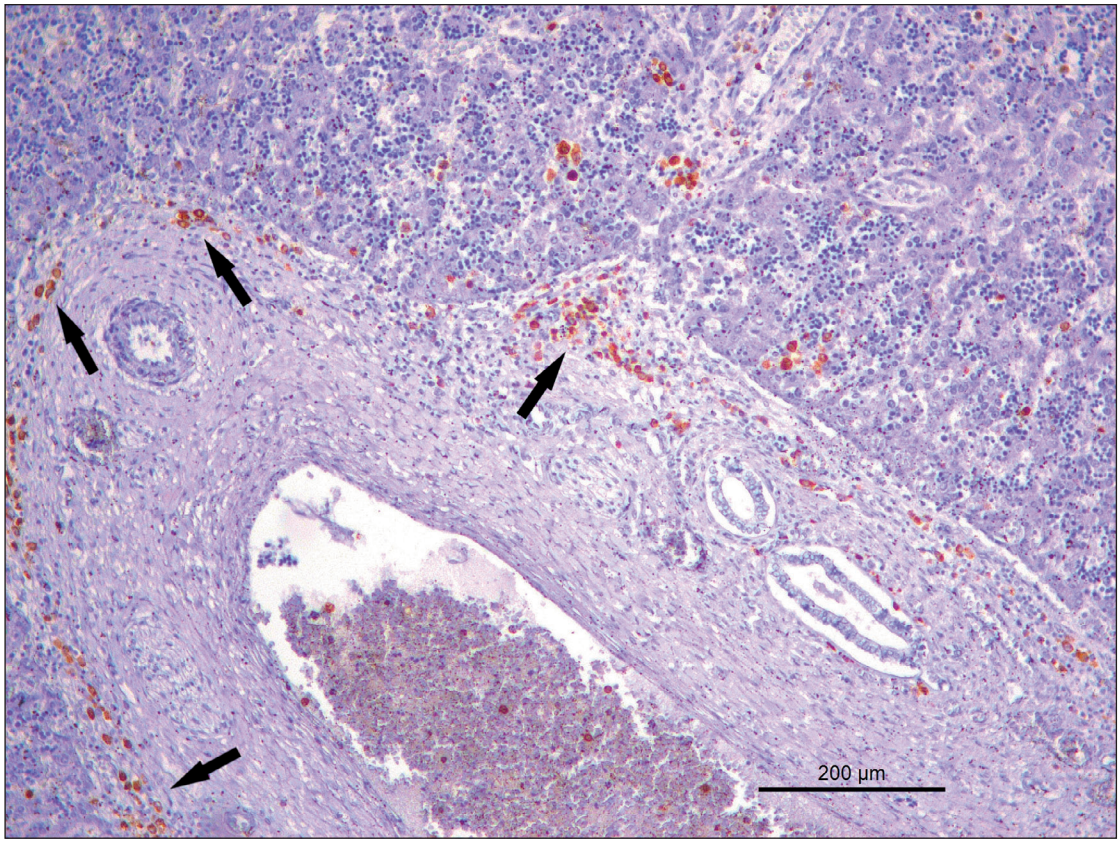

Figure 2. Arrows show Myeloperoxidase-positive cell clusters in the stroma at the periphery of a portal tract at 28 weeks of gestation (MPO; 10x; scale bar 200 µm).

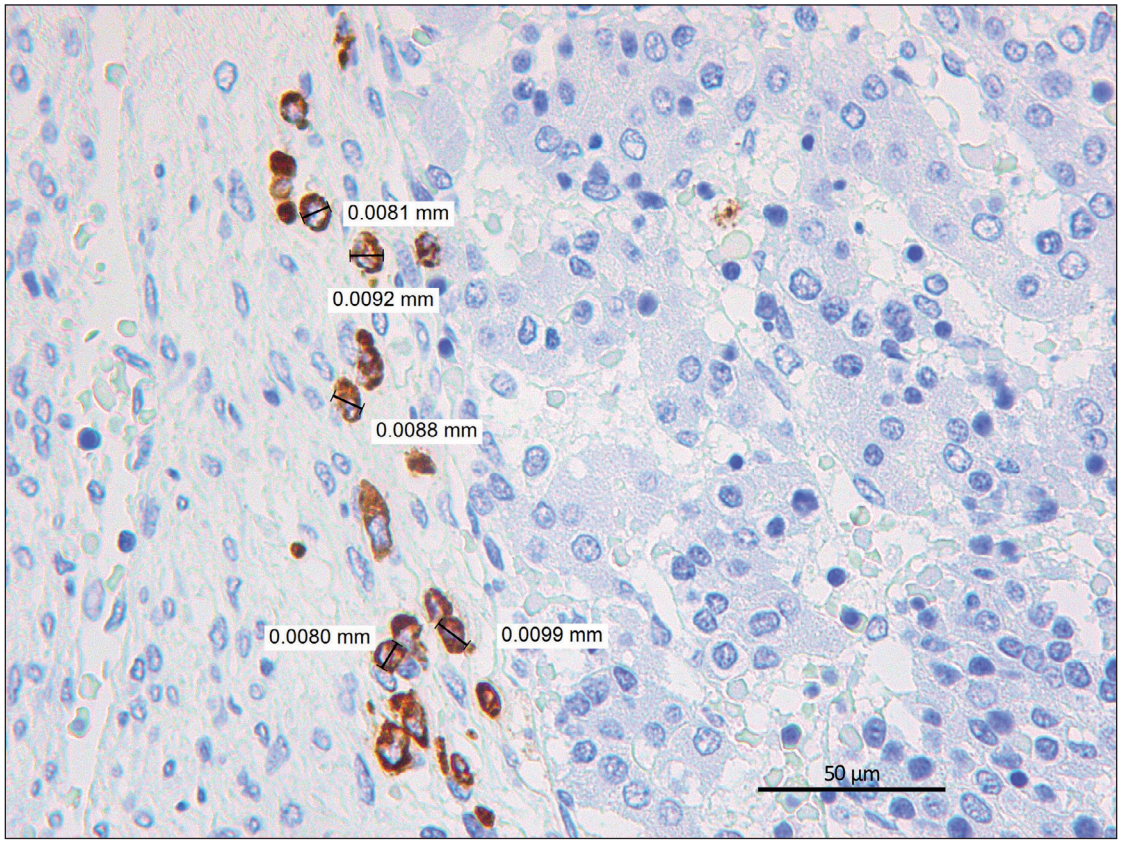

Figure 3. Myeloperoxidase-positive cells dimensions (MPO; 40x; scale bar 50 µm).

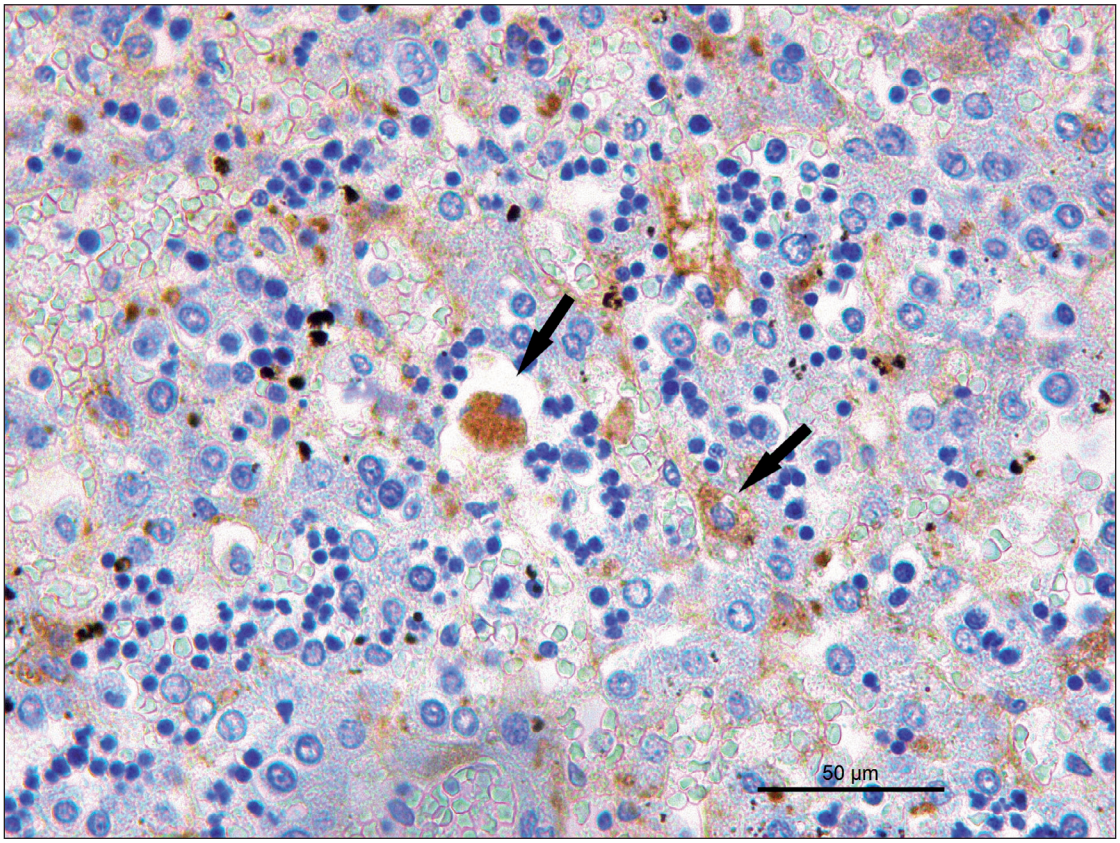

Figure 4. Arrows show Factor-VIII-positive megakaryoblasts localized in the hepatic parenchyma (Factor-VIII; 40x; scale bar 50 µm).

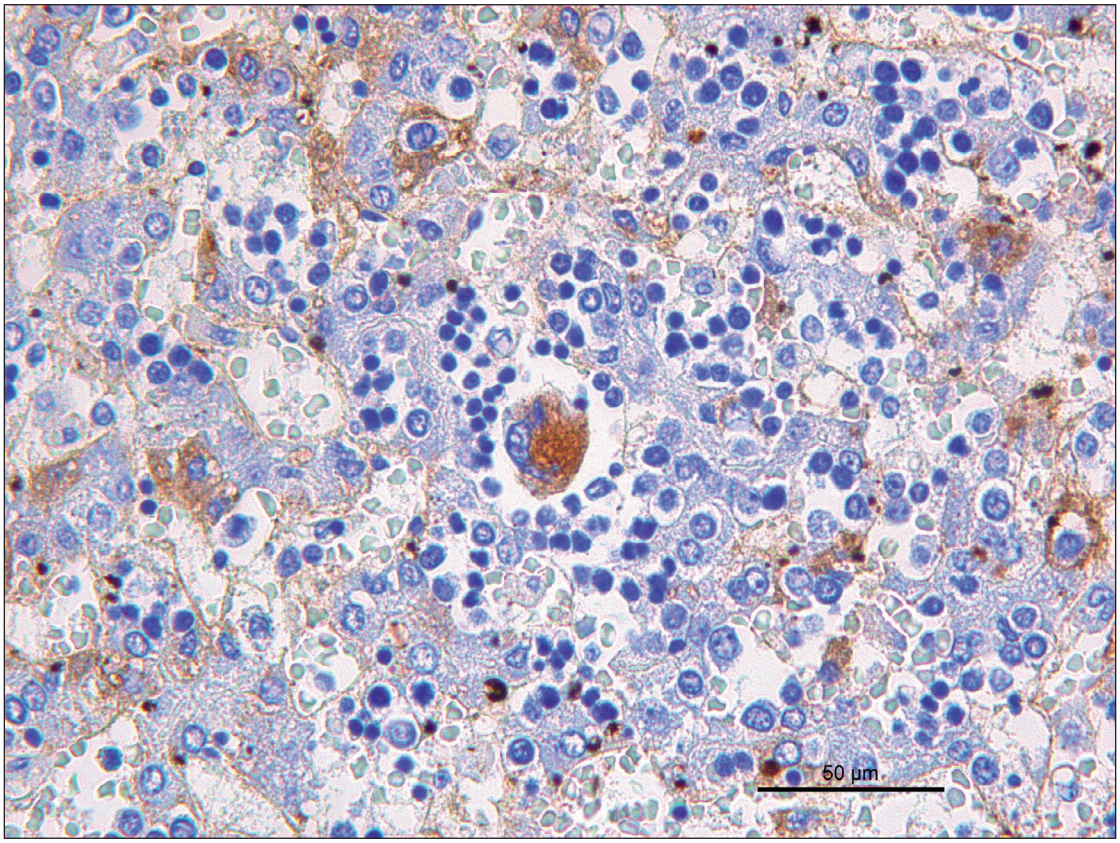

Figure 5. Factor-VIII-positive and mature megakaryocyte in the hepatic parenchyma (Factor-VIII; 40x; scale bar 50 µm).

embryos at 10 to 14 weeks of gestation. Mature megakaryocytes appeared only in specimens from fetuses at 21 to 29 weeks of gestation. Overall, however, immature megakaryocytic cells greatly surpassed in numbers the mature ones.

CD7 (T-lymphoid lineage) and CD79a (B-lymphoid lineage)

Cells positive for CD7 and CD79a were relatively scarce in every fetal liver sample observed: both types of cells were observed in the hepatic parenchyma close to hepatoblasts and almost always isolated from each other (**Fig. 6**). An exception to this pattern has been highlighted in two different fetal liver specimens with a gestational age of 10 and 12 weeks, that showed CD79a-positive cells organized in small clusters of 2-4 cells, localized both in the parenchyma and in the portal tracts (**Fig. 7**).

Terminal Deoxynucleotidyl Transferase

The marker of precursors, Terminal Deoxynucleotidyl Transferase, was found negative in all liver specimens analyzed.

CD34

In six out of nine specimens, we were able to identify CD34-positive cells. These cells appeared as isolated elements among hepatoblasts and showed no particular localization in the hepatic parenchyma (**Fig. 8**).

Discussion

Each fetal liver analyzed in this study showed a hematopoietic activity coherent with gestational age, and consistent with the length of each stage described in previous works by our group [20]. However, the overlap of different morphological patterns, representing different stages in hematopoietic activity, in fetuses from 10 to 12 weeks, suggests how liver hematopoiesis is a constantly evolving process. Also, since the time between 10 and 12 weeks of gestation represents a transitional period between stage II and stage III hematopoiesis [20], these observations could represent livers transitioning from stage II to stage III hematopoiesis. The presence of specimens at both stage III and IV hematopoiesis after 23 weeks, a time where hepatic hematopoiesis should be in

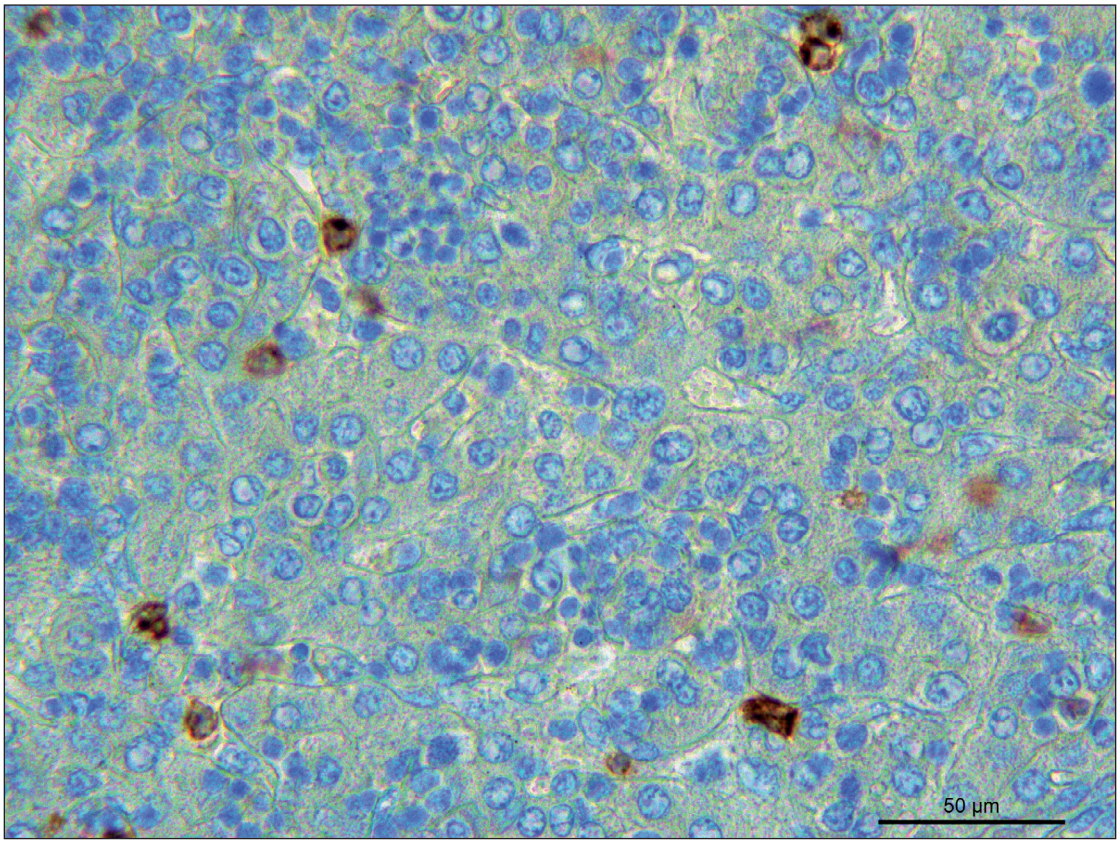

Figure 6. CD7-positive cells in the hepatic parenchyma at 21 weeks of gestation (CD7; 40x; scale bar 50 µm).

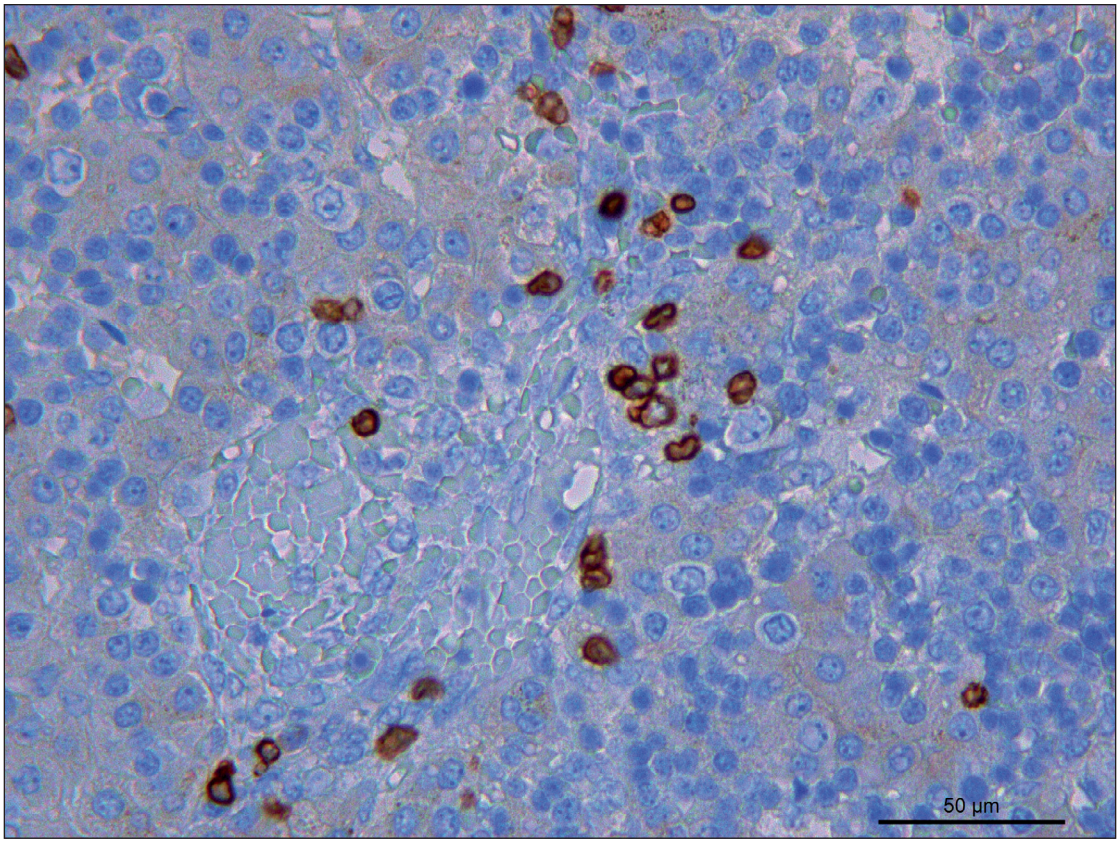

Figure 7. Clusters of CD79a-positive cells in the hepatic parenchyma of a 10 weeks embryo (CD79a; 40x; scale bar 50 µm).

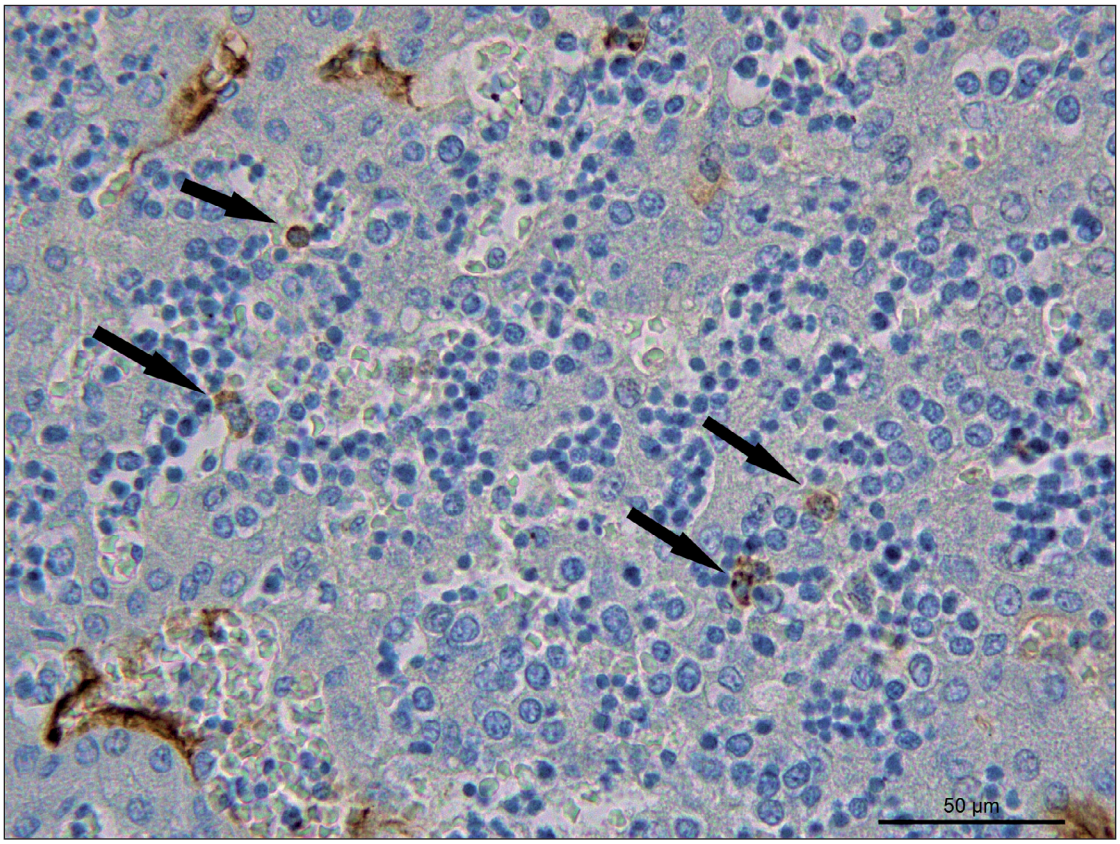

Figure 8. Arrows indicate single CD34-positive cells in the hepatic parenchyma at 21 weeks of gestation (CD34; 40x; scale bar 50 µm).

decline and likely at stage IV [20], suggests that the whole hematopoietic process in the liver could be influenced by a wide range of endogenous and exogenous factors acting on the developing fetus. These unknown factors could lead to inhibition or stimulation of the hematopoietic process as a whole, thus stimulating liver hematopoiesis to continue at higher gestational ages, or ceasing at earlier ones, with wide interindividual variability.

The degree of reactivity for Glycophorin A also closely followed the stage of hematopoiesis. Higher positivity was indeed found in specimens with a higher hematopoietic activity, which also had overall big foci dimensions. Those two findings further confirm how erythropoiesis comprises the greatest fraction of fetal liver hematopoiesis, as also suggested by other works [12, 21]. It is also of interest to note how the myeloid/erythroid ratio in the fetal liver is inverted compared to the adult bone marrow. While in the bone marrow the normal ratio is considered 3:1 or 4:1, in the fetal liver this ratio is at least 1:3, which further confirms the overall prevalence of erythropoiesis during fetal life.

As previously reported in this study, myelopoiesis was found to be highly compartmentalized. Our results are mostly consistent with older works that reported the localization of myeloid cells in the stroma of portal tracts and scattered in the liver parenchyma [14]. The periportal localization of Myeloperoxidase-positive cells supports the hypothesis that in the liver a periportal stem cell niche may exist, similar to the perivascular stem cell niche in the bone marrow [22]. This particular niche is also where mesenchymal stem and progenitor cells could reside; their ability to regulate hematopoiesis in the bone marrow has been previously proved [22]. Moreover, cells with a morphology comparable to that of mesenchymal stem/progenitor cells have already been identified in the fetal liver in close contact with developing granulocytes [23]. Since Myeloperoxidase-positive cells were also identified in the hepatic parenchyma and in a subcapsular position, two other different HSC niches, with the aim of producing myeloid cells, could be present in the fetal liver. The hypothesis of multiple hematopoietic niches in the fetal liver surely requires further study. However, as already proposed by our group [23], these myeloid cells located in different sites of the fetal liver could represent different cells of the myelomonocytic lineage, such as granulocytic-only or monocytic-only developing cells, supported by substantially different niches in their development.

The presence of megakaryocytes in the fetal liver was to be expected, as they produce platelets for the developing fetus; the smaller Factor-VII-reactive cells represent megakaryoblasts. The finding that, in all specimens, most Factor-VIII-positive cells were small, with a megakaryoblast-like morphology, could, however, be of interest. Since Factor-VIII-positive cells with a mature morphology appeared only at advanced gestational ages, the more numerous megakaryoblasts should be able to still produce platelets during gestation, achieving a physiological platelet count in the fetus. As suggested by other works, these cells are indeed able to produce platelets, but they could represent diploid instead of polyploid megakaryocytes [24]. This could explain the characteristic morphology of these cells. The fact that these cells appear smaller, and with a non-lobulated nucleus, is likely determined by the fact that they have not gone through endomitosis, required during megakaryocyte maturation. It also seems that these kinds of cells are able to produce fewer platelets than their mature counterparts and that these platelets present altered functions [25]. Further study on liver megakaryopoiesis is certainly needed, yet these findings are already coherent with the fact that preterm neonates retain dysfunctional platelets in their circulating blood as they are born [26-28].

The absence of lymphoid precursors that stained with Terminal Deoxynucleotidyl Transferase could either mean that those cells were too few to be identifiable or were not present in the fetal liver at all. It may seem then, that the liver has no role in fetal lymphopoiesis. As reported, however, a small but still relevant quantity of T and B lymphocytes was found, suggesting that B and T lymphocytes could either make their way to the liver at a certain time or be produced in the liver itself. The presence of cell clusters of the B-lymphoid lineage in the portal stroma is, however, a clue that a certain degree of B lymphopoiesis occurs in the human fetal liver, supported by the fact that B lymphopoiesis in the liver has already been observed [12, 29, 30].

Surprisingly, CD34-reactive hematopoietic stem and progenitor cells could not be found in every specimen. This finding contrasts with previous studies evidencing CD34 reactivity in the developing human liver at 7 weeks of gestation [20, 31] and confirms the hypothesis that CD34 positive cells might represent the sign of very primitive (early) hematopoiesis in

the human liver. Given the overall good degree of hematopoiesis in every specimen, they should have been present in all fetal livers. However, it should be noted that probably not all hematopoietic stem and progenitor cells express the CD34 antigen and that these cells could be equally able to give rise to cells from all lineages [32, 33]. It could then be possible that the fetal liver hematopoietic niches host slightly different populations of HSCs, with different hematopoietic abilities.

Overall, the study of hepatic hematopoiesis during the intrauterine life with immunohistochemical techniques helps to gain further knowledge on how each cell lineage behaves during its development in the human liver. It simultaneously reveals the complexities of the hematopoietic process during gestation. More research is certainly needed to further verify previous findings. At the same time, hepatic hematopoiesis could be an efficient tool to better understand the functioning of HSC niches in the bone marrow, as some similarities between hematopoiesis in the two organs surely exist. Furthermore, a deeper understanding of the hematopoietic process during gestation could in future contribute to better understand hematologic disorders in at-term and preterm neonates.

Declaration of interest

The Authors declare that there is no conflict of interest.

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