CD117^{hi} expression identifies a human fetal hematopoietic stem cell population with high proliferation and self-renewal potential

Fetal hematopoiesis occurs in multiple embryonic niches during development: after their emergence in the aorta/gonad/mesonephros (AGM) region, hematopoietic stem cells (HSC) migrate to the fetal liver (FL) where they actively proliferate and expand, before fetal bone marrow (BM) colonization.[1,2] In contrast, adult hematopoiesis takes place primarily in BM, and adult HSC are quiescent.[3] At the moment, very little is known about the changes in cell surface markers and regulators of HSC amplification and self-renewal occurring during human FL development: we described a transient CD34$^+$CD45$^+$CD144$^+$ HSC population in the first trimester (T1) FL,[4] and CD90/GPI-80 and CD143 were shown to allow HSC enrichment in the second trimester (T2) FL.[5,6] However, no surface marker allowing HSC enrichment of CD34$^+$CD38$^-$ population both in T1 and T2 FL has been described so far. Deciphering regulatory mechanisms that control HSC expansion and self-renewal during FL development could provide important clues to expand HSC for clinical use, and to highlight new fetal signatures which persist abnormally during childhood, leading to pediatric leukemias.[7]

We focused on CD117 (c-KIT), widely used in the mouse to isolate HSC-enriched populations in the AGM, FL and placenta during development,[8,9] and in adult bone marrow.[10] In humans, CD117 also marks the first HSC in the AGM,[11] and the SCF/KIT signaling pathway regulates self-renewal properties of fetal HSC and maintenance of quiescent adult HSC, with many downstream effectors identified as important regulators of the change of HSC proliferative and self-renewal behavior during development.[12] Using CD34$^+$CD38$^-$ as the referent HSC-enriched population,[2] we sorted CD34$^+$CD38$^-$CD45$^+$CD117^{hi} cells from T1 and T2 FL, and showed that CD117^{hi} expression allows isolation of an HSC-enriched population with high proliferation and self-renewal potential during hepatic hematopoiesis in human development.

We first looked for CD117^{hi} expression in the CD34$^+$CD38$^-$ fraction of T1 FL. Since CD34 also marks endothelial cells, we added CD45 to ensure to be looking at the hematopoietic population. As shown in Figure 1, a CD34$^+$CD38$^-$CD45$^+$CD117^{hi} (hereafter called CD117^{hi}) population could be detected as early as 6 weeks of gestation (WG) in T1 FL. It represents 0.35±0.07% of mononuclear cells between 6 and 9 WG and 0.21±0.05% between 9 and 12 WG. This CD117^{hi} population is still present in the T2 FL (0.15±0.07%) (Figure 1). Fluorescence-activated cell sorting (FACS) analysis showed that the CD117^{hi} population from T1 or T2 FL are CD45RA$^-$[13] and did not overlap with the described populations enriched in HSC during FL development or in the umbilical cord blood, such as the CD144^{lo}CD45^{lo},[4] the CD90$^+$GPI80$^+$,[5] and the CD34$^+$CD143$^+$[6] populations,

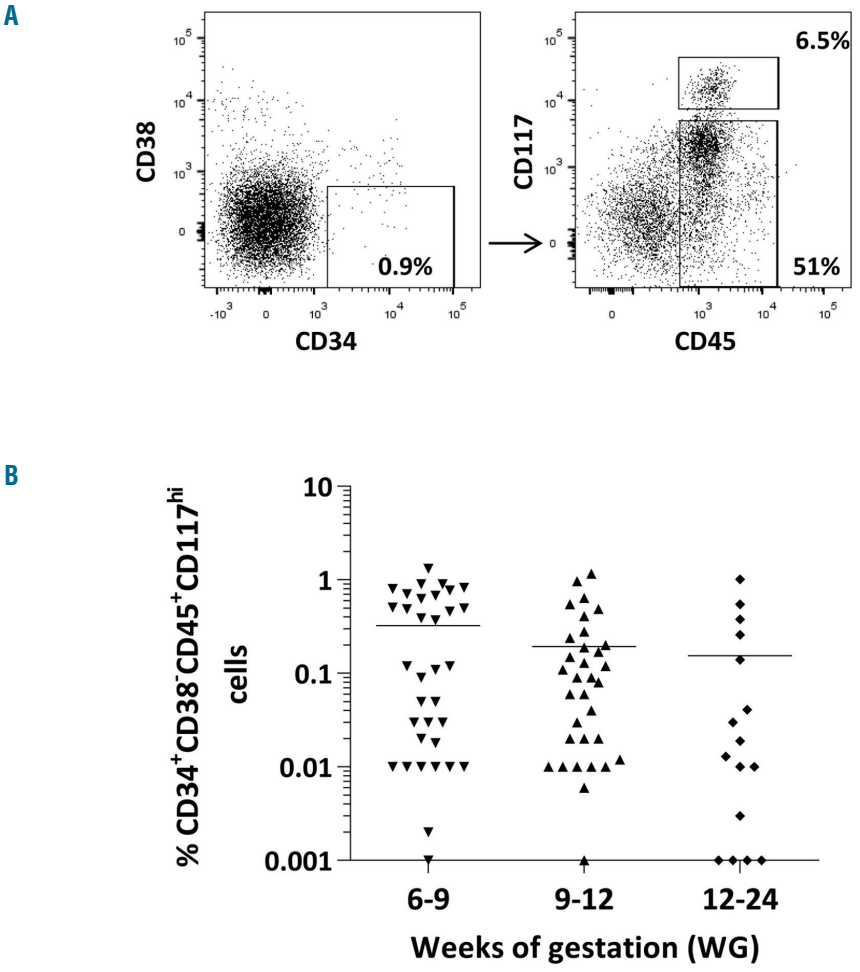
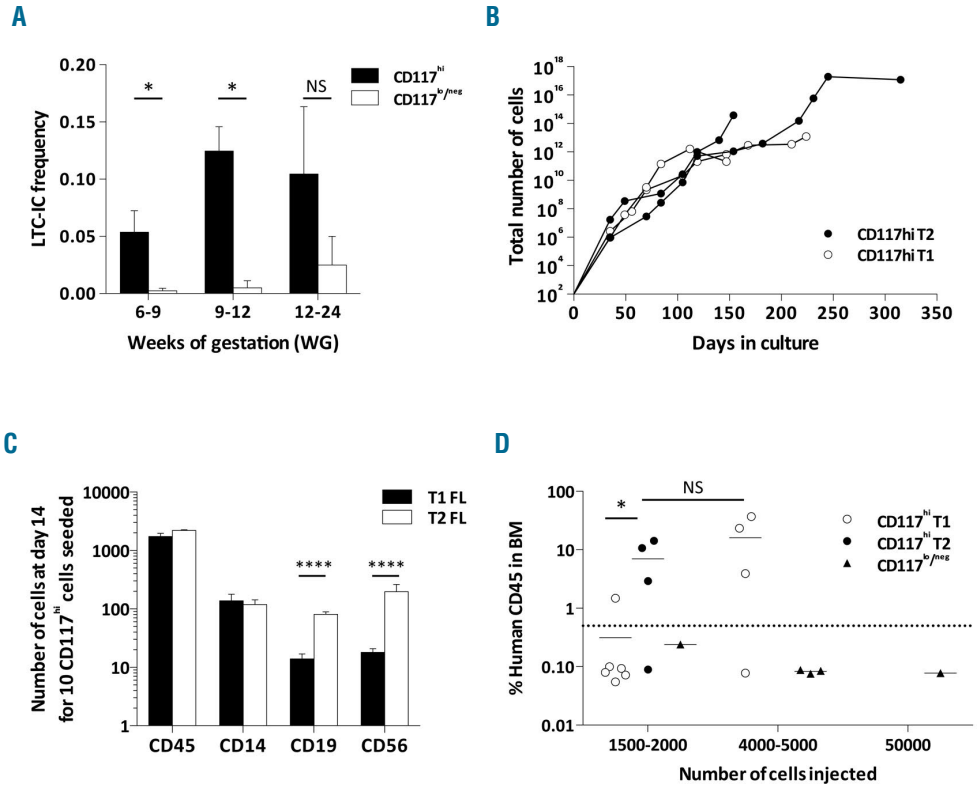


Figure 1. Presence of CD117^{hi} population during human fetal liver (FL) development. (A) Gating strategy to sort CD34$^+$ CD38$^-$ CD45$^+$ CD117^{hi} (CD117hi) population from a first trimester (T1) FL (9 weeks of gestation - WG), as a representative example. (B) FACS analysis of T1 (6-9 WG, n=31; 9-12 WG, n=30) and second trimester (T2) (12-24 WG, n=16) FL samples for the presence of CD117^{hi} cells. Percentage of CD117^{hi}cells represents the % of total FL mononuclear cells.

or CD90^{+}CD49f^{+}[14] (*Online Supplementary Figure 1*).

Long term culture – initiating cell (LTC-IC) assays were performed to check whether the CD117^{hi} population is enriched in hematopoietic stem and progenitor cells (HSPC). LTC-IC frequency of CD117^{hi} population is already high at 6-9 WG (0.053±0.024), increases at 9-12 WG (0.119±0.038) and during T2 (0.148±0.097) (Figure 2A). The CD117^{hi} LTC-IC frequency is always higher than the CD34^{+}CD38^{-}CD45^{+}CD117$^{lo/neg}$ (CD117$^{lo/neg}$) LTC-IC frequency whatever the stage of FL development studied (Figure 2A). An extended LTC-IC assay demonstrated that the T1 or T2 FL CD117^{hi} population produced cobblestone area forming cells (CAFC) and hematopoietic cells (HC) for at least five months, with an important amplification of hematopoietic progeny (1.10^{11} to 1.10^{18}) (Figure 2B). In contrast, the CD117$^{lo/neg}$ population was exhausted within one month. In addition, *in vitro* lympho-myeloid differentiation assay showed that CD117^{hi} population from T1 or T2 FL was able to give rise to both myeloid and lymphoid progeny, with an increase in lymphoid production at T2 (Figure 2C). Therefore, CD117^{hi} population presents HSC features *in vitro*. To further confirm this high proliferative and self-renewal property, we tested the *in vivo* capacity of CD117^{hi} cell population to generate long-term multilineage grafts into NOD SCID gamma (NSG) immunodeficient mice. Then 1500 to 5000 sorted CD117^{hi} cells and 5000 to 50000 sorted CD117$^{lo/neg}$



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Human FL stage	PRIMARY RECONSTITUTION	SECONDARY RECONSTITUTION		
	% hCD45 cells in BM	Mouse #	Number of hCD45^{+} cells injected (x10^{6})	% hCD45 cells in BM
T1	23.3	1	2	3.4
	37	2	2	23.6
T2	10.7	1	2	0.9
	14.2	2	2	16.8

Figure 2. FL T1 and T2 CD117^{hi} population presents *in vitro* and *in vivo* features of hematopietic stem cells (HSC). (A) *In vitro* comparison of long term culture – initiating cell (LTC-IC) frequency of CD117^{hi} cells with CD117$^{lo/neg}$ cells isolated from T1 FL (6-9 WG, n=4 and n=2, respectively; and 9-12 WG, n=3 and n=3, respectively) and T2 FL (n=6 and n=3, respectively). LTC-IC frequencies were evaluated after coculture of serial half-dilutions of sorted cells on MS-5 stroma. (B) Total number of hematopoietic cells (HC) obtained after extended LTC-IC (e-LTC-IC) cultures on MS-5 of CD117^{hi} cells from T1 (n=2, white circles) and T2 FL (n=2, black circles). (C) Lympho-myeloid differentiation assay. Total number of CD45^{+}, CD14^{+} (myeloid), CD19^{+} (B-lymphoid) and CD56^{+} (NK) cells obtained from 10 CD117^{hi} cells seeded after 14 days of co-culture, from T1 (n=3, white bars) and T2 (n=3, black bars) FL. (D) Long-term hematopoietic reconstitution of NSG mice by CD117^{hi} cells. 1500 to 2000 (T1 and T2 FL), and 4000 to 5000 (T1 FL) CD117^{hi} cells were injected into sublethally irradiated NSG mice, and mice were tested for the presence of human CD45^{+} cells in their BM 16 to 20 weeks after transplantation. CD117$^{lo/neg}$ cells from T2 FL (1500 to 2000, 4000 to 5000 and 50000 cells) were also injected as controls. (E) Secondary reconstitution of sublethally irradiated NSG with primary bone marrow from NSG injected with FL T1 (n=2) and FL T2 (n=2) CD117^{hi} cells. *P<0.05.

cells were injected intravenously, and BM human chimerism was analyzed by flow cytometry after 16 to 20 weeks. As shown in Figure 2C, T1 and T2 CD117^{hi} cells gave rise to long-term engraftment into NSG mice even when as little as 1500-2000 cells were injected, while no engraftment could be observed with CD117$^{lo/neg}$ cells. When 1500-2000 cells were injected, T2 FL CD117^{hi} cells were more potent than T1 FL CD117^{hi} (3 out of 4 NSG engrafted with a mean engraftment of 7% for T2, compared to 1 out of 6 NSG with 1.5% engraftment for T1), probably due to a higher HSC frequency in CD117^{hi} cells from T2 FL, as suggested by LTC-IC frequency. Yet,

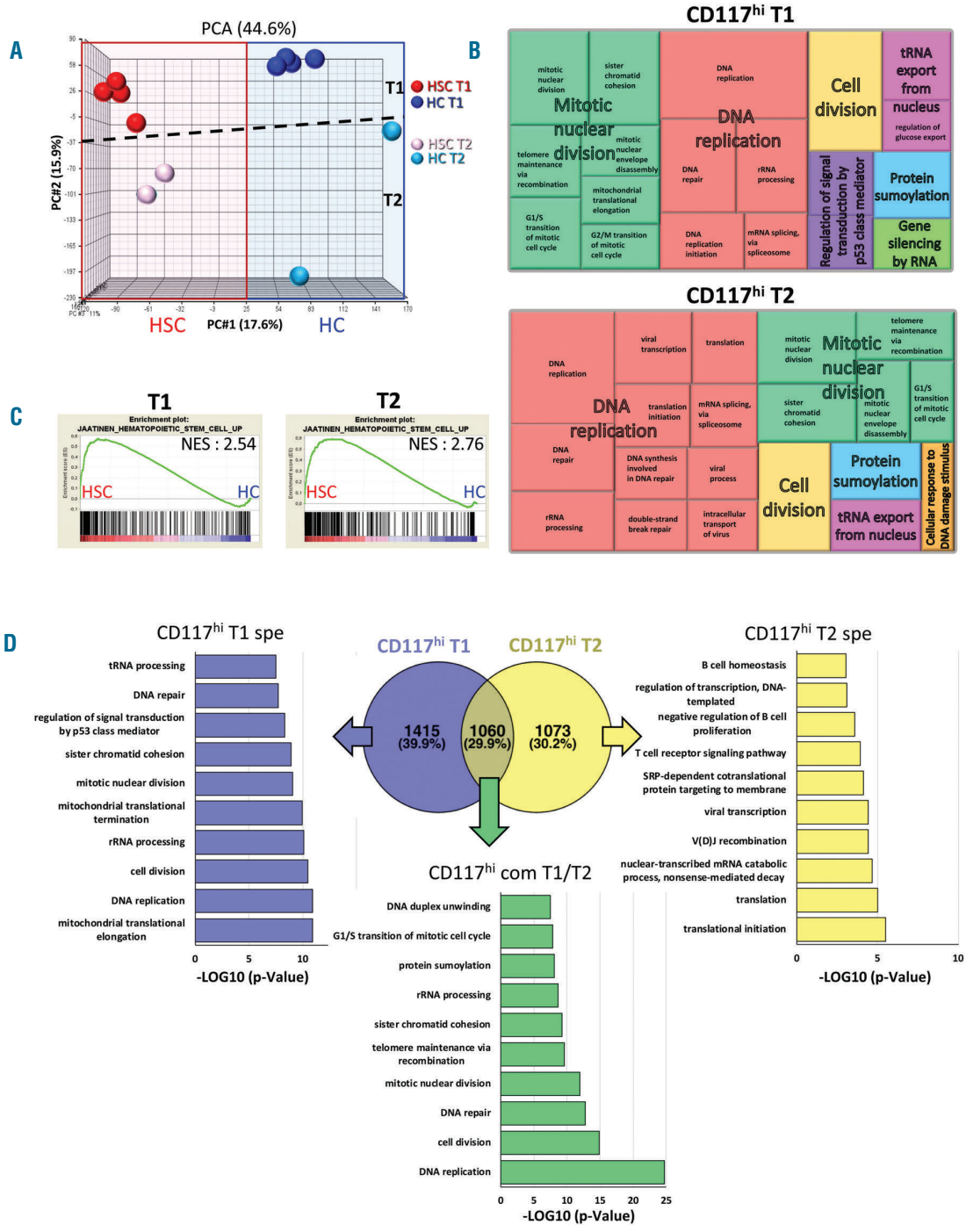

Figure 3. T1 and T2 FL CD117^{hi} cells exhibit common biological processes (BP) associated with proliferation and division and HSC molecular signature. (A) Principal composant analysis (PCA) of the whole gene expression set of sorted T1 and T2 CD117^{hi} (HSC) and CD117$^{lo/neg}$ (HC) populations. Each ball represents one sorted cell population. (B) REVIGO representation of the top 20 enriched gene ontology (GO) BP terms for CD117^{hi} HSC up-regulated differentially expressed genes (DEG) in T1 (upper panel) and T2 (lower panel) FL. GO enrichment analysis was performed using DAVID (database used for annotation, visualization and integrated discovery) (see the *Online Supplemental Table S4* for GO terms). Each rectangle is a single cluster representative, and representatives are joined into "superclusters". The size of the rectangles reflect the *P*-value of GO terms. (C) Gene set enrichment analysis (GSEA) for HSC. NES = normalized enrichment score. (D) Venn diagram of T1 *versus* T2 CD117^{hi} up-regulated genes and top 10 enriched GO BP for genes common for T1 and T2 (green), specific for T1 (blue) and specific for T2 (yellow).

a good level of engraftment of T1 CD117^{hi} FL cells was obtained when 4000-5000 cells were injected (3 of 4 NSG, mean engraftment 16%). All reconstituted mice presented multilineage hematopoietic engraftment in their BM, with the presence of human CD45^{+} HC in the spleen and thymus (*Online Supplementary Figure 2A*). Interestingly, 50±6.4% of human CD34^{+}CD38^{-} HSPC present in the BM were still CD117^{hi} (*Online Supplementary Figure 2A*). Secondary transplants of human CD45^{+} BM cells from primary NSG mice demonstrated that whether isolated from T1 or T2 FL, CD117^{hi} cells could be serially transplanted (Figure 2D). BM multilineage engraftment was also observed, with 16±9% CD117^{hi} cells still present in the CD34^{+} CD38- fraction (*Online Supplementary Figure 2B*). All of these results indicate that T1 and T2 FL CD117^{hi} cells present characteristic features of HSC with extensive self-renewal potential.

To further characterize this novel CD117^{hi} population of HSC from T1 and T2 FL, we performed transcriptomic analysis. CD117^{hi} and CD117$^{lo/neg}$ populations were sorted from T1 and T2 FL to perform a pair-wise comparison between HSC and HC. Principal component analysis (PCA) using cell populations as observations and the whole gene expression set (29 413 genes) as variables showed that first principal component (PC1) corresponded to the contrast between CD117^{hi} HSC and CD117$^{lo/neg}$ HC, while the second principal component (PC2) corresponded to the contrast between T1 and T2 (Figure 3A).

The pair-wise comparison of CD117^{hi} and CD117$^{lo/neg}$ transcriptomes demonstrated 2475 and 2133 differentially expressed genes (DEG) in CD117^{hi} HSC in T1 and T2 FL, respectively (one-way ANOVA, *P*-values <0.05, fold-change >1.5). Gene ontology (GO) revealed that both T1 and T2 FL CD117^{hi} HSC up-regulated DEG are enriched in biological processes (BP) involved in cell proliferation, such as DNA replication, mitotic nuclear division and cell division (Figure 3B and *Online Supplementary Table 4*). Gene set enrichment analysis (GSEA) confirmed these BP (*Online Supplementary Figure 3*), and additionally showed that both T1 and T2 CD117^{hi} specific sets of genes were also enriched in "Jaatinen hematopoietic stem cells UP" (Figure 3C) (126 and 150 genes out of 294 for T1 and T2, respectively). Among genes shared by T1 and T2 CD117^{hi}, key regulators involved in HSC development and maintenance were up-regulated (*Online Supplementary Table 5*). These data were confirmed by RT-qPCR for *MYB, HLF, PROM1* and *ANGPT1* (*Online Supplementary Figure 4A*).

To further investigate molecular differences between FL CD117^{hi} HSC at T1 and T2, we performed a Venn diagram of T1 *versus* T2 DEG. As shown in Figure 3D, 1060 genes were common for T1 and T2 CD117^{hi} HSC, while 1415 were specific for T1 CD117^{hi} HSCs and 1073 for T2 CD117^{hi} HSC (Figure 3D). GO analysis showed that T1 and T2 common genes as well as T1 CD117^{hi} specific genes were highly enriched in DNA replication, cell division and DNA repair BP, while T2 CD117^{hi} specific genes were linked to BP associated with lymphopoiesis and translation (Figure 3D and *Online Supplementary Table 6*). This latter observation corroborates the results of *in vitro* lympho-myeloid differentiation of T2 CD117^{hi} HSC (Figure 2C). RT-qPCR analysis of a few genes from the major histocompatibility complex, such as *HLA-DOA, HLA-DQA1* and *CIITA*, and from T-cell differentiation/activation pathways such as PIK3CD[15] and RUNX2[16] confirmed that they were expressed specifically in T2 CD117^{hi} cells (*Online Supplementary Figure 4B*).

In summary, our study highlights for the first time a marker allowing HSC enrichment of CD34^{+}CD38^{-} cells during human FL development. The CD117^{hi} HSC population presents a high amplification and a long-term self-renewal ability that could help to isolate key genes for *in vitro* maintenance and expansion of HSC. Using this marker to sort HSC from a Fanconi T2 FL, we could report for the first time that a profound HSC defect is already observed during human fetal development, accompanied by a decreased expression of important HSC transcriptional regulators and markers.[17]

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References

1. Ivanovs A, Rybtsov S, Ng ES, Stanley EG, Elefanty AG, Medvinsky A. Human haematopoietic stem cell development: from the embryo to the dish. Development. 2017;144(13):2323-2337.
2. Oh IH, Lau A, Eaves CJ. During ontogeny primitive [CD34(+) CD38(-)] hematopoietic cells show altered expression of a subset of genes associated with early cytokine and differentiation responses of their adult counterparts. Blood. 2000;96(13):4160-4168.
3. Babovic S, Eaves CJ. Hierarchical organization of fetal and adult hematopoietic stem cells. Exp Cell Res. 2014;329(2):185-191.
4. Oberlin E, Fleury M, Clay D, et al. VE-cadherin expression allows identification of a new class of hematopoietic stem cells within human embryonic liver. Blood. 2010;116(22):4444-4455.
5. Prashad SL, Calvanese V, Yao CY, et al. GPI-80 defines self-renewal ability in hematopoietic stem cells during human development. Cell Stem Cell. 2015;16(1):80-87.

6. Sinka L, Biasch K, Khazaal I, Peault B, Tavian M. Angiotensin-converting enzyme (CD143) specifies emerging lympho-hematopoietic progenitors in the human embryo. Blood. 2012;119(16):3712-3723.
7. Marshall GM, Carter DR, Cheung BB, et al. The prenatal origins of cancer. Nat Rev Cancer. 2014;14(4):277-289.
8. Sanchez MJ, Holmes A, Miles C, Dzierzak E. Characterization of the first definitive hematopoietic stem cells in the AGM and liver of the mouse embryo. Immunity. 1996;5(6):513-525.
9. Gekas C, Dieterlen-Lievre F, Orkin SH, Mikkola HK. The placenta is a niche for hematopoietic stem cells. Dev Cell. 2005;8(3):365-375.
10. Okada S, Nakauchi H, Nagayoshi K, Nishikawa S, Miura Y, Suda T. In vivo and in vitro stem cell function of c-kit- and Sca-1-positive murine hematopoietic cells. Blood. 1992;80(12):3044-3050.
11. Ivanovs A, Rybtsov S, Anderson RA, Turner ML, Medvinsky A. Identification of the niche and phenotype of the first human hematopoietic stem cells. Stem Cell Reports. 2014;2(4):449-456.
12. Kent D, Copley M, Benz C, Dykstra B, Bowie M, Eaves C. Regulation of hematopoietic stem cells by the steel factor/KIT signaling pathway. Clin Cancer Res. 2008;14(7):1926-1930.
13. Mayani H1, Dragowska W, Lansdorp PM. Characterization of functionally distinct subpopulations of CD34+ cord blood cells in serum-free long-term cultures supplemented with hematopoietic cytokines. Blood. 1993;82(9):2664-2672.
14. Notta F, Doulatov S, Laurenti E, Poeppl A, Jurisica I, Dick JE. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. Science. 2011;333(6039):218-221.
15. Okkenhaug K, Bilancio A, Emery JL, Vanhaesebroeck B. Phosphoinositide 3-kinase in T cell activation and survival. Biochem Soc Trans. 2004;32(Pt 2):332-335.
16. Hu G, Chen J. A genome-wide regulatory network identifies key transcription factors for memory CD8(+) T-cell development. Nat Commun. 2013;4:2830.
17. Domenech C, Maillard L, Rousseau A, et al. Studies in an early development window unveils a severe HSC defect in both murine and human Fanconi anemia. Stem Cell Reports. 2018;11(5):1075-1091.