FETAL/EMBRYONIC HEMATOPOIETIC PROGENITORS AND THEIR IMPACT ON ADULT DISEASES

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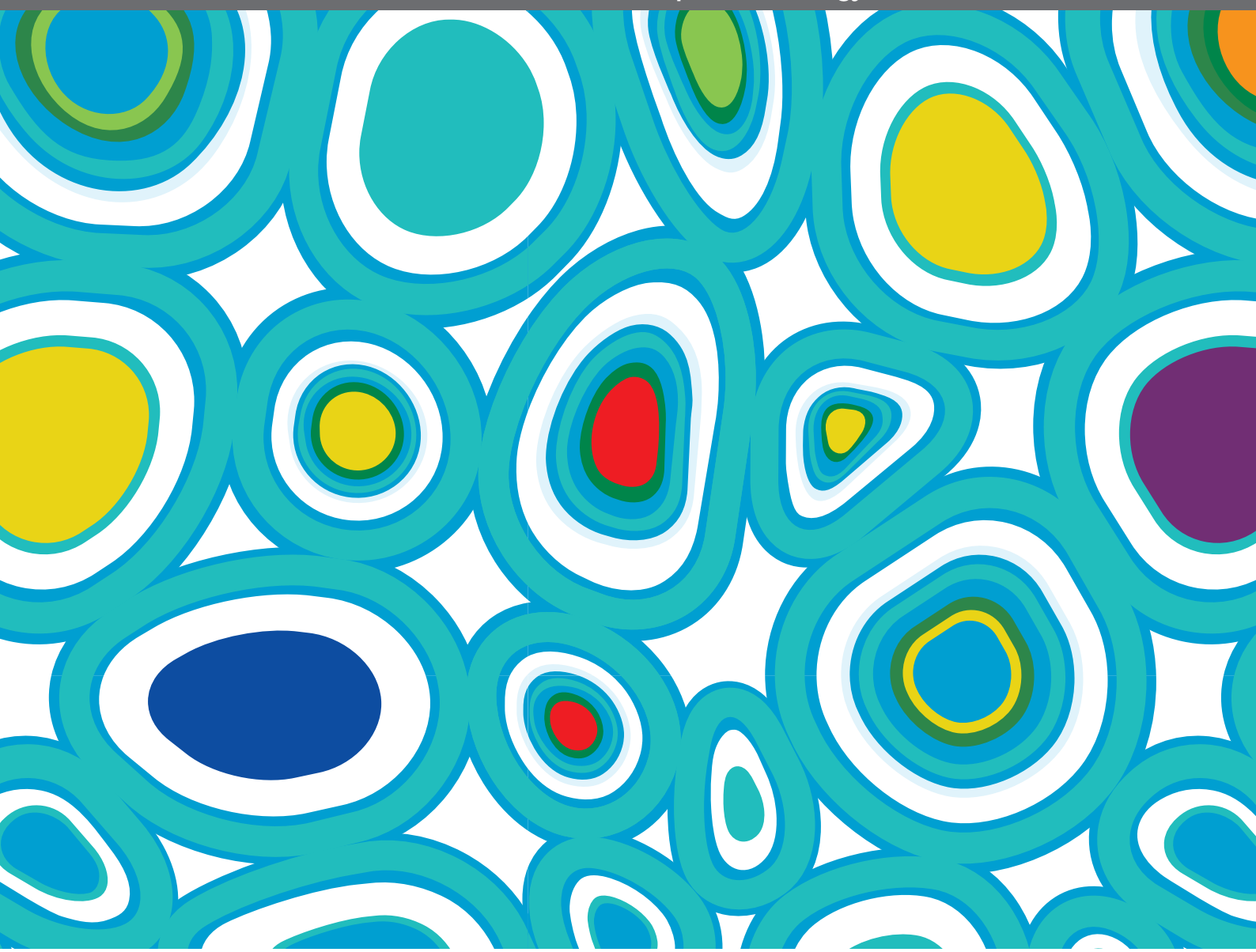

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FETAL/EMBRYONIC HEMATOPOIETIC PROGENITORS AND THEIR IMPACT ON ADULT DISEASES

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EDITORIAL
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Editorial: Fetal/Embryonic Hematopoietic Progenitors and Their Impact on Adult Diseases

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Editorial on the Research Topic

Fetal/Embryonic Hematopoietic Progenitors and Their Impact on Adult Diseases

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Despite enormous progress boosted by significant technological advances of the last decade, a complete understanding of hematopoietic development has not yet been achieved. "Primitive" and "definitive" terms have been used for many years to describe the different waves of transient and persistent blood cells generated during ontogeny. However, it is now clear that this terminology is not adequate to address the surprising complexity of the developing hematopoietic system. Gaining additional insight into embryonic and fetal hematopoiesis will not only be relevant for textbook biology, but will carry important implications for the understanding of blood diseases, including genetic conditions and pediatric leukemia. This Research Topic comprises 14 articles including reviews and primary research articles focusing on different aspects on the biology of fetal blood cells, ranging from basic hematopoiesis to technological advances and translational studies.

NEW ADVANCES IN DEVELOPMENTAL HEMATOPOIESIS: INCREASING LAYERS OF COMPLEXITY

An elegant review by Elsaid et al. delivers a comprehensive evolutionary perspective on hematopoietic development and shows that the concept of "layered" hematopoiesis is a successful conserved design introduced very early in evolution. Authors focus in particular on lymphoid cell development, which until few years ago was thought to exclusively derive from HSC activity. This view had implications in the readouts of *in vitro* or *ex vivo* experiments aimed at assessing the presence of HSCs; however, this concept has been recently challenged by the discovery of HSC-independent lymphoid progenitors within the embryo. Indeed, the fetal lymphoid compartment develops in sub-waves, which may, intriguingly, perform specific functions, similar to macrophages (see below).

A review from Neo et al. primarily focuses on mouse models and provides a useful and detailed overview of knockout and inducible fate mapping systems used to study hematopoietic development, which not only showcases its intricacies, but serves as a reminder of the importance of taking into account different and complementary experimental models when interpreting these data. The authors also review the specific functions of HSC-independent

hematopoietic cells. Tissue resident macrophages (TRM) are derived from yolk sac erythro-myeloid progenitors (EMP), and exert a multitude of functions during development, including support for organogenesis, for vascular network formation and direct support for HSC development. Importantly, HSC-independent waves persist in the adult and perform unique functions, not limited to macrophages but also including mast- and lymphoid cells.

One of the reasons why developmental hematopoiesis is less studied than its adult counterpart is that cells of interest are not easily available. Indeed, research on human hematopoietic development has historically been hampered by the scarce availability of source material. For this reason, human pluripotent stem cells (hPCS) represent an important option, discussed by Gutierrez-Aguera et al. in a Perspective article contained in this Topic. These Authors provide an important comparison on the two most used hPSC hematopoietic differentiation protocols, which will be undoubtedly useful for many researchers in the field. For the same availability issue, single cell technologies were initially applied to the study of adult blood cells, but developmental hematopoiesis was soon to follow. Karlsson et al. here provide a timely review on how the advent of single cell technologies, which began with qPCR studies and followed up with transcriptomics, impacted and revolutionized developmental hematopoiesis, allowing to resolve cell heterogeneity and differentiation trajectories with unprecedented clarity.

MACROPHAGES: KEY PLAYERS OF TISSUE REMODELING FROM THE EMBRYO TO THE ADULT

In their review Wu and Hirschi discuss the developmental origins of tissue-resident macrophages, describing how macrophages emerge along the three main waves of hematopoiesis, during primitive hematopoiesis, erythro-myeloid progenitor (EMP) generation, and definitive hematopoietic stem cell (HSC)-mediated hematopoiesis and how these stages were characterized by using different mouse models. Furthermore, they briefly outline macrophages molecular regulation in specific tissues and their impact on embryonic development and postnatal homeostasis, focussing in particular on angiogenesis, erythropoiesis, neurogenesis and osteogenesis.

On this last particular topic Yahara et al. review the heterogeneity and role of macrophages and osteoclasts during bone homeostasis and fracture repair, describing the signaling mechanisms leading to their recruitment at the site of damage and their effective role in bone regeneration. They also highlight new findings about the developmental origin of macrophage and osteoclasts and how a population of EMP-derived embryonic macrophages persist in the adult, acting independently from the HSCs-derived peripheral monocytes.

The concept of the persistence of EMP derived cells in the adult and their key role in physiological and pathological tissue homeostasis is also discussed in the review from Mass and Gentek. Focusing on macrophages and mast cells, they present emerging evidence that demonstrate how the different ontogeny is related to different cell roles. Moreover, in support of the Developmental Origins of Health and Disease theory (DOHaD), they highlight how perturbation of EMP-derived macrophage and mast cells programming and differentiation, due to somatic mutation of early maternal or environmental adverse events, can lead to a wide variety of lifelong diseases in the adult, spanning from allergy to neurological disorders and cancer.

ERYTHROPOIESIS: SUBSEQUENT WAVES AND NEW WINDOWS FOR THERAPEUTIC INTERVENTION

Beside myeloid cells, EMPs generate the second embryonic wave of erythroid cells, which acts as a bridge between the first yolk sac-derived Primitive Red Blood cells and the establishment of HSC-dependent adult hematopoiesis. Similar to HSCs, EMPs originate from the hemogenic endothelium, through the endothelial to hematopoietic transition (endoHT).

The receptor tyrosine kinase KIT is a known key regulator of definitive erythropoiesis but it is also expressed in the hemogenic endothelium. This evidence raises the question of its possible functional role in endoHT. Fantin et al. combine mouse genetics and single cell transcriptomic analysis to address this point. Results show that Kit is dispensable for endoHT and for EMPs immediate differentiation. Instead, after EMPs seed the fetal liver, Kit loss significantly reduces EMPs-derived erythropoiesis to the benefit of the alternative myeloid lineage, uncovering the role of Kit in EMPs downstream erythroid commitment.

In humans, the different waves of erythropoiesis are accompanied by the production of different types of hemoglobin: embryonic HbE, fetal HbF and adult HbA. The switch from HbF ($\alpha 2\gamma 2$) to HbA ($\alpha 2\beta 2$) has relevant clinical interest because the persistence of HbF after birth ameliorates β-thalassemia and Sickle Cell Disease (SCD), the most common monogenic diseases worldwide. Barbarani et al. focus on the regulatory networks controlling γ-globin expression in normal and aberrant conditions. Interestingly, in some cases, such as in Hereditary Persistence of Fetal Hemoglobin (HPFH), postnatal γ-globin expression is caused by mutations within the β-locus or within modifiers genes in adult cells. Instead, in the particular case of juvenile myelo-monocytic leukemia (JMML), a rare aggressive pediatric cancer, high HbF together with other fetal red cells traits, suggest a fetal origin of cancer cells, uncovering the cellular heterogeneity underlying the postnatal HbF phenotype.

Early fetal stages of hematopoiesis may represent a potential window for therapeutic intervention for hematological diseases. In their article, Villaverde Cortabarria et al. discuss the feasibility of *in utero* stem cell transplantation (IUSCT) to cure Sickle Cell Disease. Hematopoietic stem cell transplantation (HSCT) is the only SCD cure but it is greatly limited by the availability of matched donors. The option of prenatal intervention, before fetal immune system maturation, could significantly

enhance allogenic engraftment, donor-specific tolerance, and lifelong chimerism without immunosuppression. Beside immune response, the host-donor cells competition within the niche represents a second critical obstacle for IUSCT. Despite the scientific, technical, and ethical challenges posed by IUSCT, the perspective to offer a definitive cure for SCD preventing the occurrence of anemia and of major organs damage, warrants further investigation.

HEMATOLOGICAL MALIGNANCIES: FROM CELL OF ORIGIN TO TREATMENT

Leukemia is one of the most frequent malignancies in children, dominated by acute lymphoblastic leukemia of the B cell lineage, B-ALL. Even though prognosis has improved markedly the last decades, with survival rates reaching 90%, some subgroups still have dismal prognosis. There is ample evidence that the disease initiates *in utero* and this is the main topic of the review by Cazzola et al. The cell of origin and initiation of the disease, in relation to embryonic hematopoiesis, as well as the need of refined models to study the disease are considered. Moreover, the fact that the disease may initiate in an HSC-independent progenitor, a largely unexplored area of research, is also highlighted.

A minor fraction (10–15%) of pediatric ALL are of T cell origin, a leukemia that usually affect slightly older children than B-ALL. Evidence suggest that T-ALL may also have a prenatal origin, and this is investigated further in the study by Ding et al. When overexpressing Notch pathway, a common mutation in T-ALL, embryonic cells from the paraaortic splanchnopleura (P-Sp) but not from the yolk sac, were shown to give rise to T-ALL when transplanted into mice. Overall the results suggest that there may be a prenatal origin of T-ALL.

In contrast to B-ALL, which has highest incidence in children, acute leukemia of myeloid lineage increases with age and is the most common acute leukemia in adults. Histone deacetylase 8 (HDAC8) has been shown to be overexpressed in some subtypes of Acute Myeloid Leukemia (AML) and its role in normal hematopoiesis as well as possible therapeutic target is the focus of the study by Spreafico et al. By using zebrafish embryos overexpressing Hdac8 an expansion of hematopoietic stem and progenitor cells (HSPCs) was seen. The phenotype could be reversed by an HDAC8 inhibitor, that was shown to induce p53-mediated apoptosis. The HDAC8 inhibitor was also tested in AML cell lines and all together these data indicate HDAC8 as a possible future target for treatment.

Another therapeutic target is the JAK-STAT pathway, a signaling pathway associated with many different hematological malignancies. The role of JAK-STAT in normal as well as malignant hematopoiesis is the topic of the review by Fasouli and Katsantoni. Therapeutical implications, indirect as well as direct inhibition of JAK-STAT pathway and combination with other therapies are also discussed in the review.

CONCLUSIONS

Collectively, our Research Topic highlights that research in hematopoietic development is more active than ever. We think that this field is a perfect example of translation and multidisciplinarity, where discoveries made by basic scientists can bear relevance for clinicians and for the understanding of human disease—and vice versa. It is now clear that cells considered until few yers ago as embryonic-confined transient populations can in fact persist after birth, where they functionally contribute to adult tissues in a range of different ways. We anticipate that this topic will continue to be intensively studied in the upcoming years.

AUTHOR CONTRIBUTIONS

All authors equally contributed to the article and approved the submitted version.

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HDAC8: A Promising Therapeutic Target for Acute Myeloid Leukemia

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Histone deacetylase 8 (HDAC8), a class I HDAC that modifies non-histone proteins such as p53, is highly expressed in different hematological neoplasms including a subtype of acute myeloid leukemia (AML) bearing inversion of chromosome 16 [inv(16)]. To investigate HDAC8 contribution to hematopoietic stem cell maintenance and myeloid leukemic transformation, we generated a zebrafish model with Hdac8 overexpression and observed an increase in hematopoietic stem/progenitor cells, a phenotype that could be reverted using a specific HDAC8 inhibitor, PCI-34051 (PCI). In addition, we demonstrated that AML cell lines respond differently to PCI treatment: HDAC8 inhibition elicits cytotoxic effect with cell cycle arrest followed by apoptosis in THP-1 cells, and cytostatic effect in HL60 cells that lack p53. A combination of cytarabine, a standard anti-AML chemotherapeutic, with PCI resulted in a synergistic effect in all the cell lines tested. We, then, searched for a mechanism behind cell cycle arrest caused by HDAC8 inhibition in the absence of functional p53 and demonstrated an involvement of the canonical WNT signaling in zebrafish and in cell lines. Together, we provide the evidence for the role of HDAC8 in hematopoietic stem cell differentiation in zebrafish and AML cell lines, suggesting HDAC8 inhibition as a therapeutic target in hematological malignancies. Accordingly, we demonstrated the utility of a highly specific HDAC8 inhibition as a therapeutic strategy in combination with standard chemotherapy.

Keywords: HDAC8, AML, PCI-34051, zebrafish, p53, WNT

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INTRODUCTION

Acute myeloid leukemia (AML) is a group of heterogeneous malignant hematological disorders underlain by genetic and epigenetic changes in hematopoietic stem cells (HSCs) and myeloid progenitors causing an imbalance between survival, proliferation and differentiation. The net effect of all changes is the accumulation of unfunctional myeloid cells, termed blasts, in the bone marrow. AML is the most frequent acute leukemia type in adults and, currently, it is curable in 35–40% of patients under 60 years of age and only in 5–15% of patients older than 60 years (Döhner et al., 2015).

Histone deacetylase 8 (HDAC8) is a ubiquitously expressed class I HDAC (Buggy et al., 2000; Hu et al., 2000; Van Den Wyngaert et al., 2000). Unlike other class I HDACs, it localizes both in the nucleus and in the cytoplasm (Li et al., 2014), lacks the C-terminal protein-binding domain

(Somoza et al., 2004) and is characterized by a peculiar negative regulation of its activity by cAMP-dependent protein kinase (PKA) (Lee et al., 2004), which suggests a functional specialization. HDAC8 has been demonstrated to target non-histone proteins, such as the structural maintenance of chromosome 3 (SMC3) cohesin protein, retinoic acid induced 1 (RAI1) and p53, thus regulating diverse processes (Deardorff et al., 2012; Wu et al., 2013; Olson et al., 2014). *HDAC8* is either overexpressed or dysregulated in cancers, such as neuroblastoma, breast cancer, colon cancer (Nakagawa et al., 2007; Oehme et al., 2009; Park et al., 2011) and hematological malignancies. In particular, *HDAC8* expression was found to be increased in primary cells from childhood acute lymphoblastic leukemia patients (Moreno et al., 2010), in adult T cell leukemia/lymphoma (Higuchi et al., 2013) and human myeloma cell lines (Mithraprabhu et al., 2014). HDAC8 was demonstrated to interact with CBFβ-SMMHC fusion protein, resulting from the inversion of chromosome 16 [inv(16)] (Durst et al., 2003). The interaction of both HDAC8 and p53 with inv(16) fusion protein leads to increased deacetylation and consequent inhibition of p53, which promotes survival and proliferation of inv(16)+ AML CD34+ cells (Qi et al., 2015). Interestingly, high *HDAC8* expression was detected not only in inv(16)+ AML CD34+ cells, but also in non-inv(16)+ AML CD34+ cells, suggesting a more general involvement of HDAC8 in AML development (Qi et al., 2015). The role of HDAC8 in AML onset is further supported by a recent finding of it playing a crucial role in maintaining long-term HSC under stress condition by inhibiting p53 (Hua et al., 2017).

Histone deacetylase inhibitors (HDACi) possess an anti-cancer activity through the induction of apoptosis and cell cycle arrest (Eckschlager et al., 2017) in solid and hematological tumors (Ceccacci and Minucci, 2016; Imai et al., 2016). However, the use of HDACi is still limited due to the safety issues as side effects, including fatigue, diarrhea and thrombocytopenia, have been observed following their administration (Subramanian et al., 2010). Such toxicity is most likely related to the lack of selectivity of most of these drugs that act as pan-HDACi. In order to improve the outcome of the therapy and reduce side effects, compounds targeting specific HDAC isoforms are needed.

The distinctive structure of HDAC8, in comparison to others class I HDAC family members, allowed the development of high specific HDAC8 inhibitor PCI-34051 (hereafter PCI) (Balasubramanian et al., 2008), previously tested in T-cell lymphoma (Balasubramanian et al., 2008) and AML (Qi et al., 2015). The aim of this project was to explore the feasibility of HDAC8 inhibition as a therapeutic approach in AML. To this end, we generated a zebrafish (*Danio rerio*) model for Hdac8 overexpression that displayed a hematopoietic phenotype characterized by an increase in the hematopoietic stem/progenitor cells (HSPCs) population that could be rescued by PCI treatment. In parallel, we assessed the response of AML cell lines (OCI-AML5, HL60, PLB985, THP-1, and AML193) to PCI. We observed that PCI elicits apoptosis in THP-1 cell line and in the zebrafish embryos overexpressing Hdac8, while it induces cell cycle arrest in p53-null HL60 cells, prompting a search of alternative mechanisms explaining PCI action in the absence of p53. We, thus, demonstrated an involvement of the canonical Wnt signaling. Our results suggest that selective inhibition of HDAC8 by PCI may be a valuable therapeutic approach for the treatment of AML patients.

MATERIALS AND METHODS

Zebrafish Embryos

Zebrafish (*D. rerio*) were maintained at the University of Milan, Via Celoria 26 – 20133 Milan, Italy (Autorizzazione Protocollo n. 295/2012-A – December 20, 2012) and Cogentech s.c.a.r.l. via Adamello 16 – 20139 Milan, Italy (Autorizzazione Protocollo n. 007894 – May 29, 2018). Zebrafish strains AB, *Tg*(*CD41:GFP*), *Tg*(*TOPdGFP*) and $p53^{M214K}$ (Dorsky et al., 2002; Berghmans et al., 2005; Lin et al., 2005) were maintained according to international (EU Directive 2010/63/EU) and national guidelines (Italian decree No 26 of the 4th of March 2014). Embryos were staged and used until 5 days post fertilization, a time windows in which zebrafish is not considered an animal model according to national guidelines (Italian decree No 26 of the 4th of March 2014). Embryos were staged as described in Kimmel et al. (1995) and raised in fish water (Instant Ocean, 0.1% Methylene Blue) at 28°C in Petri dishes, according to established techniques. Embryonic ages are expressed in hours post fertilization (hpf) and days post fertilization (dpf). To prevent pigmentation, 0.003% 1-phenyl-2-thiourea (PTU, Sigma-Aldrich, St. Louis, MI, United States) was added to the fish water. Embryos were anesthetized with 0.016% tricaine (Ethyl 3-aminobenzoate methanesulfonate salt, Sigma-Aldrich) before proceeding with experimental protocols.

Zebrafish Microinjection and Treatment

Injections were carried out on 1- to 2-cell stage embryos. Zebrafish *hdac8* full-length mRNA was injected at the concentration of 500 pg/embryo as previously described (Bottai et al., 2019). As a control the membrane red fluorescent protein (*mrfp)* coding mRNA was injected at the same concentration. Alternatively, in double immunofluorescence staining analyses, we injected water as a control. For canonical Wnt inhibition, zebrafish *dkk1b* mRNA was injected at the concentration of 50 pg/embryo (Mazzola et al., 2019). PCI treatment were done in 24-well plates, 30 embryos/well. PCI was added to fish water at the concentration of 150 μM PCI and embryos were kept at 28°C in the dark for 24 h. Equal concentration of DMSO was used as a control.

FACS Analysis in Zebrafish

Embryos dissociation was achieved as described in Bresciani et al. (2018). FACS analysis were performed on *Tg*(*CD41:GFP*) zebrafish embryos at 3 dpf as previously described (Ma et al., 2011; Mazzola et al., 2019). We used Attune NxT (Thermo Fisher Scientific, Waltham, MA, United States) instrument equipped with software Kaluza (Beckman Coulter, Brea, CA, United States) for the analysis. AB wild-type embryos were used to set the gate to exclude auto-fluorescence of cells. The gate for GFP low/high cells was set on control *Tg(CD41:GFP)* embryos to distinguish a GFP_{low} population representing around 0.2% of total cells, as

previously reported (Mazzola et al., 2019), and applied to all categories analyzed.

Immunofluorescence

Embryos were fixed overnight in 4% paraformaldehyde (Sigma-Aldrich) in PBS at 4°C. For single-color staining, we used rabbit anti-GFP 1:500 (NC9589665, Torrey Pines Biolab, Houston, TX, United States) as primary antibody and Alexa Fluor 488-conjugated goat anti-rabbit IgG 1:400 (A11008, Invitrogen Life Technologies, Carlsbad, CA, Untied States) as secondary antibody. For dual staining, we took advantage of mouse anti-GFP 1:2000 (MAB3580, Merck-Millipore, Burlington, MA, United States), rabbit anti-histone H3 (phospho S10) 1:200 (ab5176, Abcam, Cambridge, United Kingdom), and rabbit anti-cleaved caspase 3 1:100 (9664, Cell Signaling Technologies, Danvers, MA, United States) as primary antibodies and Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa 546-conjugated goat anti-rabbit IgG 1:400 (A11001 and A11010, Invitrogen Life Technologies) as secondary antibodies. Embryos were equilibrated and mounted in 85% glycerol solution in PBS and imaged using a "TCS-SP2" confocal microscope (Leica, Wetzlar, Germany), with 20× oil immersion 9 objective, 488 nm argon ion and 405 nm diode lasers. Single stack images were acquired for each sample. Images were processed using Adobe Photoshop software. Quantification was performed by using the ImageJ software. For dual staining, we counted the total number of both GFP^+ cells and double positive cells. The percentage of double positive cells was calculated as the ratio of double positive/total GFP^+.

Reverse Transcription and Real-Time Quantitative PCR

Total RNA was extracted from cells or zebrafish whole embryos (at least 30 embryos) with NucleoZOL reagent (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions and treated with RQ1 RNase-free DNase (Promega, Madison, WI, United States). cDNA was synthetized using the GoScript Reverse Transcription Kit (Promega), as specified by the manufacturer's instructions. qPCR analyses were performed with the GoTaq qPCR Master Mix (Promega) on the Bio-Rad iQ5 Real Time Detection System (Bio-Rad, Hercules, CA, United States) and Quantum Studio 5 (Thermo Fisher). Gene expression changes were calculated with the ΔΔCt method. We used *GAPDH* for AML cells and *rpl8* and β-*actin* for zebrafish as internal control. Primer sequences are list in **Supplementary Table 1**.

Cell Lines PCI Treatment

OCI-AML5, HL60, PLB985, THP-1, and AML193 cell lines were originally obtained from ATCC/DSMZ repositories and since stored at the internal cell line bank at the Department of Experimental Oncology, IEO. Cell lines undergo regular authentication and mycoplasma testing. Cells were seeded at 10^4 cells/well in 96-well plates in 100 μl of growth medium and allowed to grow for 24 h prior to treatment commencement. PCI was dissolved in DMSO, diluted in the appropriate culture medium and added into plates, as indicated. The concentration range of PCI has been determined based on published data and ranged between 3.12 and 50 μM (Balasubramanian et al., 2008; Qi et al., 2015). Seventy-two hours later, CellTiter-Glo assay (Promega) was performed as indicated in the manufacturer's instructions and read on GloMax (Promega) plate reader. Cells treated with DMSO (0.2% in appropriate medium) were used as a control.

In vitro Proliferation

Cells were seeded in duplicate at 10^5 cells/ml and allowed to grow for 24 h at 37°C, 5% CO_2, 95% humidity. Then cells were treated with 50 μM PCI or with 0.2% DMSO (control). At 12, 24, 48, and 72 h, both viable and dead cells were counted under an inverted-light microscope (Leica) following 0.4% trypan blue staining.

Cell Cycle Analysis

Cells were seeded at 10^5 cells/ml and then treated with DMSO or with 50 μM of PCI for 48 h. One-million of viable cells were harvested after 12, 24, and 48 h, washed once with cold PBS, fixed in 70% of ice-cold ethanol dropwise and kept on ice for 30 min. Next, cells were washed in 1% BSA in PBS and stained overnight with DNA staining solution containing 250 μg/ml of RNase and 5 μg/ml propidium iodide (PI) at 4°C. Data analysis was done using flow cytometry (FACSCelesta, FlowJo10 software).

Apoptosis Assay

Cells were seeded at 10^5 cells/ml and then treated with DMSO or with 50 μM of PCI for 72 h. A total of 50×10^4 cells were harvested after 72 h, washed once with cold PBS and then with annexin buffer. Cells were resuspended in 100 μl annexin-APC diluted 1:50 in annexin buffer and incubated 1 h at room temperature in the dark. Next, cells were washed once with annexin buffer and resuspended in 1× PBS. Cells were stained for maximum 5 min with PI solution. Data analysis was done using flow cytometry (FACSCelesta, FlowJo10 software).

Combination Treatment

Cells were seeded at 10^4 cells/well in 96-well plates and allowed to grow for 24 h prior to treatment commencement. Drug concentrations ranged from 0.78 to 100 μM and from 0.078 to 10 μM for PCI and cytarabine, respectively. Cells were treated with all concentrations of single agents and in combination setting, in which decreasing concentrations of each compound were used together (**Supplementary Figure 5**). The combination index (CI), based on the Bliss Independence model, was calculated as $CI = \frac{E_A+E_B-E_AE_B}{E_{AB}}$, where E_A indicates the effect of compound A, E_B indicates the effect of compound B and E_{AB} the effect of the combination of both compounds. $CI < 1$ indicates synergism; $CI = 1$ indicates an additive effect, while $CI > 1$ indicates antagonism (Foucquier and Guedj, 2015).

Statistical Analysis

Each experiment was performed at least twice (biological replicates). A minimum number of 15 embryos was analyzed in each imaging experiment, while RNA was extracted from a minimum of 30 animals. PCI treatment outcome was assessed

on at least 30 zebrafish embryos. For qPCR analysis on zebrafish, experiments were performed on at least three different independent experiments (batches of embryos deriving from different matings). The statistical significance was determined using two-sided Student's *t*-test when comparing two groups and one-way ANOVA test followed by Tukey *post hoc* correction when comparing three groups. One-sample *t*-test was used when control group was set to a defined value of 1. Data were considered significant if $p < 0.05$.

RESULTS

Hdac8 Overexpression in Zebrafish Leads to HSPCs Expansion and Its Inhibition Elicits Apoptosis and Rescues the Phenotype

CD34+ cells derived from inv(16)+ AML patients express high levels of HDAC8 (Qi et al., 2015). We generated a zebrafish model for Hdac8 overexpression by injecting embryos with the full-length zebrafish *hdac8* mRNA (500 pg/embryo) to assess whether HDAC8 upregulation would alter hematopoietic phenotype *in vivo*. The injection of the *hdac8*-mRNA, although increasing the *hdac8* transcript and protein levels (**Supplementary Figures S1A,B**), did not alter the general morphology of the embryo or organ size (**Supplementary Figures S1C–E**) but specifically impact on the hematopoietic phenotype. In this regard, to obtain an easy read-out of the hematopoietic phenotype, we performed *hdac8* overexpression in the *Tg*(*CD41:GFP*) transgenic line that expresses GFP protein in HSPCs, Lin et al. (2005); **Supplementary Figures S1F,G**, and we assessed the expression of the HSC transcription factor markers *c-Myb*, *gata2b* and Runx1, and of the immature myeloid cells *pu.1* that resulted upregulated following Hdac8 ectopic expression (**Supplementary Figures 1H,I**). Confocal images of the caudal hematopoietic tissue (CHT) in 3 dpf embryos (**Figure 1A**), showed an increase in HSPC population upon Hdac8 overexpression in comparison to controls (**Figures 1B-C**). This effect was specific, as we obtained a significant reduction of HSPCs in *hdac8* mRNA-injected embryos treated with PCI (**Figure 1D**). We then quantified HSPC number by enumerating GFP_{low}-HSPCs in the three categories of embryos by flow cytometry (Ma et al., 2011; Mazzola et al., 2019). GFP_{low}-HSPCs were significantly increased in *hdac8*-injected embryos compared to controls and were reduced when treated with PCI (**Figures 1E–H**). We also evaluated the effect of PCI on zebrafish HSPCs in the absence of Hdac8 overexpression as a control. Immunofluorescence and FACS analyses for the HSPCs in the *Tg*(*CD41:GFP*) line and gene expression analyses for the HSC marker *cmyb* indicated a decrease of HSPCs in PCI-treated embryos compared to control embryos (**Supplementary Figures 2A–F**). To assess whether the expansion in HSPCs population following Hdac8 ectopic expression is indicative to a pre-leukemic state and if these cells possess higher self-renewal ability compared to their differentiated counterparts, we performed dual immunofluorescence with GFP (in green) and phospho-histone H3 (PH3, in red) in the *Tg(CD41:GFP)* embryos at 3 dpf. The ectopic expression of Hdac8 induced an increased proliferation of HSPCs in comparison to controls, that was reduced following PCI treatment (**Figures 1I–L**).

Since p53 is a target of HDAC8 and studies indicate that PCI treatment determines cell cycle arrest and induction of apoptosis in *in vivo* (Oehme et al., 2009) and *in vitro* (Rettig et al., 2015) models, we verified whether PCI treatment determines apoptosis also in zebrafish (**Supplementary Figures 2G,H**). Thus, to assess PCI-mediated apoptosis specifically in the HSPC population which was expanded following *hdac8*-overexpression, we evaluated caspase-3 activation by dual immunofluorescence in Hdac8-overexpressing *Tg(CD41:GFP)* zebrafish embryos at 3 dpf treated or not with PCI. We observed an increase of caspase-3^+/GFP^+ HSPCs in PCI-treated *hdac8*-mRNA injected embryos compared to *hdac8*-mRNA-injected and control embryos (**Figures 1M–P**). This result was confirmed by the significant increase of expression levels of the p53 target genes (*baxa, bida, bbc3, cdkn1a*, and *gadd45ba*) by RT-qPCR in PCI-treated *hdac8*-mRNA injected embryos compared to both control mRNA- and *hdac8*-mRNA-injected embryos (**Figure 1Q**; Qi et al., 2015). Taken together, these results indicate that Hdac8 overexpression in zebrafish determines an expansion of HSPC population and that PCI treatment induces a block in cell expansion activating p53-mediated apoptosis.

PCI Exerts Cytostatic and Cytotoxic Effect on AML Cell Lines

To evaluate the effects of HDAC8 inhibition also in human myeloid cells, we selected five AML cell lines expressing *HDAC8* (OCI-AML5, HL60, PLB985, THP-1, and AML193). We treated them once for 72 h with decreasing concentrations of PCI and evaluated the viability using CTG luminescence assay, an indicator of metabolically active cells. PCI decreased the viability of HL60, PLB985, THP-1, and AML193 cell lines while OCI-AML5 seemed less sensitive to the treatment (**Figure 2A**).

We excluded from further analyses the PLB985 cell line since it is a sub-clone of HL60 and AML193 childhood AML cell line as all the other cell lines derive from adult AML patients. We validated HDAC8 expression levels by means of RT-qPCR and western blot analyses. HL60 and THP-1 expressed significantly higher *HDAC8* mRNA than OCI-AML5 by RT-qPCR (**Supplementary Figure 3A**). Western blot analysis also indicated higher HDAC8 protein levels in HL60 and THP-1 than in OCI-AML5 (**Supplementary Figure 3B**). To determine whether PCI elicits cytostatic of cytotoxic effect, we treated the selected cell lines with 50 μM of PCI or with DMSO as a control for 12, 24, 48, and 72 h and counted daily following trypan blue staining. No significant effect on cell growth was observed in the less-PCI-sensitive OCI-AML5 cell line (**Figure 2B**), although PCI treatment increased the levels of acetylated SMC3 (ac-SMC3), a specific HDAC8 target (**Supplementary Figure 3C**). By contrast, PCI exerted a cytostatic effect in HL60 cell line and caused cell death in THP-1 cells, as indicated by viable and dead cell

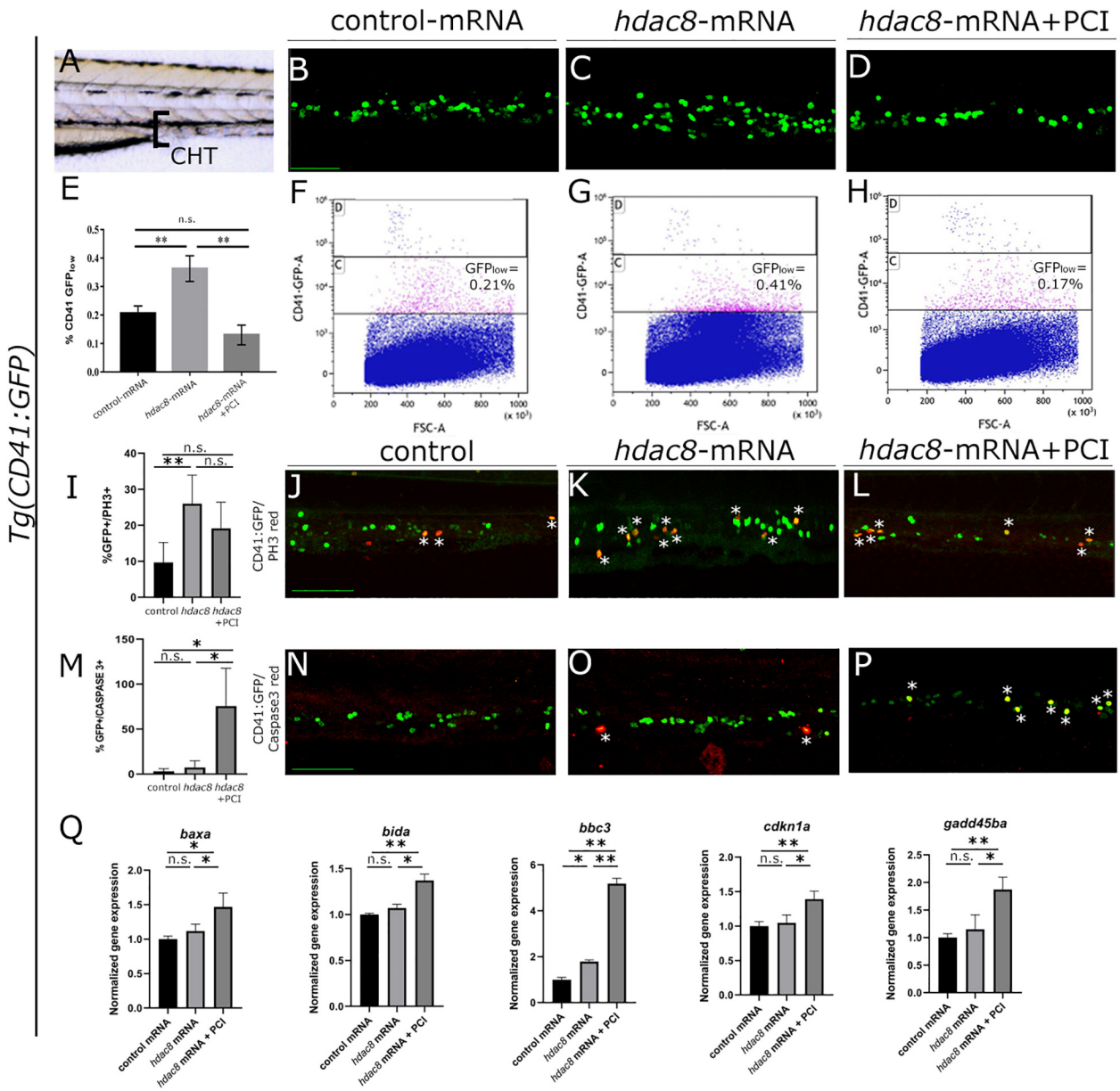

FIGURE 1 | Analysis of Hdac8 overexpression in zebrafish and apoptosis induced by PCI treatment. **(A)** Scheme of trunk-tail region of zebrafish embryos: confocal imaging was always performed on the same embryo region, comprising the tip of the yolk sack extension, between the dorsal aorta and the vein; representative images of **(B)** control; **(C)** *hdac8*-mRNA injected; **(D)** PCI-treated *hdac8*-mRNA injected *Tg(CD41:GFP)* zebrafish embryos at 3 dpf. Scale bar represents 100 μm. **(E)** Quantification by FACS of GFP_{low}-HSPCs of **(F)** control; **(G)** *hdac8*-mRNA injected; **(H)** PCI-treated *hdac8*-mRNA injected *Tg(CD41:GFP)* zebrafish embryos at 3 dpf. The results are presented as mean ± SD from three independent experiments. **(I–L)** Proliferation of HSPCs in the caudal hematopoietic tissue of the *Tg(CD41:GFP)* zebrafish line following Hdac8 overexpression and PCI treatment. GFP^+ HSPCs in green; PH3 in red; quantification in **(I)**, *N* = 6 embryos analyzed. **(M–P)** Apoptosis of HSPCs in the caudal hematopoietic tissue of the *Tg(CD41:GFP)* zebrafish line following PCI treatment. GFP^+ HSPCs in green; activated caspase-3^+ cells in red; quantification in **(M)**, *N* = 3 embryos analyzed. Asterisks indicate double positive cells (yellow). Scale bar represents 100 μm. **(Q)** RT-qPCR analyses of apoptotic markers *baxa*, *bida, bbc3, cdkn1a,* and *gadd45ba* expression in control mRNA and *hdac8*-overexpressing zebrafish embryos treated with DMSO or PCI. The results are presented as mean value ± SD from three independent experiments. *$p < 0.05$, **$p < 0.01$, n.s. = not significant, one-way ANOVA followed by Tukey *post hoc* correction.

count (**Figure 2B**), which spurred us to investigate how PCI treatment impacted cell cycle. To this end, we treated AML cell lines with 50 μM PCI for 12, 24, and 48 h and analyzed DNA content by PI staining. At 12 h PCI treatment resulted in a block in the G0–G1 phase of over 70 and 80% of HL60 and THP-1 cells, respectively, while we did not detect any variations between untreated and treated OCI-AML5 samples (**Figure 2C**). Cell cycle arrest following PCI treatment in HL60 and THP-1

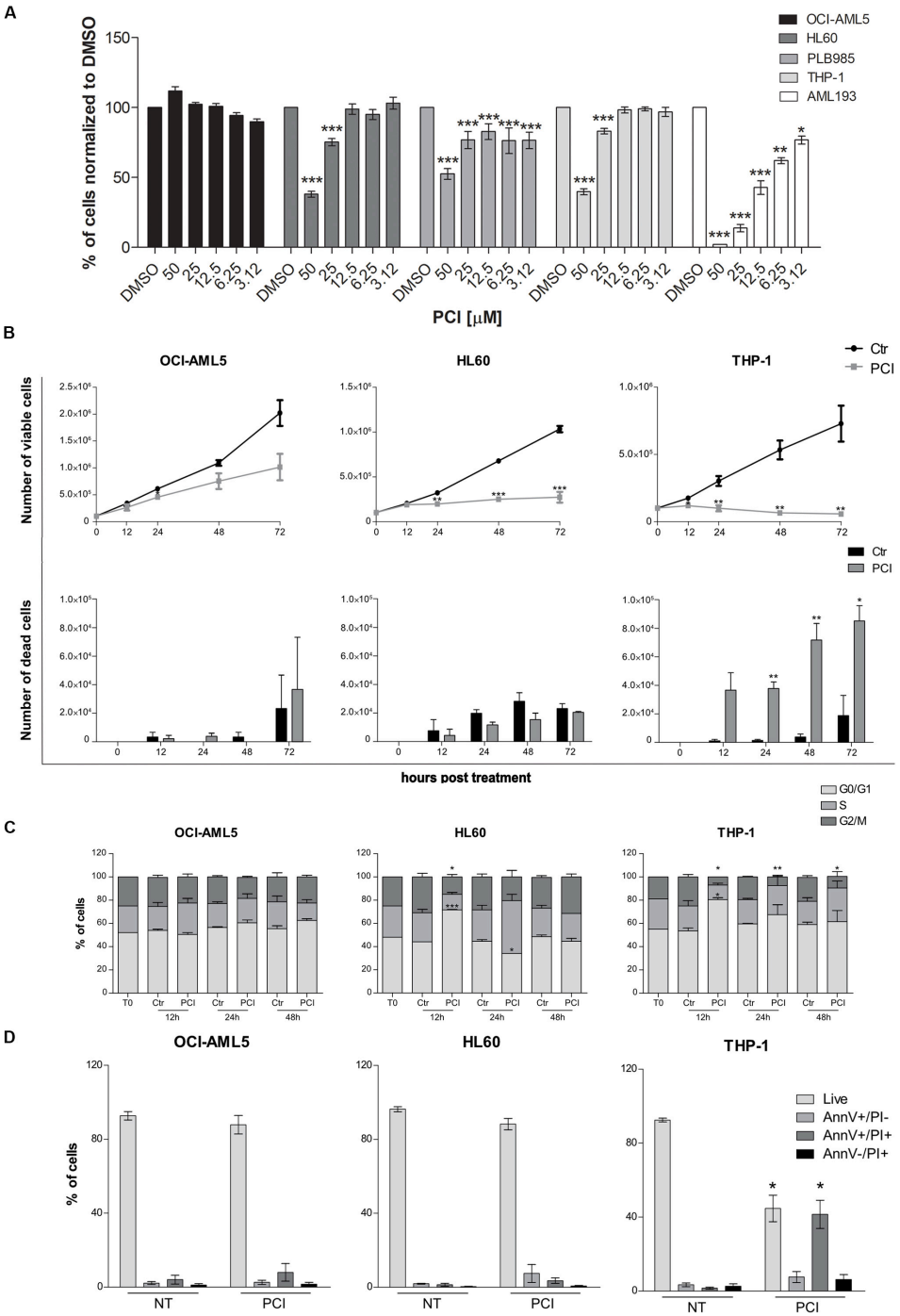

FIGURE 2 | Cytostatic and cytotoxic effect of PCI in AML cell lines. **(A)** The indicated cell lines were treated for 72 h with different concentration of PCI, or DMSO alone as a control. CTG assay was used to assess the effect of the treatment on the cell viability. The results are presented as mean ± SD from four technical replicates deriving from one independent experiment for PLB985 and AML193 cell lines, and at least two independent experiments for OCI-AML5, HL60, and THP-1 cell lines. **(B)** Effect of PCI on AML cell line growth. Cells were treated with 50 μM of PCI or with DMSO as a control. Cell viability (upper panel) and cell death (lower panel) were determined 12, 24, 48, and 72 h after treatment using trypan blue staining. The results are presented as mean ± SEM from two independent experiments for 12 h and three independent experiments for the others time points. **(C)** Cell cycle time course over 48 h of PCI treatment. Cells were treated with 50 μM of PCI or with DMSO as a control. Histogram showing cell distribution in three different cell cycle phases indicated as diverse shades of gray. The results are presented as mean ± SEM from two independent experiments. **(D)** Induction of apoptosis upon 72 h of PCI treatment. Cells were treated with 50 μM of PCI or with DMSO as a control. Histogram showing the percentage of live, AnnV+/PI, AnnV+/PI+ and AnnV–/PI+ cells indicated as diverse shades of gray. The results are presented as mean ± SEM from three independent experiments. NT, untreated; T0, time zero; AnnV, annexin V; PI, propidium iodide. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, Student's *t*-test.

cell lines was underlain by a decrease in *CyclinD1* (*CCND1*) and *CMYC* expression (**Supplementary Figure 4**). Next, we evaluated apoptosis induction using Annexin V/PI staining and we demonstrated that PCI treatment induces apoptosis only in THP-1 cells as attested by increased percentages of Annexin V^+/PI^+ positive population corresponding to late apoptotic cells at 72 h. Indeed, the HL60 cells responded to PCI inhibition but cannot undergo apoptosis lacking functional p53 (Wolf and Rotter, 1985; **Figure 2D**).

Taken together, these data indicate that PCI treatment impacts on AML cell survival causing cell cycle arrest followed by apoptosis when p53 is functional.

PCI Synergizes With Cytarabine in AML Cell Lines

Combination therapy allows for dose reduction, lowers the incidence and severity of side effects and prevents the development of resistance. We treated AML cell lines with cytarabine, an agent used at the frontline of AML treatment, alone or together with PCI to assess whether their combination resulted in synergy, additivity or antagonism as an *in vitro* indicator of a potential advantage of combination over single-agent treatment. OCI-AML5, HL60, and THP-1 cells were treated for 72 h with cytarabine at concentrations ranging from 0.078 to 10 μM and PCI at concentration range of 0.78–100 μM alone or together mixing decreasing concentrations of each compound (**Supplementary Figure 5**). Based on CI, a synergistic effect was observed for all AML cell lines when combining 0.35 μM cytarabine with 25 μM PCI. The combination of cytarabine and PCI resulted in an effect that was greater than the sum of single treatment outcomes in OCI-AML5 and HL60 cell lines, whilst showing a dramatic combination effect in THP-1 cell line at a concentration of cytarabine that alone had no effect. In detail, in THP-1 cell line the combination resulted in 47% of cell death compared to 0% of cytarabine and 23.5% of PCI in single-agent setting (**Figure 3**).

These results might suggest that cytarabine doses can be reduced in combination therapy while eliciting the same inhibitory effect on cell proliferation.

HDAC8 Inhibition Downregulates Canonical Wnt Pathway

We wished to identify a mechanism of action responsible for PCI-induced growth arrest in HL60 cells that responded to PCI inhibition without undergoing apoptosis being p53-null. Recently, we and others demonstrated that HDAC8 activates canonical Wnt pathway (Tian et al., 2015; Ferrari et al., 2019), which is frequently dysregulated in AML (Gruszka et al., 2019). We investigated whether Wnt signaling was affected by HDAC8 inhibition in AML cell line. We analyzed by RT-qPCR the expression levels of canonical Wnt pathway inhibitors *NKD1* and *PPP2R2B*, previously reported to be downregulated by forced *HDAC8* expression and upregulated following PCI treatment (Tian et al., 2015). We observed that the expression levels of both *NKD1* and *PPP2R2B* increased following PCI treatment in PCI sensitive cell lines HL60 and THP-1, indicating that HDAC8-mediated downregulation of the canonical Wnt signaling could be the cause of cell cycle arrest in these cell lines. Interestingly, the Wnt pathway was not modulated in the less-sensitive OCI-AML5 cells, confirming the specificity of HDAC8 inhibition on Wnt regulation (**Figure 4A**).

To verify that the downregulation of the Wnt pathway is p53-independent, we assessed Wnt pathway modulation in p53 mutant zebrafish embryos, which mimicked the p53-null HL60 condition. Thus, we took advantage of a homozygous zebrafish $p53^{M214K}$ mutant line, which lacks functional p53 (Berghmans et al., 2005), and we observed that, although the expression of the apoptotic markers *bida* and *cdkn1a* was not affected as expected in a p53 null background, a decrease of the expression of Wnt inhibitors *axin2*, *nkd1,* and *ppp2r2b* was observed in Hdac8-overexpressed mutant embryos compared to controls while PCI treatment restored the expression of Wnt inhibitors (**Figure 4B**). To further demonstrate that Hdac8 modulates Wnt signaling, we evaluated the regulation of canonical Wnt signaling by assessing the levels of active- and total-β catenin by western blot techniques (**Supplementary Figure 6**), and we used a zebrafish canonical Wnt reporter transgenic line *Tg(TOPdGFP)* (Dorsky et al., 2002). Following *hdac8* overexpression, canonical Wnt signaling was increased also in the HSPCs in the CHT region, while it was switched off following PCI administration (**Figure 4C** and **Supplementary Figure 7**). Interestingly, a similar reduction in the HSPCs in the CHT region of *Tg(TOPdGFP)* or *Tg(CD41:GFP)* zebrafish embryos was achieved following inhibition of the canonical Wnt signaling in *hdac8*-mRNA-injected embryos by means of co-injection of the *dkk1b* transcript (50 pg/embryo) (Mazzola et al., 2019; **Figure 4D,E** and **Supplementary Figure 7**). Taken together, these data demonstrate that HDAC8 activates canonical Wnt pathway that, in turns, regulates hematopoietic cell proliferation (Richter et al., 2017; Mazzola et al., 2019). PCI administration downregulates Wnt signaling and reduces HSPCs, an important finding as Wnt downregulation is a clinical treatment currently in use for AML patients.

DISCUSSION

Aberrations in epigenetic regulators contribute to cancer, including leukemia insurgence, hence, the use of epigenetic modifiers may comprise a promising therapeutic approach (Nakagawa et al., 2007; Zhang et al., 2012). Epigenetic defects are generally reversible, as opposed to genetic changes, providing a strong rationale for a pharmaceutical intervention. Low level of acetylation due to high expression of HDACs (Nakagawa et al., 2007; Wang et al., 2016) is one of the most frequent epigenetic modifications found in cancer cells. HDACs are more expressed in hematological malignancies including AML than in normal hematopoietic cells (Bradbury et al., 2005; Marquard et al., 2009), and we demonstrated that forced expression of Hdac8 in zebrafish embryos induced an increase in HSPC number that can be rescued with the use of a specific HDAC8 inhibitor. HDACi have been used as therapeutic agents in AML, myelodysplastic syndromes, lymphoma, and chronic lymphoblastic leukemia (Melnick and Licht, 2002; Altucci and Minucci, 2009; Gloghini et al., 2009);

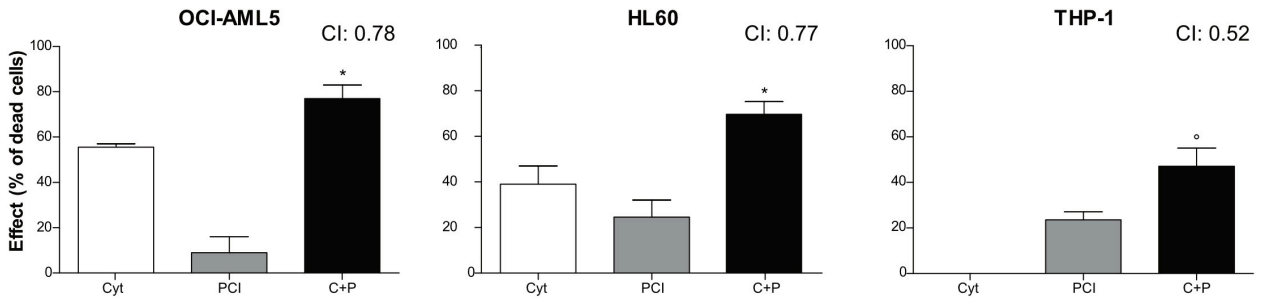

FIGURE 3 | Combination treatment with cytarabine + PCI. OCI-AML5, HL60, and THP-1 cell lines were treated for 72 h with single compound and their combination. CTG assay was used to assess the effect of each treatment on the cell culture viability. The bar graph represents the effect of one of the eight concentrations tested. CI values are shown for each combination. The results are presented as mean ± SEM from two independent experiments. Asterisk (*) indicates significance of combination treatment versus PCI, circle (°) indicates significance of combination treatment versus cytarabine. One symbol, $p < 0.05$, Student's *t*-test. Cyt and C, cytarabine; P, PCI; CI, combination index.

however, monotherapy elicits modest effects. There are several possible reasons behind this failure. For example, HDACi exert different outcomes depending on timing of administration and differentiation stage of the tumor (Kuendgen et al., 2011; Garcia-Manero et al., 2012; Novotny-Diermayr et al., 2012; Romanski et al., 2012; Xie et al., 2012; Candelaria et al., 2017; Huang and Zong, 2017; Young et al., 2017). In addition, the vast majority of preclinical and clinical studies deploying HDACi for anti-cancer treatment involved unselective inhibitors targeting all HDACs (pan-HDACi) with broad spectrum of side effects and toxicity, thus calling for exploitation of agents that specifically block individual HDACs. PCI is a specific small molecule inhibitor endowed with 200-fold higher selectivity for HDAC8 than for other HDACs; it is more effective and less toxic than pan-HDACi (Balasubramanian et al., 2008).

We demonstrated that HSPCs are sensitive to selective HDAC8 inhibition both in a zebrafish embryological context as well as in adult derived-AML cell lines. This is in agreement with findings of Qi et al. (2015) that showed that leukemic cells bearing inv(16) linked to high expression levels of HDAC8 (5–12 times that of CD34+ cells from healthy donors) were particularly responsive to treatment with PCI, although also non-inv(16) AML blasts showed a degree of sensitivity. Since the hierarchy of HSPCs is finely tuned during development until adulthood and subjected to different regulatory cues, our demonstration that the HDAC8 inhibition is effective on HSPCs during embryogenesis and in the adult, provides a common mechanism against HSPCs self-renewal and amplification and an attractive therapeutic treatment for the future.

We found that PCI elicits cytostatic or cytotoxic effect in AML sensitive cell lines. Mechanistically, sensitive cells undergo cell cycle arrest, followed by apoptosis when expressing p53. Cell cycle arrest in the G0/G1 phase and induction of p21WAF1/CIP1 expression was previously observed in neuroblastoma cells upon HDAC8 silencing, while an increase of cells in G2/M phase of the cell cycle was reported in hepatocellular cancer cells treated with PCI (Tian et al., 2016). p53 is a known HDAC8 target and its aberrant deacetylation by HDAC8 disables p53 function and promotes leukemic transformation (Wu et al., 2013). HDAC8 knockout or pharmacological inhibition effectively restores p53 acetylation and activity inducting apoptosis in inv(16)$^+$AML CD34$^+$ cells (Qi et al., 2015). Similarly, we observed p53-dependent apoptosis specifically in CD41-GFP$^+$ hematopoietic compartment following PCI treatment of zebrafish embryos overexpressing Hdac8. This population was increased in zebrafish embryos upon Hdac8 overexpression. Our findings are consistent with literature data demonstrating that HDAC8 regulates HSPC survival under hematopoietic stress by modulating p53 activity (Liu et al., 2009; Asai et al., 2011, 2012). Our experiments show that THP-1 cells underwent apoptosis, while the p53-null HL60 cell line remained blocked in the G0/G1 phase of the cell cycle. This led us to consider alternative mechanisms of growth arrest elicited by PCI treatment. Studies show that canonical WNT signaling is activated by HDAC8 (Tian et al., 2015; Ferrari et al., 2019). We now show that canonical Wnt pathway is significantly downregulated both in cell lines and in zebrafish embryos following HDAC8 inhibition. Downregulation of canonical Wnt pathway by PCI has been described in a model of hepatocellular cancer, in which HDAC8 physically interacts with chromatin modifier EZH2 to repress Wnt antagonists, activating Wnt pathway. PCI treatment, instead, reduced active β-catenin and *cyclin D1* expression in this system. We showed that PCI downregulates Wnt signaling independently of p53 status; however, it does not kill cells unless p53 is functional (Tian et al., 2016).

We explored the possibility of performing combination treatment and combined cytarabine with PCI. The two compounds synergize in all cell lines treated, including the less-PCI-sensitive OCI-AML5 cells. Although we failed to reduce the concentration of PCI, in THP-1 cell line the synergy was observed when combining a dose of cytarabine that alone elicits no effect. This may indicate that AML patients with high HDAC8 and functional p53 may particularly benefit from this combination. Indeed, a recent study reported the efficacy of HDAC8 inhibition in combination with FLT3 inhibitor in suppressing FLT3-ITD$^+$ AML cells, thus sustaining the potential of combination treatment employing HDACi and standard chemotherapy (Long et al., 2020). Phase II and III

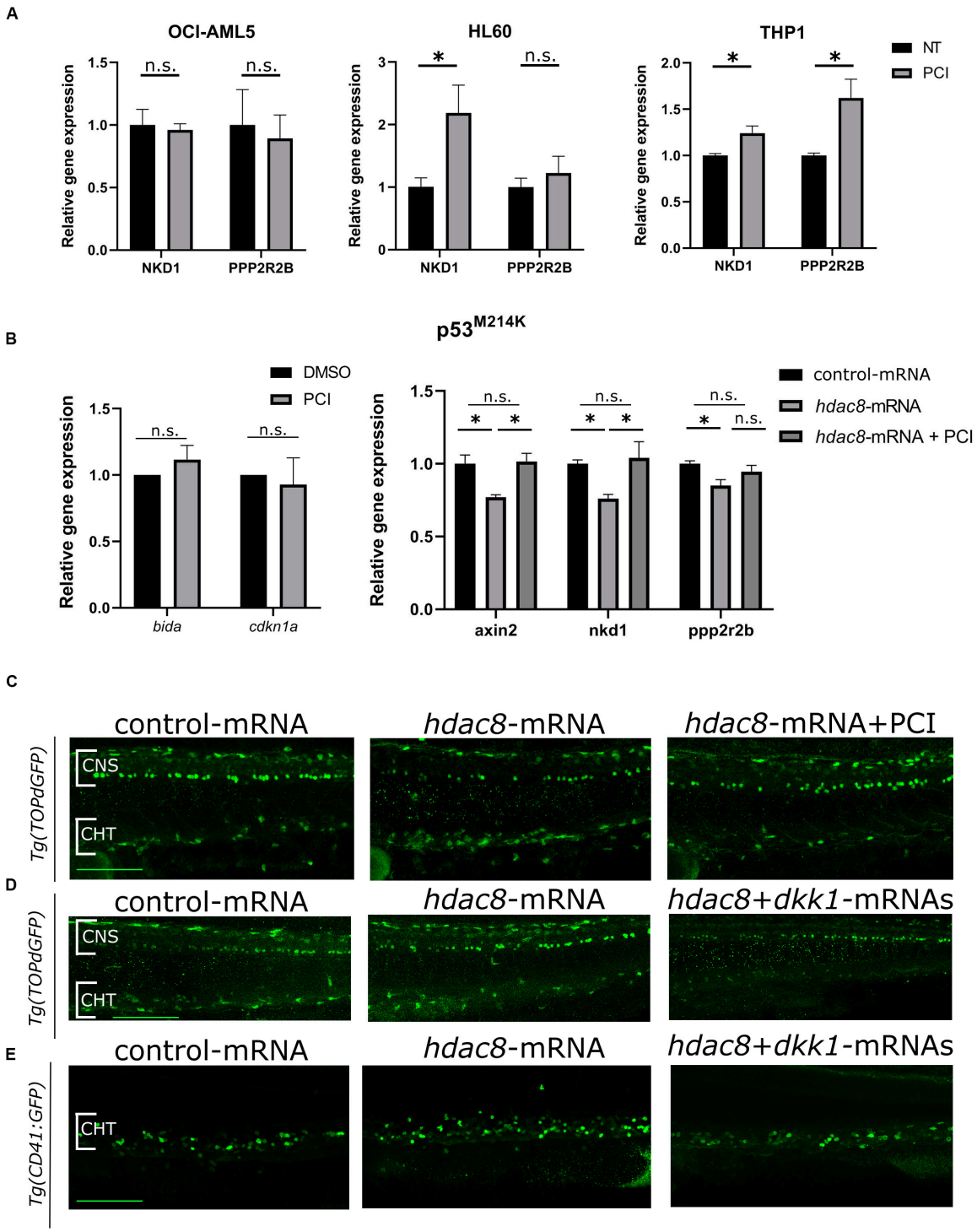

FIGURE 4 | Canonical Wnt pathway modulation by HDAC8. **(A)** Canonical Wnt pathway modulation by PCI in OCI-AML5, HL60, and THP-1 cell lines. *NKD1* and *PPP2R2B* Wnt inhibitors were analyzed by RT-qPCR. Results are presented as mean ± SD from three independent experiments. $*p < 0.05$, Student's *t*-test. **(B)** WNT pathway modulation by PCI in zebrafish mutant $p53^{M214K}$. *bida* and *cdkn1a* apoptotic genes and *axin2*, *nkd1* and *pppr2r2b* WNT target genes were analyzed by RT-qPCR. Results are presented as mean ± SD from three independent experiments. n.s.: not significant, $*p < 0.05$; One sample *t*-test for apoptotic genes, one-way ANOVA followed by Tukey *post hoc* correction for canonical WNT pathway genes. **(C)** Representative images of canonical Wnt modulation by *hdac8* overexpression and PCI on HSPCs in the CHT of the Wnt reporter line *Tg(TOPdGFP)*. **(D,E)** Representative images of canonical Wnt modulation by *hdac8* overexpression and Wnt inhibition by *dkk1b* injection on HSPCs in the CHT of the Wnt reporter line *Tg(TOPdGFP)* and of the HSPCs reporter line *Tg(CD41:GFP)*. NT, not treated; CNS, central nervous system; CHT, caudal hematopoietic tissue. Scale bar represents 100 μm.

clinical trial results confirm that HDACi act more efficiently when combined with conventional chemotherapy. However, more studies are needed to understand the precise mechanism of action of the combination.

Taken together, our study validates the preclinical potential of specific inhibition of HDAC8 as a potent therapeutic approach in AML.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

AP, AM, CB, AG, and MA conceived and designed the experiments. MS, MM, and GD performed the experiments on zebrafish. AG and DV performed the experiments on AML cells. AQ and MT performed the FACS analyses on zebrafish. MS, MM, GD, and AP analyzed the data on zebrafish. AG, DV, and CB analyzed the data on AML cells. MS, AG, DV, and AP wrote the manuscript. AP, AM, and AG supervised the manuscript drafting. AP supervised the research project. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00844/full#supplementary-material

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REVIEW
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Hematopoiesis: A Layered Organization Across Chordate Species

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The identification of distinct waves of progenitors during development, each corresponding to a specific time, space, and function, provided the basis for the concept of a "layered" organization in development. The concept of a layered hematopoiesis was established by classical embryology studies in birds and amphibians. Recent progress in generating reliable lineage tracing models together with transcriptional and proteomic analyses in single cells revealed that, also in mammals, the hematopoietic system evolves in successive waves of progenitors with distinct properties and fate. During embryogenesis, sequential waves of hematopoietic progenitors emerge at different anatomic sites, generating specific cell types with distinct functions and tissue homing capacities. The first progenitors originate in the yolk sac before the emergence of hematopoietic stem cells, some giving rise to progenies that persist throughout life. Hematopoietic stem cell-derived cells that protect organisms against environmental pathogens follow the same sequential strategy, with subsets of lymphoid cells being only produced during embryonic development. Growing evidence indicates that fetal immune cells contribute to the proper development of the organs they seed and later ensure life-long tissue homeostasis and immune protection. They include macrophages, mast cells, some γδ T cells, B-1 B cells, and innate lymphoid cells, which have "non-redundant" functions, and early perturbations in their development or function affect immunity in the adult. These observations challenged the view that all hematopoietic cells found in the adult result from constant and monotonous production from bone marrow-resident hematopoietic stem cells. In this review, we evaluate evidence for a layered hematopoietic system across species. We discuss mechanisms and selective pressures leading to the temporal generation of different cell types. We elaborate on the consequences of disturbing fetal immune cells on tissue homeostasis and immune development later in life.

Keywords: hematopoieisis, lymphopoieis, embryo, evo devo biology, layered

LAYERED HEMATOPOIESIS: A HISTORICAL PERSPECTIVE

Hematopoiesis, the process by which blood cells are produced, has been considered to be initiated by hematopoietic stem cells (HSC) that develop through multiple differentiation intermediates and give rise to all blood lineages. Recent studies have challenged this view by showing that specialized embryonic-derived subsets persist throughout life.

During embryogenesis, successive waves of mesoderm-derived hematopoietic progenitors contribute to the formation of erythroid, myeloid, and lymphoid lineages. In the 1920s, studies on embryonic hematopoiesis revealed that the first blood cells appear in the yolk sac (YS): red blood cells were found in clusters surrounded by an endothelial layer, named blood islands. The order of events that resulted in the formation of these structures led embryologists to hypothesize that both hematopoietic and endothelial cells had a common origin, a bipotent cell designated hemangioblast (see **Table 1**; Choi et al., 1998). In the 1970s, Moore and Owen (1967) showed that the embryonic day (E) 7 chicken YS generated mostly erythroid and also myeloid and lymphoid progeny after transplantation into irradiated embryos, pointing to a YS origin of HSCs. In mammals, the first single-cell assays showed the existence of multilineage progenitors in the YS, shortly after the beginning of circulation (Moore and Metcalf, 1970). Subsequent studies using chick–quail or chick–chick chimeras challenged the view that YS was the source of HSCs and showed that long-lasting hematopoietic potential was only found in intra-embryonic progenitors (Dieterlen-Lievre, 1975; Lassila et al., 1978). In mouse, culture of YS vs. the intra-embryonic aorta-gonad-mesonephros (AGM) region before the establishment of circulation demonstrated that the origin of HSCs is exclusively intra-embryonic (Cumano et al., 1996). It is now accepted that three waves of hematopoietic progenitors are generated in the mammalian embryo. The first wave is generated during gastrulation in the YS blood islands (Godin and Cumano, 2002). Soon after, a second wave of progenitors emerges in the YS vascular plexus (Frame et al., 2016). These two hematopoietic waves, which generate primitive and definitive erythrocytes, respectively, provide the oxygen needed for embryo survival as well as megakaryocytes and myeloid cells that are important for tissue remodeling and hemostasis. The third wave occurs in the great vessels of the embryo and generates HSCs that will migrate to the fetal liver (FL) where they expand (Ema and Nakauchi, 2000). This pool of HSCs will sustain hematopoiesis throughout adult life, generating not only erythroid and myeloid cells but also lymphoid cells.

The notion that YS populations are only produced and necessary during embryonic development has recently been challenged. Inducible lineage-tracing mouse models formally demonstrated that YS progenitors generate unique tissue-resident macrophage populations that persist throughout adulthood (Ginhoux et al., 2010; Gomez Perdiguero et al., 2015). These studies challenged the dogma that HSCs were the source of all hematopoietic lineages in the adult and provided evidence for the concept of a "layered" hematopoietic system.

Embryonic hematopoiesis is thus characterized by the partial overlap of distinct waves of progenitors that transit through different organs in embryonic and adult life. Each wave is well-orchestrated in time and space, each serving specific internal and external environmental needs. We will speculate on the selective advantages of a layered system across evolution that relates to hematopoiesis in general and to lymphopoiesis.

Abbreviations: a, anterior; AGM, aorta, gonads, mesonephros; BM, bone marrow; BMP4, bone morphogenic protein 4; c, cortical; CFC, colony-forming cells; CHT, caudal hematopoietic tissue; CLP, common lymphoid progenitor; CS, Carnegie stage; DA, dorsal aorta; DETC, dendritic epidermal T cells; DLP, dorsal lateral plate; dpf, days post-fertilization; E, embryonic day; EHT, endothelial-to-hematopoietic transition; EMP, erythromyeloid progenitors; Ery-P, primitive erythroid; Epo, erythropoietin; ES, embryonic stem; FL, fetal liver; HE, hemogenic endothelium; hpf, hours post-fertilization; HSC, hematopoietic stem cells; HIAC, hematopoietic intra-aortic clusters; ICM, intermediate cell mass; IL, interleukin; LMP, lympho-myeloid-restricted progenitors; LTi, lymphoid tissue inducer; m, medullary; p, posterior; PAF, para-aortic foci; PBI, posterior blood islands; PLM, posterior-lateral mesoderm; RA, retinoic acid; RBI, rostral blood islands; Shh, sonic hedgehog; TdT, terminal deoxynucleotidyl transferase; TEC, thymic epithelial cell; TSP, thymic seeding progenitors; VBI, ventral blood islands; YS, yolk sac.

EMBRYONIC HEMATOPOIESIS IN DIFFERENT SPECIES: A COMMON STRATEGY TO MAKE BLOOD

Foreword

Hemocytoblasts or primordial HSCs, derived from mesoderm, generate multiple hematopoietic cell types and are found across species starting in coelomic invertebrates. Key transcription factors that modulate the divergence of different hematopoietic cell types are conserved. For example, orthologs of *Gata*, *Fog*, and *Runx* have been identified in *Drosophila*. These transcription factors regulate the production of plasmatocytes, which exert phagocytic activity, granulocytes, with metabolic activity and immune function, and oxygen-transporting red blood cells (Evans et al., 2003; Hartenstein, 2006). In contrast, Metazoa without coelom are devoid of well-defined mesoderm and have a gelatinous matrix between ectoderm and endoderm enclosing large numbers of amoebocytes. These cells move using pseudopodia and fulfill functions that differ among species, ranging from defending the organism against pathogens, digesting food, or disposing of waste.

A major accomplishment in the transition of invertebrate to vertebrate metazoans was the development of a closed circulatory system and that of a centralized pumping organ, the heart, which ensures a rapid distribution of oxygen and nutrients to tissues, and the deployment of immune surveillance in the organism. Most invertebrates rely on myoepithelial cells with contractile capabilities to ensure that cells transit from the hemal spaces (fluid-filled sinus without a lining) and channels to the tissues, collectively designated as an open circulatory system (Muñoz-Chápuli et al., 2005; Hartenstein, 2006). However, there are several exceptions to this rule. For example, annelids have a closed

TABLE 1 | Glossary.

Hemangioblast. A bipotent hematopoietic and endothelial progenitor. The most common example is yolk sac blood islands in mice where primitive erythrocytes and the endothelial cells lining these structures are thought to have a common progenitor. In the mouse embryo, multiple progenitors contribute to blood island formation, and therefore, their common origin remains to be formally demonstrated.
Endothelial-to-hematopoietic transition (EHT). The process whereby cells with morphology, phenotype, and spatial position of endothelial cells convert into a hematopoietic cell. This process has been identified to be at the origin of erythromyeloid bipotent cells in the vasculature of the yolk sac and of multipotent hematopoietic stem cell progenitors in the dorsal aorta. This conversion has been clearly visualized in the zebrafish embryo and is independent of cell division. In the fish embryo, newly converted single hematopoietic cells appear to rapidly egress into the space between the vein and the artery. By contrast, in mammalian and chicken embryos, newly converted cells extensively expand *in situ* forming hematopoietic intra-aortic clusters budding into the lumen, before being released into blood circulation.
Hemogenic endothelium. Designates the endothelial cells in the yolk sac and dorsal aorta that have the capacity to convert into a hematopoietic cell through a process named EHT.
Hematopoietic intra-aortic clusters (HIAC). Clusters of hematopoietic cells adjacent to the endothelium protruding in the lumen of the dorsal aorta after being generated through EHT. They are found in birds and mammalian embryos, but not in zebrafish.
Primitive wave. The first hematopoietic cells with a given identity. Designates the first hematopoietic cells that generate primitive erythrocytes. Primitive erythrocytes derive from erythroid progenitors in the YS blood islands and are large nucleated cells that express embryonic hemoglobins Hbb-bh1 and Hbb-y. This concept also includes macrophages and megakaryocytes.
Definitive wave. Refers to the hematopoietic cells that generate definitive erythrocytes. These are enucleated cells that express embryonic and adult forms of hemoglobin (Hbb-bh1 and Hbb-b1) but lack Hbb-y hemoglobin expression. They can originate from either erythromyeloid progenitors that derive from the YS or from hematopoietic stem cells of intra-embryonic origin, during embryonic or post-natal life, respectively.
Somatic recombination. Genomic recombination that occurs in somatic cells. It is the process whereby the genes coding for the antigen receptors in T and B lymphocytes are assembled from the random assortment of variable (V), diversity (D), and joining (J) elements on immunoglobulin heavy chain, TCRβ and TCRδ chains, or from V and J elements in Ig light chains, TCRα and TCRγ chains.
T cell negative selection. Elimination of T lymphocytes expressing a self-reactive antigen receptor occurring in the thymus. Negative selection is mediated through the interaction of immature thymocytes with thymic epithelial cells located in the thymic medulla (mTEC) and dendritic cells (DC) that express a large collection of tissue-specific peptides.
Antigen receptor repertoire. Collection of the diverse antigen receptors expressed by B or T lymphocytes and generated by somatic recombination (see above). The diversity of the antigen receptor repertoire is further increased by trimming of the ends of the segments and addition of nucleotides without templated (N sequence addition) prior to the joining.
Tolerance. The absence of reactivity toward a given antigenic determinant. Often referred to self-tolerance, it designates the absence of lymphocytes that recognize antigen determinants from the organism where they reside. Defective tolerance induction results in autoimmune disorders.

circulatory system with pumping muscular blood vessels. Molluscs, instead, have an open circulatory system and one (or several) centralized hearts composed of cavities (atria and ventricles).

The emergence of a closed circulatory system with a centralized heart is linked to the appearance of vascular endothelial cells, the origin of which has long been a matter of debate. It was hypothesized that endothelial cells originate from the amoebocytes that, in acoelomatic invertebrates, adhere to the basement membrane that lines the hemal cavities (Muñoz-Chápuli et al., 2005). This hypothesis links the development of endothelial and hematopoietic cells throughout vertebrate evolution. We analyzed here different model systems of chordates in which independent generations of hematopoietic cells and different primary hematopoietic organs have been documented (**Table 2**).

Cephalochordates (Amphioxus)

The amphioxus are invertebrates that together with Tunicates stand in close phylogenetic proximity to vertebrates (Delsuc et al., 2006; **Figure 1**). They appear, therefore, to be the model of choice to understand the evolution of a circulatory system. Developmental studies in amphioxus demonstrated that their cardiac region is decentralized and that blood is pumped by contractile vessels throughout life. By contrast, analysis of orthologs of *Pax2/5/8*, together with those of key vessel and hematopoietic development markers *Flk1* and *Scl*, identified specific expression in an AGM-like region, thus pointing to a similar origin of hematopoietic cells in amphioxus and vertebrates (Pascual-Anaya et al., 2013). This hypothesis was reinforced by experiments showing that the treatment of amphioxus larva with retinoic acid (RA) inhibited the expression of hematopoietic genes, reminiscent of what was observed in zebrafish larva and mouse embryonic stem (ES) cells where treatment with RA inhibited primitive hematopoiesis (Pascual-Anaya et al., 2013). Altogether, these data indicate that despite lacking a centralized heart, amphioxus developed a process that resembles hemogenic endothelium (HE) and endothelial-to-hematopoietic transition (EHT) (see **Table 1**). These studies were performed up to the stage of 2 day-old larva and, therefore, do not allow to assert whether invertebrates also generate hematopoietic progenitors at independent sites.

Tunicates

Tunicates are currently considered as the closest relatives to vertebrates (Delsuc et al., 2006; **Figure 1**). Unlike cephalochordates, they have a centralized pumping organ (the heart) and an established network of blood vessels (Rosental et al., 2020). They have, therefore, in the sessile adult animal, circulating blood cells composed of myeloid cells. They also have a progenitor compartment located in the endostyle, tentatively considered as a functional equivalent to the bone marrow (BM) (Rosental et al., 2018). Cells endowed with cytotoxic activity that reject allografts could represent the equivalent population to NK cells. There is presently no experimental evidence on the origin of these cells.

TABLE 2 | Models to study hematopoiesis.

Species	Advantages	Disadvantages	Methodologies used for the study of hematopoiesis
Mouse	Broad availability of transgenic and gene-deficient strains	Species-specific differences in basic biology (e.g., replicative rate, DNA damage response, etc.) Inbred mouse strains do not account with genetic diversity; the choice of a specific genetic background can influence the observed phenotype	*In vitro* clonal assays (CAFCs, LTC-IC, CFU assays) Flow cytometry phenotyping Functional repopulation assays (competitive and non-competitive transplantation assays) Lineage tracing models Clonal analysis of lineage fate in native hematopoiesis (Sun et al., 2014) Single-cell transcriptomics and proteomic analysis
Human	Extensively characterized hematopoietic system Higher translational value for clinical applications	Limited sources of human hematopoietic cells and tissues Limited accessibility to steady-state human hematopoiesis: limited studies of human hematopoietic cells on their natural microenvironment; no clonal tracking possible out of transplantation setting *In vivo* xenotransplantation murine models only capture part of the cell-intrinsic properties of human hematopoiesis Cell-extrinsic aspects of human hematopoiesis are difficult to access and study *In vivo* assays are time-consuming	Characterization of hematopoietic populations by surface markers expression—flow cytometry (Notta et al., 2011, 2016) Evaluation of differentiation potential—*in vitro* colony-forming assays (Notta et al., 2016) *In vivo* functional repopulation assays in immunodeficient mice—xenograft models (Kamel-Reid et al., 1989; Beer and Eaves, 2015) Repopulation dynamics of HSCs in humans—post-transplantation clonal tracking (Scala and Aiuti, 2019) Single-cell transcriptomics and proteomic analysis
Zebrafish	Rapid and external development Embryo optical transparency Easy high-resolution optical imaging in live animals Large-scale genetic and chemical screens Several transgenic lines available (reviewed in Stachura and Traver, 2016)	Lack of antibodies for phenotypic characterization Lack of knock-in technologies Need to establish breeding standards; Inbreed and outbreed depression	Genome targeting (ZFNs, TALENs, CRISPR, and morpholino-mediated gene knockdown) to produce mutants of interest (reviewed in Sertori et al., 2016) Major blood lineages isolation by size and granularity using FACS (Traver et al., 2003) Hematopoietic cell transplantation (Traver et al., 2003, 2004; Hess et al., 2013) Stromal culture assays (Stachura et al., 2011; Wolf et al., 2017) Clonal methylcellulose assays (Stachura et al., 2011) Parabiotic embryos for cell migration and homing studies (Demy et al., 2013) High-resolution time-lapse live imaging (e.g., Bertrand et al., 2010; Kissa and Herbomel, 2010) Xenotransplantation (Hess and Boehm, 2016; Parada-Kusz et al., 2018) *In vivo* lineage tracing (e.g., Murayama et al., 2006; Jin et al., 2007; He et al., 2020)
Axolotl	Neoteny (no metamorphosis) Regeneration without scar tissue formation	Lack of antibodies for phenotypic characterization Gene manipulation difficult to perform Long periods of generation	Transplantation (Lopez et al., 2014)
Xenopus	Large embryo size Lineage tracing strategies Available chimeric procedures to determine cell origin	Lack of antibodies for phenotypic characterization Gene manipulation difficult to perform	Chimeras (Du Pasquier et al., 1989) Lineage tracing of blastomeres (Ciau-Uitz et al., 2000)
Chicken	Large egg size Amenable to surgical manipulation Quail–chicken chimeric system	Lack of antibodies for phenotypic characterization Lack of growth factors for *in vitro* cultures Gene manipulation technologies difficult to perform	Quail–chicken and chicken–chicken chimeras (Le Douarin, 1969) Corio-allantoid transplantation (Yvernogeau and Robin, 2017) Lineage tracing (Jaffredo et al., 2000)

DIC, differential interference contrast; FACS, fluorescence-activated cell sorting; TALENs, transcription activator-like effector nucleases; ZFNs, zinc finger nucleases.

TABLE 3 | Hematopoietic waves.

Species	Adult hematopoietic sites	Embryonic hematopoietic waves	Location	Potential
Mouse	Bone marrow Spleen	Primitive	YS	Primitive erythrocytes, macrophage colony-forming cells, and megakaryocyte colony-forming cells (Palis et al., 1999)
		Definitive	YS	Erythromyeloid progenitors (Palis et al., 1999; Bertrand et al., 2005)
			AGM	HSC (Cumano et al., 1996; Medvinsky and Dzierzak, 1996)
Human	Bone marrow	Primitive	YS	Primitive erythrocytes and myeloid cells (Tavian et al., 1999)
		Definitive	YS	Erythromyeloid progenitors (Migliaccio et al., 1986)
			AGM	HSCs (Tavian et al., 1996)
Zebrafish	Kidney marrow (pronephros) Thymus	Primitive	VLM, RBI	Primitive macrophages and granulocytes (Herbomel et al., 1999)
			PLM, ICM	Primitive erythrocytes (Detrich et al., 1995; Thompson et al., 1998)
		Definitive (transient)	PBI	Erythromyeloid progenitors (mammalian EMP-like) (Bertrand et al., 2007)
				Lympho-myeloid and myeloid progenitors* (He et al., 2020) CD4 Tαβ lymphocytes (HSC-independent) (Tian et al., 2017)
		Definitive	VDA	
				HSC (Bertrand et al., 2010; Kissa and Herbomel, 2010)
Axolotl	Spleen Thymus	?	?	HSC? (Lopez et al., 2014)
Xenopus	Liver periphery (HSC) Spleen Bone marrow (GMP and lymphocytes) Thymus	Primitive Definitive Definitive (HSC)	aVBI pVBI DLP	Primitive erythrocytes (Ciau-Uitz et al., 2010) Definitive erythrocytes (Ciau-Uitz et al., 2010) HSC (Ciau-Uitz et al., 2010)
Chicken	Bone marrow Thymus Bursa of Fabricius	Primitive	YS	Macrophages and erythrocytes
		Definitive	Dorsal aorta	HSC (Yvernogeau and Robin, 2017)

*BM, bone marrow; ICM, intermediate cell mass; HSCs, hematopoietic stem cells; PBI, posterior blood island; PLM, posterior-lateral mesoderm; VDA, ventral dorsal aorta; VLM, ventrolateral mesoderm; YS, yolk sac; VBI, ventral blood islands; DLP, dorsal lateral plate; GMP, granulocyte-macrophage progenitor. *Erythroid potential was not assessed for these progenitors.*

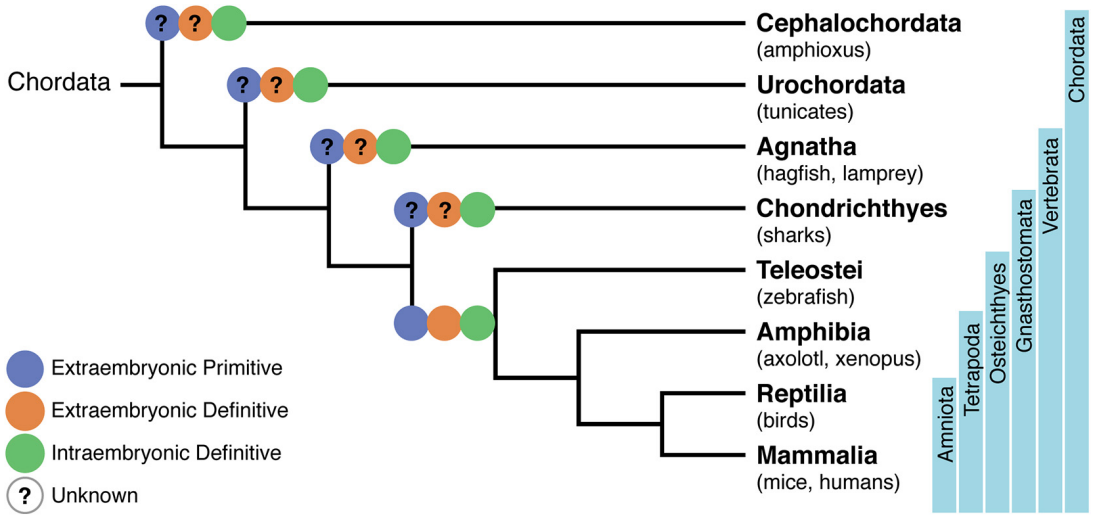

FIGURE 1 | Phylogenetic tree of chordates showing the evolutionary relationships of the hematopoietic system. A phylogeny representing Cephalochordata to Amniota, illustrating the development of a layered hematopoietic system with (i) yolk sac (YS) or extra-embryonic primitive hematopoietic cell generation in blue, (ii) YS definitive hematopoietic cell generation in orange, and (iii) intra-embryonic definitive hematopoiesis in green. Osteichthyes (which include zebrafish, axolotl, Xenopus, birds, mice, and humans) were the first where the distinct origins of hematopoietic cells have been demonstrated. Lack of information on specific processes are represented with a question mark.

Agnathes or Jawless Chordates (Lampreys and Hagfish)

Lampreys possess circulating erythrocytes, myeloid and lymphoid cells, and were the most ancient vertebrates to show a robust adaptive immunity with the capacity to reject allografts, to produce specific antibodies, and to develop immunological memory. The molecular basis of this sophisticated immune system is a unique set of "building blocks" that encode lymphocyte antigen receptors (Pancer et al., 2004). They differ from those in higher vertebrates but are assembled in a similar manner, undergoing a process of somatic recombination (see **Table 1**) and exerting similar functions, illustrating an unprecedented example of parallel evolution. An independent site for T cell development, equivalent in function to the mammalian thymus, has been identified in the gills (Bajoghli et al., 2011). Lampreys have three different loci of the "building blocks" that encode antigen receptors of three distinct lymphoid cells reminiscent of the mammalian B, αβ-T, and γδ-T cells, indicating strong selective pressures for non-redundant functions of these different cell types (Boehm et al., 2018).

Lampreys develop through a complex life cycle with a larval stage, called ammocoetes, a metamorphosis that lasts for several months and an adult stage. In ammocoetes, the first hematopoietic cells are found in the typhlosole, which is a longitudinal fold of the intestinal inner wall, and also in the adipose tissue of the nephric fold. During metamorphosis, blood cell formation is displaced from these sites to the supra-neural body, a unique organ equivalent to the mammalian BM. The typhlosole is composed of mesenchymal cells located close to the dorsal aorta, and that form blood islands where the first hematopoietic cells are found. These hematopoietic cells appear by morphology to belong to the myeloid, erythroid, and lymphoid lineages and many of them are actively proliferating (Amemiya et al., 2007). Although it has proven difficult to identify, in lampreys, equivalent anatomical sites to the higher vertebrate YS or dorsal aorta (DA), it is possible that the first hematopoietic cells are generated in the typhlosole blood islands.

Chondrichthyes or Cartilaginous Fish (Sharks)

Sharks are the most primitive organisms to have a functional adaptive immune system that is, unlike Agnathes, similar to that found in mammals. It is also in cartilaginous fish where the spleen is found for the first time in phylogeny and where hematopoietic progenitors were first detected and identified (Manca et al., 2018). It appears, therefore, that the spleen is the major hematopoietic organ already active in the embryo. The limited information concerning the hematopoietic development in these organisms suggests that HSC originated in the YS and transit later to the Leydig organ (located close to the esophagus) and to the spleen (Manca et al., 2019). In summary, there are different sites where hematopoiesis occurs over time, but the emergence of hematopoietic cells at multiple sites have not been documented.

Teleosts or Bony Fish (Zebrafish)

Zebrafish (*Danio rerio*) is the most well-studied teleost species and has been an important model to study hematopoietic development, given that it is uniquely suitable for large-scale mutagenesis experiments, genome editing, chemical screenings, and high-resolution live imaging (**Table 2**).

Similar to other vertebrates, zebrafish hematopoiesis develops through successive waves emerging at different locations (**Table 3** and **Figure 2**). Contrary to mammals, birds, and other teleosts, zebrafish primitive hematopoiesis initiates intra-embryonically,

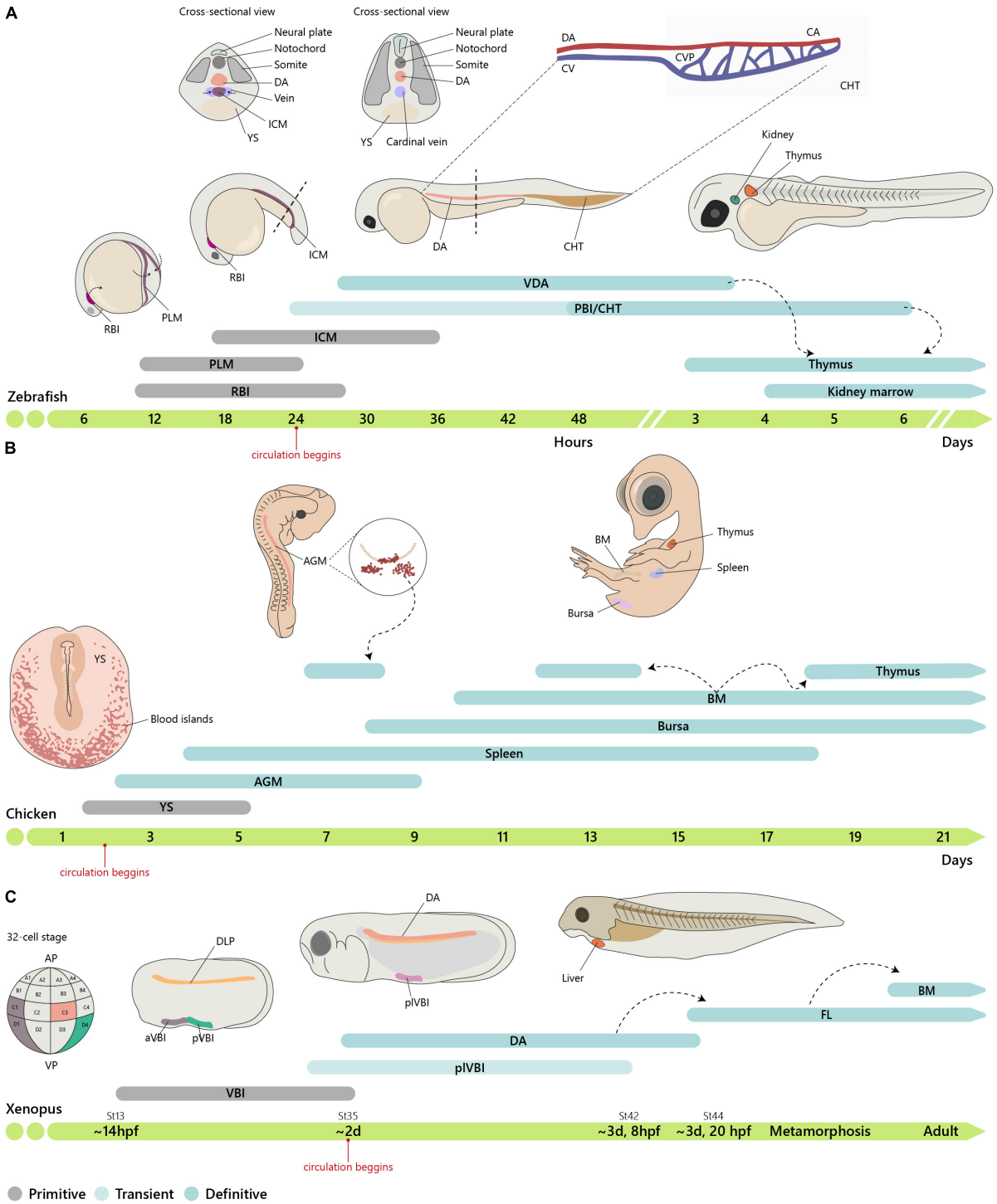

FIGURE 2 | Embryonic origin of the hematopoietic system. **(A)** Timeline of hematopoietic development in zebrafish. In zebrafish, primitive hematopoiesis occurs in the RBI and ICM region generating primitive macrophages and erythrocytes, respectively. The CHT, consisting of the CA (the continuation of the DA as it enters the tail), CV, and an endothelial network in between, the CVP, hosts a niche for HSC expansion and differentiation, reaching a peak at around E6. **(B)** In chicken, primitive hematopoiesis *at* E1.5 occurs in the YS blood islands. IAHCs are first detected at E2.25, reach a peak at E3, and gradually decrease, being residual at E5.5. PAF cells are detected at E2.5, rapidly surpassing the number of HIAC and last until around E9. **(C)** In Xenopus, the first hematopoietic site is the VBI (YS equivalent). Subsequent generation occurs after progenitor cells from the DLP migrate to the midline where they coalesce to give rise to the dorsal aorta (AGM). Cells from the two waves colonize the liver, which is the definitive site of hematopoiesis in both larval and adult stages. Xenopus were staged according to Nieuwkoop and Faber. See http://www.xenbase.org/anatomy/alldev.do for equivalences to dpf. AGM, aorta-gonad-mesonephros; aVBI, anterior VBI; BM, bone marrow; CA, caudal artery; CHT, caudal hematopoietic tissue; CVP, caudal vein plexus; DA, dorsal aorta; ICM, intermediate cell mass; FL, fetal liver; *PAF, para-aortic foci;* PBI, posterior blood island; PLM, posterior-lateral mesoderm; pVBI, posterior VBI; plVBI, posterior-lateral VBI; RBI, rostral blood islands; YS, yolk sac; VBI, ventral blood island.

in a structure known as intermediate cell mass (ICM). This structure results from the migration of two posterior bilateral stripes of lateral mesoderm (posterior-lateral mesoderm, PLM) to the trunk midline. It is in this structure where primitive erythroid progenitors and endothelial cells were found (Detrich et al., 1995; Thompson et al., 1998). Hematopoietic commitment, defined by the expression of the erythroid-specific transcription factor *Gata1*, occurs as early as the 2-somite stage [around 11 h post-fertilization (hpf)] in the PLM. Another site of primitive hematopoiesis was identified in the rostral blood island (RBI) region of the anterior mesoderm. This site produces macrophage-like cells that migrate into the yolk syncytial layer and disseminate through different tissues (Herbomel et al., 1999).

The DA resembles the mammalian AGM and was first suggested to harbor hematopoietic stem cell activity based on the identification of transcripts for the zebrafish orthologs of *cmyb*, *lmo2* (Thompson et al., 1998), and *runx1* (Kalev-Zylinska et al., 2002). Co-expression of these markers with vascular markers (*fli1*, *flk1*, and *flk4*) suggested they are hemangioblasts (Thompson et al., 1998). Challenging this idea, the emergence of definitive HSCs in the DA was directly visualized as an EHT mechanism where cells in endothelial position and morphology adopted a spherical shape and migrated ventrally toward the caudal vein (Bertrand et al., 2010). This process did not require cell division and EHT was taken as direct evidence for HSC originating from HE (Kissa and Herbomel, 2010).

The endothelial lineage branch to create HE before cells migrate across the ventral somites to reach the ventral aspect of the dorsal aorta (Kobayashi et al., 2014). This process involves the expression of adhesion molecules (Jam) that ensure the required strength of Notch signaling delivered by the somite cells (Kobayashi et al., 2014). Attempts to dissect the molecular events that shape EHT identified Runx1 as an essential player inducing the survival of newly generated hematopoietic cells (Kissa and Herbomel, 2010). Bmp4 is expressed in the ventral aspect of the dorsal aorta and promotes the hematopoietic stem cell program, whereas the expression of Shh in the roof maintains the arterial program (Wilkinson et al., 2009). More recently, it was shown that myeloid cells (Espín-Palazón et al., 2014) and metabolic alterations that promote inflammasome-induced IL1β-signaling in macrophages enhance HSC production, and inflammasome inhibition results in decreased hematopoietic generation (Frame et al., 2020). Taken together, these observations indicate that the EHT is a complex process involving several key molecular players and different cell types. The sequence of events and the precise molecular requirements essential for EHT remain largely incomplete, and attempts to recreate the conditions to promote EHT *in vitro* are still being developed (Gomes et al., 2018).

Contrary to mammals and birds, intra-aortic clusters were not observed in zebrafish and newly formed HSCs did not directly enter circulation (**Figure 3**). HSCs migrate and reside transiently in the posterior region in the tail, called caudal hematopoietic tissue (CHT) (Murayama et al., 2006). At earlier stages, before 36 hpf, this region, which also generates hematopoietic progenitor cells, independently of HSCs (discussed below), is more commonly referred as posterior blood island (PBI) and corresponds to the ventral portion of the tail immediately caudal to the yolk tube extension (Bertrand et al., 2007). Like the mammalian FL, the CHT environment promotes the expansion and differentiation of newly formed HSCs. This niche is modulated by the incoming hematopoietic cells that remodel the vascular niche (Tamplin et al., 2015). Migration of the hematopoietic cells toward the CHT depends on CxCL8/CXCR1 chemokine signaling that also promotes residency (Blaser et al., 2017); Klf6a promotes their maintenance and expansion in the CHT through CCL25b-Ccr7 chemokine signaling (Xu et al., 2015).

Fate-mapping studies revealed erythromyeloid progenitors (EMP), analogous to the mammalian EMP, arise at 24–30 hpf in the vascular plexus of the PBI (Bertrand et al., 2007). These cells have both erythroid and myeloid differentiation potentials but lack lymphoid potential. In mice, it is currently accepted that the microglia (brain-resident macrophages) are exclusively derived from the YS (Ginhoux et al., 2010; Gomez Perdiguero et al., 2015). By contrast, in zebrafish, embryonic/larval and adult microglia were shown to have different sources: the RBI, the YS equivalent for the former and the ventral wall of the DA for the latter (Xu et al., 2015). These differences may be species-specific and/or reflect different timing in the establishment of the blood–brain barrier.

Fate-mapping analysis using high temporal–spatial resolution cell labeling techniques identified two waves of T lymphopoiesis. One, which is HSC-independent, originates in the DA and PBI regions and generates predominantly CD4 αβ T cells found only in the larval stage. A later HSC-dependent wave develops from progenitors restricted to the DA and gives rise to various types of T cells from the larval stage up to adulthood (Tian et al., 2017). Lymphopoiesis is initiated in the thymus after 3 days post-fertilization (dpf), with the onset of *rag1* expression, although the first progenitor immigrants arrive in the thymus by 54 hpf (Kissa et al., 2008). Live imaging of these progenitors revealed that besides extravasation from the nearest vessels, most of them migrate to the thymus through the mesenchyme with remote sites of extravasation (Kissa et al., 2008). By 4 dpf, hematopoietic cells seed the kidney marrow (pronephros), the BM counterpart in the zebrafish, where they establish the adult hematopoietic system (Murayama et al., 2006).

Amphibians (Axolotl and *Xenopus*)

Two major amphibian models have contributed to our understanding of biological processes. One is the urodele *Axolotl mexicanum*, a model of tissue regeneration without scar tissue formation. The other is the anuran *Xenopus laevis* (allotetraploid) and *tropicalis* (diploid) that are the most-studied amphibian model systems for vertebrate embryonic development, cell and molecular biology, immunology, and, more recently, evolutionary diversification following genome duplication.

Axolotl

The *Axolotl mexicanum* has been used to study tissue regeneration without fibrosis. Although little is known of

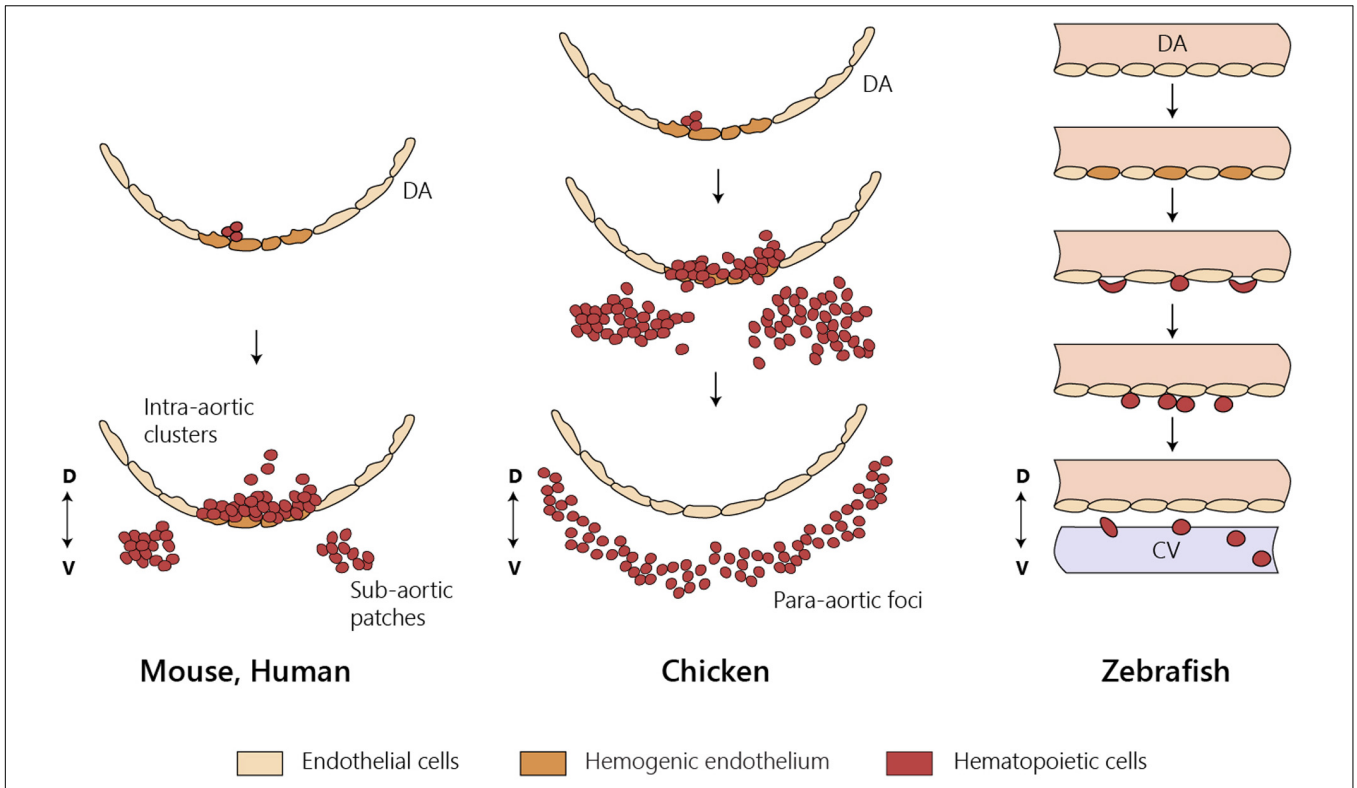

FIGURE 3 | HSC emergence at the dorsal aorta in mice, chicken, and zebrafish. Intra-embryonic hematopoietic clusters (IAHCs) containing newly formed HSCs in birds and in mammals. IAHC originate from PAF in chicken and sub-aortic patches in mice. Contrary to mammals and birds, IAHCs are not observed in zebrafish and newly formed HSCs do not enter directly into circulation. CV, caudal vein; D, dorsal; DA, dorsal aorta; V, ventral; PAF, para-aortic foci.

its hematopoietic system, this is nevertheless an interesting model to study hematopoiesis because, unlike *Xenopus*, adult animals retain many embryonic features (neoteny) and do not undergo a clear stage of metamorphosis. The main hematopoietic organs were recently identified (Lopez et al., 2014). Using cell transplantation assays with minimal graft-vs.-host reaction, it was shown that the liver is the first site of hematopoiesis. High expression of the lymphocyte-specific enzyme terminal deoxynucleotidyl transferase (TdT) was found transiently in the liver and later being restricted to the spleen (Golub et al., 2004) where it remains throughout life. So, like in other model systems lacking hematopoietic BM, the spleen is the adult hematopoietic organ. These recent advances, however, did not indicate the origin of the hematopoietic cells that colonize these organs.

Frogs

The *Xenopus* model has been extensively used to study hematopoiesis because larval chimeras are easy to make and early embryos have large cells that can be marked and traced in fate-mapping studies (Ciau-Uitz and Patient, 2016). Natural polyploids have also been used as models to study the regulation of gene duplication and gene silencing and to identify donor and recipient cells in transplantation experiments (Du Pasquier et al., 1989). Transplantation and chimera experiments indicated that hematopoietic cells originate first in the ventral blood islands (VBI) and later in the tissues originated from the dorsal lateral plate (DLP) (Chen and Turpen, 1995). Lineage tracing experiments established that these two territories are independent. Thus, blastomeres from 32-cell stage embryos that contribute to the DLP and to adult HSCs do not contribute to VBI, and conversely, blastomeres that contribute to VBI do not participate in the generation of adult HSCs (Ciau-Uitz et al., 2000). The VBI, the equivalent site to the mammalian YS, comprises an initial anterior region (aVBI) where primitive erythrocytes are first found along with myeloid cells and, at later stages, a posterior VBI (pVBI) that contains definitive erythrocytes, myeloid cells, and, similar to what was found in the zebrafish, also lymphocytes (Smith et al., 1989; Turpen et al., 1997). A third wave of hematopoietic generation occurs in the AGM, formed by the midline migration of the DLP, with the emergence of HSC and hematopoietic intra-aortic clusters (HIAC) formation.

It has been proposed that in aVBI, hemangioblast-like cells are at the origin of the first hematopoietic cells (Ciau-Uitz et al., 2010). By contrast, hemangioblast-like cells were not found in the pVBI, and hematopoietic cells are produced through an EHT process similar to that operating in fish, birds, and mammals in the DA and large arteries. Thus, hematopoiesis in frogs appears to occur in three independent waves similar to bony fish, birds, and mammals. Recently, a fourth myeloid cell generation has

been described to occur in the mesenchyme posterior to the DLP (Imai et al., 2017).

The peripheral region of the liver was shown to be the primary hematopoietic organ throughout life containing HSC and erythroid progenitors, whereas the BM contains myeloid progenitors that respond to myeloid growth factors (Yaparla et al., 2019). Lymphocytes are produced in the thymus and the spleen in juvenile frogs, whereas the BM and the thymus have lymphopoietic activity in the adult (Greenhalgh et al., 1993).

Birds

The large size and the easy manipulation of the avian fertilized egg allowed the construction of xenogeneic and congenic chimeras. The quail–chicken chimera system devised by Nicole Le Douarin combined with a rigorous identification of tissues originated from each species provided a reliable lineage-tracing tool (Le Douarin, 1969). Seminal experiments using this chimeric system established the intra-embryonic origin of HSC at a time when the YS was the consensual source of definitive hematopoiesis (Dieterlen-Lievre, 1975). Similar experiments using congenic chicken strains reinforced this notion (Lassila et al., 1978). The DA was soon designated as the site of origin of the hematopoietic progenitors of intra-embryonic origin because it harbored intra-aortic hematopoietic clusters (IAHC) comprising hematopoietic cells (Le Douarin and Dieterlen-Lièvre, 2013). IAHCs are the origin of another hematopoietic cell structure called para-aortic foci (PAF) formed later below the endothelial cell layer (**Figure 3**). A similar structure was described in the mouse and designated sub-aortic patches (Manaia et al., 2000). Cells within the IAHC or PAF contain HSC able of long-term reconstitution of the hematopoietic system (Dunon et al., 1998). Although it is presently difficult to phenotypically distinguish cells from the two structures, it is thought that PAF are an intermediate site where HSC mature before colonizing the thymus, the bursa of Fabricius, and the BM (Yvernogeau and Robin, 2017). The quail–chicken chimera system was also used to demonstrate the YS origin of most macrophages found in the central nervous system (microglia) (Cuadros et al., 1993).

Another important contribution from the Dieterlen-Lièvre group was the observation that two independent sources of endothelial cells contributed to the formation of the DA (Pardanaud et al., 1996). Thus, the roof and the sides of the DA are formed by endothelial cells originated in the somites whereas the floor of the DA, where hematopoietic cell generation occurs, is of splanchnopleural origin (ventral mesoderm). After hematopoietic cell generation, the endothelial cells in the floor of the DA are replaced by somite-derived cells similar to the remaining vessel wall (Pouget et al., 2006). This replacement process might not be evolutionarily conserved across vertebrates and is currently under investigation in other species. Additional experiments showed that expression of *Runx1* and activation of the Notch signaling pathway in HE required contact with the underlying mesenchymal cells. Both signals are essential for EHT, reinforcing the notion that EHT is an exceptional phenomenon that occurs under unique developmental conditions (Richard et al., 2013). Cell-labeling experiments targeting endothelial cells in embryos prior to IAHC emergence provided further evidence for an endothelial origin of the emerging hematopoietic cells (Jaffredo et al., 2000).

In summary, the chicken hematopoietic development also appears to occur in layers, although the precise contribution of YS to hematopoiesis has not been determined and no EMPs have been identified, either because they do not exist or because their identification was hampered by the absence of antibodies specific for the different hematopoietic lineages.

Mice

The first hematopoietic cells arise in the mouse YS around E6.5–E7 in blood islands (Moore and Metcalf, 1970). Colony-forming assays identified bipotent erythroid/megakaryocyte (Xu et al., 2001; Tober et al., 2007) and macrophage progenitors (Palis et al., 1999) within these structures. Primitive erythroid progenitors (Ery-P) are exclusively present in the YS from E7.5 to E8.5 (Palis et al., 1999) and generate primitive erythrocytes. These primitive erythrocytes are larger than their BM counterparts and lack adult globin expression (Wong et al., 1986). Similar to other non-mammalian species, mouse Ery-P maintain their nucleus for several days (Kingsley et al., 2004). In contrast to those found in adult BM, primitive macrophages do not differentiate through a monocyte stage but directly from monopotent macrophage precursors (Mac-CFC/YS-Mp) (Bertrand et al., 2005).

The second hematopoietic wave starts at E8.5, with EMPs emerging from HE in the recently formed YS vascular bed, through a process of EHT (Goldie et al., 2008; Bertrand et al., 2010; McGrath et al., 2015; Frame et al., 2016; Kasaai et al., 2017). These progenitors proliferate extensively during the following 48 h and are found in circulation by E9 and in the hepatic primordium by E10.5 (Palis et al., 1999). This second wave generates definitive erythroid progenitors, the first mast cells, and bipotent macrophage granulocyte progenitors. Embryonic definitive erythrocytes can be found in circulation around E11.5–E12.5 and are distinguished from primitive erythrocytes by their small size (similar to adult erythrocytes) and the absence of nucleus. They originate from a novel population of progenitors of YS-EMP origin, recently identified, that ensures erythrocyte production and oxygenation up until birth (Soares-da-Silva et al. bioRxiv 2020.02.27.968230; doi: https://doi.org/10.1101/2020.02.27.968230).

Of note, the second hematopoietic wave generates tissue-resident macrophages (Gomez Perdiguero et al., 2015; Hoeffel et al., 2015). They have self-renewal ability and persist throughout life (Merad et al., 2002; Ajami et al., 2007; Jenkins et al., 2011).

The third and last wave of hematopoietic cell generation starts around E9.5, when multipotent progenitors emerge through EHT in the embryo DA and other large blood vessels (de Bruijn et al., 2000; Zovein et al., 2008; Boisset et al., 2010; Yzaguirre and Speck, 2016). Opposite to the first two hematopoietic waves, this third wave generates the progenitors of HSC and is the only capable to give rise to lymphocytes (Cumano et al., 1996; Medvinsky and Dzierzak, 1996). Similar to zebrafish, Notch signaling in the

mouse AGM is determinant for hematopoietic cell generation (Kumano et al., 2003), and YS-derived macrophages, which are the most represented mature cells in this location, have been shown to enhance HSC emergence (Mariani et al., 2019). The total number of hematopoietic cells generated in the AGM has been estimated to be in the order of several hundreds of cells (Godin et al., 1999; Ganuza et al., 2017). According to some authors, HSC can also be generated in extraembryonic structures such as the placenta (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). HSC then migrate to the FL where they mature, proliferate [expanding by more than 30-fold (Ema and Nakauchi, 2000)], and differentiate. Although adult and embryonic HSCs produce all major blood cell types, some specific lymphoid lineages are only produced during embryonic development, namely, the dendritic epidermal T cells (DETC) ($V\gamma5^+$) (Ikuta et al., 1990), lymphoid tissue inducer (LTi) cells (Eberl et al., 2004), and a subset of IL-17-producer γδ T cells ($V\gamma6^+$) (Haas et al., 2012).

Humans

The development of the human hematopoietic system also follows a layered strategy. The first hematopoietic cells are found in the YS [starting at around E16–18, Carnegie stage (CS) 7–8]. At this stage, $CD45^+$ cells are found in the cardiac cavities and mark the onset of circulation (Tavian et al., 1999). Whereas in mice the generation of hematopoietic cells and their detection in the FL and placenta are largely overlapping, in humans, the spatial–temporal progression of hematopoiesis is better discriminated (Ivanovs et al., 2017). In the YS, few megakaryocytes and macrophages are found in addition to erythrocytes. Although a population of EMP has not been formally identified, data suggests a second wave of hematopoietic cell emergence in the YS at around CS 13–15. IAHC emerge in the DA between CS 13 and 17 and $CD34^+CD45^+$ progenitors colonize the FL by CS 13, suggesting that HSC initiate liver colonization as they are generated (Tavian et al., 1996; Ivanovs et al., 2011). $CD34^+CD45^{lo}$ HSC are found in the placenta only after the 9th week of gestation (4 weeks after the first $CD34^+$ cells reach this site). For this reason, the placenta is not considered a site of HSC generation in humans. The BM formation in humans marks the end of the embryonic period (CS 23 or E56) and colonization by HSC is initiated shortly after, although the exact time of HSC activity in the BM remains undetermined. Like in the mouse, human HSC initially express endothelial and hematopoietic markers that together with the detection of IAHC suggest a generation through an EHT. Curiously, and unlike in mice, the umbilical artery does not appear to be a site of hematopoietic cell generation in humans (Ivanovs et al., 2011).

An Evolutionarily Conserved Design

From the comparison of hematopoietic development across species, it appears that the sequential generation of different hematopoietic progenitors was an evolutionarily successful strategy. Establishment of circulation was the key step toward the establishment of a vertebrate hematopoietic system.

Paradoxically, the first hematopoietic cells to be produced are differentiated rather than multipotent progenitors and fulfill the most basic functions like oxygen delivery, hemostasis, and tissue remodeling. Overall, despite some variations, distinct YS and DA hematopoietic generations are well conserved. Although controversial (Ueno and Weissman, 2006), the notion that bipotent hemangioblast-like cells contribute to hematopoietic cell generation is associated with that of primitive erythrocytes, megakaryocytes, and macrophages that occur in the YS blood islands.

With tissue complexity, subsequent hematopoietic waves have to fulfill increasingly specialized functions, and for that, multipotent progenitors are better adapted to generate a diverse array of cells with specific and diversified functions.

Remarkably, gene expression analysis in cephalochordates led to the identification of HE (Pascual-Anaya et al., 2013), which places EHT process as ancient and highly conserved through phylogeny. EHT appears to be the mechanism operating in the generation of multipotent progenitors in late YS and in the AGM and the only one to contribute to HSC production.

There is a consistent contribution of YS to the microglia and other tissue-resident macrophages and to primitive erythrocytes (Godin and Cumano, 2002). It is also generally accepted that YS hematopoietic cells do not contribute to the lymphoid compartment, although some minor and transient lymphocyte subsets have been suggested to emerge in the YS (discussed below). Other sites of hematopoietic cell generation, the most discussed being the placenta (Gekas et al., 2005; Ottersbach and Dzierzak, 2005; Rhodes et al., 2008), have been proposed but are not consensual, and the allantois that was hypothesized to be an alternative hematopoietic site in the chicken was recently shown to have no contribution to HSC generation (Yvernogeau and Robin, 2017). Recent evidence points to a role of YS-derived macrophages in the generation of HSC (Mariani et al., 2019). It is tempting to speculate that a selective pressure for an early generation of this unique type of macrophages lies not only in their capacity to ensure general tissue homeostasis, as the embryo develops, but also in their contribution to HSC emergence. It remains to be determined whether the unique properties of YS-derived macrophages are cell autonomous or whether they are modulated by cues received from the environment where they reside.

As different hematopoietic cells emerge, they transit through different primary hematopoietic organs: the FL and later the BM or their equivalents in the different species. Due to the pronounced differences in cell composition of the primary hematopoietic organs, it is to be expected that the cues that hematopoietic progenitors receive are substantially different in distinct organs and that this may influence their development. A recent example of this selective pressure is the lower requirements for erythropoietin (Epo) of embryonic erythrocytes compared to those in BM. They develop in the FL where Epo concentrations are lower than those produced in the kidney of adults. The recent discovery that these embryonic erythrocytes are of YS origin and outcompete those of HSC origin in the FL exemplifies how the environment can select for progenitors of different origins in space and time (Soares-da-Silva et al. bioRxiv 2020.02.27.968230; doi: https://doi.org/10.1101/2020.02.27.968230). The generally low concentration

of other cytokines (i.e., IL-7) in FL also impacts lymphocyte development (see below).

LYMPHOCYTE DEVELOPMENT: A STRATEGY DESIGNED FOR LIFE-LONG TISSUE HOMEOSTASIS AND IMMUNE PROTECTION

Lymphopoiesis, the process of lymphocyte generation, is the best-documented system where successive waves of progenitors differentiate and migrate to specific tissues thus generating the basis of a layered developmental organization. In this section, we will describe the development of embryonic lymphoid lineage populations and why they are selectively produced within an embryonic environment resulting in a diverse adult lymphoid compartment.

B Cell Development

The "layered immune system" concept was initially proposed by Herzenberg (1989), with the identification of functionally distinct "lineages" of B cells that are generated during ontogeny. B-1a cells, which reside mainly in the peritoneal and pleural cavities and produce natural antibodies in a T cell-independent manner, are usually obtained from embryonic precursors and contribute to innate-like immunity (reviewed in Herzenberg, 2000; Montecino-Rodriguez and Dorshkind, 2012; Elsaid et al., 2019). There are different explanations for the unique capacity of fetal progenitors to generate specific lymphoid cells (**Figure 4**).

The identification of a specific B1 progenitor only found in the embryo led to the hypothesis that these cells could derive from progenitors distinct from those that generate classical B-2 B cells. Recent studies show that innate-like B and T cells preferentially originate from developmentally restricted progenitors (Beaudin et al., 2016). These cells are exclusively found in fetal and neonatal stages, suggesting that they represent a distinct lineage from HSC.

However, experiments of cellular barcoding coupled with transplantation showed that FL HSCs can generate both B-1a and B-2 cells and that the capacity to generate B-1a cells is lost during development and upon transplantation (Kristiansen et al., 2016). Moreover, it was recently shown that antigen receptor specificity is sufficient to induce cell proliferation and acquisition of the B-1 phenotype and function (Graf et al., 2019). Lin28b, a regulator of fetal hematopoiesis, is important in the generation of B1a lineage, and loss of its expression controls the switch from neonatal to adult B cell production (Vanhee et al., 2019). Low levels of IL-7, a cytokine crucial for adult B cell development, in FL favors the generation of B1, highlighting the impact of the microenvironment on the preferential production of particular cell types (Wong et al., 2019).

Thymopoiesis

In the thymus, T lymphocyte differentiation occurs from hematopoietic progenitors migrating from either the FL or the BM at later developmental stages in a process called "thymopoiesis." In general, thymopoiesis resembles B lymphopoiesis in many ways, as it is also a stepwise process of rearrangement of antigen receptor genes and selection to ensure self-tolerance (Abramson and Anderson, 2017; Hosokawa and Rothenberg, 2020). The thymus produces T lymphocytes that are important for protection against environmental pathogens. Thymus architecture is key to T cell tolerance and a normal immune system. The first thymic seeding progenitors (TSP) that colonize the embryonic thymus display a unique capacity to generate innate and innate-like lymphocytes. These cells are required to shape the thymic architecture, thus ensuring life-long efficient T cell tolerance and avoiding autoimmunity (Elsaid et al., 2020).

The layered organization of thymopoiesis has been reported in all species studied from zebrafish to humans. During development, multiple waves of hematopoietic progenitors derived from distinct anatomical sites colonize the thymus. These waves give rise to T cells that fulfill distinct functions at distinct developmental stages. We will discuss below embryonic thymopoiesis across species and how cells from different waves contribute to diverse T cell compartments.

Zebrafish

Fate-mapping analysis using high temporal–spatial resolution cell labeling techniques identified two waves of T cell progenitors: an early HSC-independent, from the DA and PBI regions, generating predominantly αβ T cells that do not persist beyond the larva stage and a late HSC-dependent T lymphopoiesis, restricted to the DA, giving rise to various types of T cells from the larval stage up to adulthood (Tian et al., 2017). This first wave shares two properties with the corresponding population in mice: they are important in thymic architecture and they differentiate fast (Hess and Boehm, 2012). In-depth characterization of the phenotype and function of T cells generated from this HSC-independent precursor is presently not available.

Frogs

Xenopus laevis have two major hematopoietic organs, the liver, which is predominant in the larvae, and the spleen, the major hematopoietic organ in adult animals. B cells are produced in the liver in larval stages and the spleen takes over that function after metamorphosis (Du Pasquier et al., 1989). The diversity of the B cell repertoire is apparent in larvae and it is achieved through the combinatorial rearrangement of a large number VH, DH, and JH segments. Somatic hypermutation also operates in *Xenopus* larvae possibly as a response to the complex biota of their environment. Interestingly, terminal deoxynucleotidyl transferase (TdT) expression and N sequence additions are only detected after metamorphosis (Lee and Hsu, 1994), indicating further diversification of the antigen receptor repertoire (see **Table 1**) in adult animals.

Two waves of TSP, predominantly of DLP origin, have been identified during the larval stages. The first wave generates a transient population of T lymphocytes, whereas the progeny of the second wave persists in the adult (Turpen and Smith, 1989). It has been reported that T and B cells can also originate in the VBI (Smith et al., 1989). A third wave of progenitors enters the

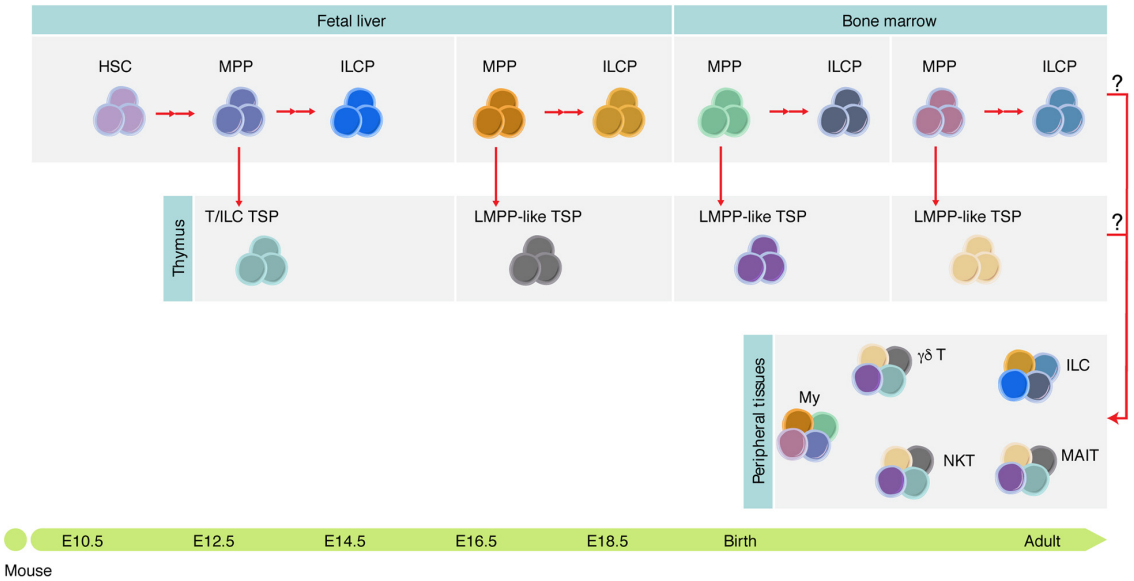

FIGURE 4 | Tissue-resident immune cells are mosaic of cells derived at different developmental stages. Different hematopoietic progenitors are generated during development, some of which contribute to the adult tissue-resident immune cell compartment diversity. It is still unknown which functions these cells play during development and whether they persist in adulthood. ILCP, ILC progenitor; My, myeloid cells; NKT, NK T cells; MAIT, mucosal-associated invariant T cells.

thymus at the beginning of metamorphosis and provides T cells throughout adult life.

In conclusion, T lymphopoiesis in frogs is organized in waves, and B and T lymphocytes with a limited repertoire diversity are produced early in embryonic life. The exposure of frog and fish larvae to a biota-rich environment compared to chicken and mammals might account for some differences in timing and the type of lymphocyte responses in the different species.

Birds

An interesting and unique property of the chicken lymphoid development is that B cells develop in a unique environment called bursa of Fabricius where they acquire a diverse antigen receptor repertoire (Reynaud et al., 1987). Sequential transplantation of thymic lobes demonstrated for the first time that the embryonic thymus is discontinuously seeded by hematopoietic cells. In total, three waves of hematopoietic cells reach the thymus: the first at E6.5 and the third at E18, 1 day before hatching (Coltey et al., 1987). It was subsequently shown that the first wave of TSP is derived from cells located in the para-aortic clusters shortly after their generation in the DA. These observations raised the possibility that, in chickens, the first wave of TSP is composed of multipotent hematopoietic cells. The refractory periods where no hematopoietic progenitors reach the thymus are consequent to the absence of circulating progenitors because the injection in circulation of cells derived from para-aortic foci during refractory periods resulted in efficient thymic colonization (Dunon et al., 1998, 1999).

Mice

Two waves of distinct TSP colonize the fetal thymus where they contribute to thymic organogenesis. First wave TSP are first detected around E12.5 and persist until E15.5. These progenitors differentiate rapidly, exhibit low proliferative capacity, express transcripts related to the ILC lineage, and are the only ones with the capacity to generate Vγ5$^+$ T cells and LTi cells. A second wave of more immature hematopoietic progenitors colonize the thymus after E15 and ensure thymopoiesis thereafter (**Figure 5**; Ramond et al., 2014; Elsaid et al., 2020).

The thymic rudiment is detected around E11.5 in the mouse embryo (Takahama et al., 2017). The thymic epithelial cells (TEC) divide into cortical TEC (cTEC) and medullary TEC (mTEC). Aire$^+$ mTECs ensure the expression of tissue-specific peptides that mediate T cell negative selection (see **Table 1**). Vγ5$^+$ T cells and LTi cells, a subset of group 3 innate lymphoid cells (ILC3), are required to establish a mature compartment (*Aire*-expressing) of mTEC (Roberts et al., 2012). In line with this work, Elsaid et al. (2020) showed that the first wave of TSP generates invariant γδ T cells and LTi within a thymic microenvironment and that temporal depletion of this wave resulted in loss of (Aire)-expressing mTEC at birth **(Figure 4)**. The crosstalk between the progeny of the first TSP and immature mTEC ensures that mature Aire$^+$ mTEC are in time and place to induce tolerance (see **Table 1**) in the nascent αβ T cell repertoire during the neonatal period.

The first wave of TSP originates from a particular subset of FL common lymphoid progenitor (CLP), no longer detected after E15, that preferentially arises in the poor IL-7 environment of the FL (Berthault et al., 2017).

Before HSC activity, lympho-myeloid-restricted progenitors (LMP) co-expressing lymphoid- and myeloid-associated genes were reported in YS (Böiers et al., 2013) and proposed to be the origin of the first wave of TSPs (Luis et al., 2016). These studies challenged the notion that the first TSP derived from HSC

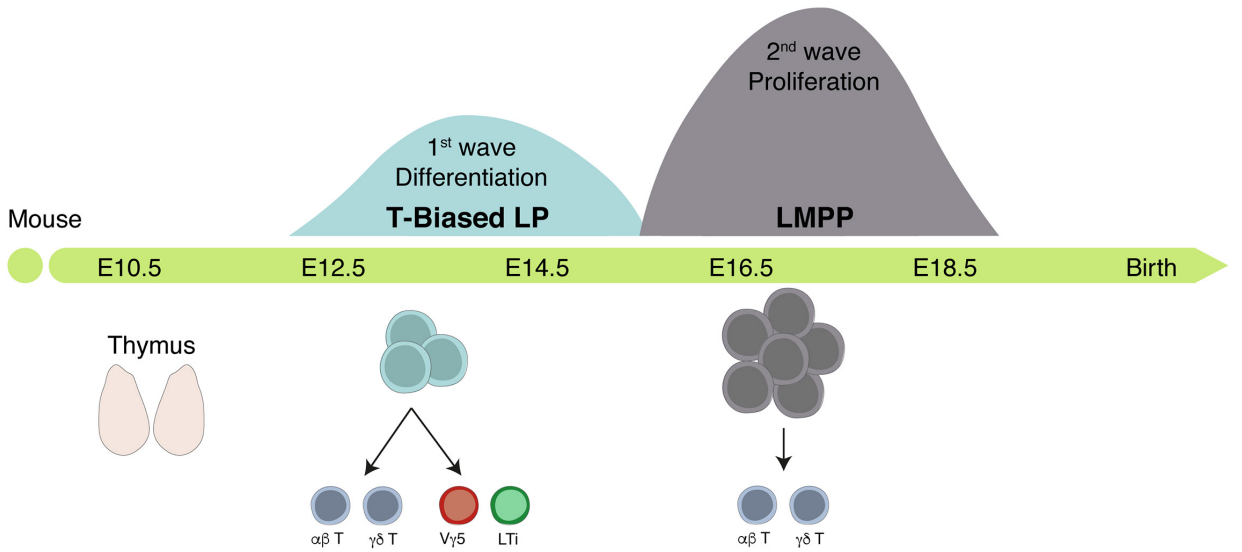

FIGURE 5 | The fetal thymus is colonized by two successive waves of hematopoietic progenitors. Scheme of the timeline of thymus colonization in the mouse. Area of the Gaussian curves is proportional to the number of progenitors in each wave. The first wave generates few cells whereas the second expands before differentiation but lacks the capacity to generate LTi and Vγ5$^+$ T cells.

relied on the analysis of reporter and lineage tracing mouse lines. Corroborating these observations, single-cell transcriptional and epigenetic analysis of IAHC detected a population of lympho-myeloid-primed progenitors, suggesting an early differentiation process or an HSC-independent generation (Zhu et al., 2020). Alternatively, or complementarily, despite a transient expression of lymphoid transcripts, the reported YS-derived LMP were devoid of lymphoid potential and therefore do not qualify as lymphoid progenitors (Elsaid et al., 2020).

Humans

Growing evidence suggests that, as in mice, the human fetal thymus is colonized by distinct waves of hematopoietic progenitors. Unlike their adult counterpart (Tieppo et al., 2020), fetal thymic progenitors expressing low levels of TdT generate invariant γδ T cells and promote tolerance (Mold et al., 2010; Ng et al., 2019). It was further shown that, like that of innate B-1 cells in mice, the generation of fetal invariant γδ T cell subsets was Lin28b-dependent. Further studies are required to identify the phenotype and functions of these fetal-derived T cells in fetal immunity and organ development.

Why a Layered Lymphoid Compartment?

Similar to what was described for hematopoietic cells, not all lymphocyte subsets are produced simultaneously nor throughout life. In the mouse, whereas lymphocytes with a highly variable receptor repertoire are produced constantly after birth, some particular subsets that comprise B1 B cells and Vγ5$^+$ and Vγ6$^+$ T cells are exclusively or preferentially produced during embryonic life and persist in adults. These subsets share some general properties: (1) they express invariant or restricted antigen receptor repertoires and are, therefore, not devoted to highly specific immune responses. Rather, they fulfill broader functions and are, therefore, commonly designated as "innate-like" cells; (2) they are produced in small numbers; and (3) rather than circulating, they reside in tissues. These lymphocyte subsets that might confer a first line of protection before adaptive immune cells are fully functional also contribute to tissue architecture and homeostasis. Due to their stage-restricted development, it is tempting to hypothesize that these particular lymphoid subsets originate from specialized cells that develop independent from HSC. The demonstration that B1 and B2 cells share a similar cellular origin (Kristiansen et al., 2016), that Vγ5-expressing T cells do not differentiate from conventional YS progenitors, and that embryonic hematopoietic progenitors often express lymphoid specific transcripts, regardless of their differentiation potential, offer alternative possibilities that need to be further investigated (Elsaid et al., 2020).

B1 cells make natural circulating immunoglobulin that provides a first line of defense against pathogens. Although B2 cells can also produce IgM, their development is delayed and transient protection is better achieved through immunoglobulins of B1 cell origin. In the absence of Vγ5$^+$ T cells in the skin, wound healing is delayed (Jameson et al., 2002). Similarly, in the absence of Vγ6$^+$ T cells, there is increased susceptibility to airway viral infections as epithelium integrity is easily compromised (Guo et al., 2018). Because IL-17, the main effector cytokine in epithelium regeneration, can also be produced by conventional T cells, γδ T cells might only be crucial in neonates and juveniles before conventional T cell immunity is mature. Both Vγ5$^+$ T cells and LTi that contribute to the maturation of mTEC in the thymus have a common origin in specialized TSP only present in the embryo. Other cell types such as CD4$^+$ T cells can produce RANKL and induce mTEC maturation. LTi are no longer found in the adult thymus, suggesting that, together with γδ T cells, they fulfill a very specific role in late gestation and around birth.

The fact that they originate from a particular progenitor cell arising specifically in the low-IL-7 FL environment (Berthault et al., 2017) and that in some mouse strains neonatal tolerance induction is required for prevention of autoimmune disease later in life (Guerau-de-Arellano et al., 2009) suggests a role of these developmental-restricted cells in the establishment of peripheral tolerance.

A selective pressure including environmental constraints appears to favor the early development of less diverse and sophisticated immune cells that are important in the establishment of basic immune functions and that confer protection before the highly complex diverse immune system is operational. An early development before large numbers of lymphoid cells circulate also ensures an efficient colonization of peripheral tissues where niches are possibly limited. Cell-autonomous mechanisms that condition the differentiation of developmentally restricted progenitors are still largely unknown. The HSC-independent origin proposed by some authors will have to be probed against a possible loss of the expression of embryo-specific genes such as loss of Lin28b and of the B1 differentiation capacity as HSC transit from FL to BM, through either an internal clock-type mechanism or environmentally induced.

CONCLUDING REMARKS

It is interesting to realize that cells usually considered as pro-inflammatory such as macrophages have been also involved in organogenesis and in maintenance of tissue integrity, throughout evolution. It follows that what is considered to be the adult inflammasome might have tissue-remodeling functions during development and that therefore both functions co-evolved. This notion can be extended to specific subsets of innate-like T cells and ILCs.

The first waves of immune cells appear to be also devoted to tissue remodeling although they can also engage in defense, namely, in species whose embryos are released in microbiota-rich environment. However, they appear to be prone to mount low inflammatory responses compatible with the integrity of tissues, as elsewhere described in *X. laevis* tadpoles.

AUTHOR CONTRIBUTIONS

MP and FS also contributed to the figures. AB, AC, FS, and RE conceptualized and assembled the manuscript. All authors reviewed the literature, discussed, contributed to writing, and approved the submitted version.

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Tissue-Resident Macrophage Development and Function

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Tissue-resident macrophages have been associated with important and diverse biological processes such as native immunity, tissue homeostasis and angiogenesis during development and postnatally. Thus, it is critical to understand the origins and functions of tissue-resident macrophages, as well as mechanisms underlying their regulation. It is now well accepted that murine macrophages are produced during three consecutive waves of hematopoietic development. The first wave of macrophage formation takes place during primitive hematopoiesis, which occurs in the yolk sac, and gives rise to primitive erythroid, megakaryocyte and macrophage progenitors. These "primitive" macrophage progenitors ultimately give rise to microglia in the adult brain. The second wave, which also occurs in the yolk sac, generates multipotent erythro-myeloid progenitors (EMP), which give rise to tissue-resident macrophages. Tissue-resident macrophages derived from EMP reside in diverse niches of different tissues except the brain, and demonstrate tissue-specific functions therein. The third wave of macrophages derives from hematopoietic stem cells (HSC) that are formed in the aorta-gonad-mesonephros (AGM) region of the embryo and migrate to, and colonize, the fetal liver. These HSC-derived macrophages are a long-lived pool that will last throughout adulthood. In this review, we discuss the developmental origins of tissue-resident macrophages, their molecular regulation in specific tissues, and their impact on embryonic development and postnatal homeostasis.

Keywords: primitive hematopoiesis, hemogenic endothelial cells, erythro-myeloid progenitors, definitive hematopoiesis, tissue-resident macrophages

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INTRODUCTION

Tissue-resident macrophages are best known as immune sentinels that sense and respond to invading pathogens and challenging surroundings; they are also essential in tissue development, remodeling, and homeostasis (McGrath et al., 2015b). During embryonic development, macrophages are one of the first blood cell lineages to emerge. In mice, embryonic/fetal macrophages are known to originate during three distinct waves of hematopoiesis: primitive hematopoiesis, erythro-myeloid progenitor (EMP) generation, and definitive hematopoietic stem cell (HSC)-mediated hematopoiesis. Each wave differs but also overlaps temporally and

spatially. Macrophages are then discretely positioned in the majority of developing organs (Davies et al., 2013).

As a consequence of diverse origins and the influence of tissue-specific microenvironments, tissue-resident macrophages are heterogeneous and exhibit tissue-specific functions during development and adulthood. Understanding the origins and development of tissue-resident macrophages will help us better understand tissue-macrophage heterogeneity, and provide insights into the fundamental differences between tissue-resident macrophages and adult monocyte-derived macrophages that reside in blood circulation (Davies et al., 2013). However, due to the complex nature of embryonic hematopoiesis, as well as the lack of specific phenotypic markers of different waves, it is very challenging to precisely delineate macrophage ontogeny. In this review, we will discuss what is known about the developmental origin of tissue-resident macrophages, their molecular regulation in specific tissues, and their functions during development and postnatally.

ORIGINS OF TISSUE-RESIDENT MACROPHAGES DURING EMBRYOGENESIS

Primitive Hematopoiesis: The First Wave of Macrophage Emergence

In mice, primitive hematopoiesis begins around embryonic day (E)7.25 (Palis, 2016). The process occurs in distinct clusters of cells in the extra-embryonic yolk sac, called blood islands (Palis, 2016; **Figure 1**). Blood islands are composed of primitive hematopoietic cells in the center and sparse endothelial cells in the periphery (Hoeffel and Ginhoux, 2018). In the blood islands, primitive hematopoiesis generates unipotent myeloid progenitors that can only give rise to the macrophage lineage (Hoeffel and Ginhoux, 2018), as well as bipotent progenitors of erythrocytes and megakaryocytes (Tober et al., 2007). These progenitors remain in the blood islands until they are released during the onset of blood circulation around E8.0 and circulate throughout the embryo proper (Hoeffel and Ginhoux, 2018).

The first embryonic-derived macrophages are detected in the yolk sac at E9.0 (Naito et al., 1989; Takahashi et al., 1989). Although they are generated after initiation of both primitive hematopoiesis and EMP production, they are thought to be produced by the earlier primitive wave, considering the time needed for myeloid progenitors to self-expand and differentiate. Yolk sac-derived macrophages continue to proliferate and colonize the developing brain by E9.5 and the rest of the embryonic tissues by E12.5 until further diluted and replaced by macrophages generated from later waves (Schulz et al., 2012; Hoeffel et al., 2015; McGrath et al., 2015b; Palis, 2016).

Unlike macrophages that are generated during the two successive waves of hematopoiesis, macrophages originating during primitive hematopoiesis are generated directly from progenitors without going through a monocyte intermediate (Naito et al., 1989; Takahashi et al., 1989). Indeed, in terms of developmental timeline, the appearance of primitive macrophages is observed prior to monocytes in the mouse embryo, and when E8.0 mouse yolk sac progenitors are cultured *in vitro*, they do not produce monocyte precursors (Naito et al., 1989; Takahashi et al., 1989). More direct evidence that macrophages derive directly from yolk sac progenitors *in vivo* have come from recent fate mapping studies. Using inducible *Runx1* promoter-driven GFP reporter mice at specific time points in development, Ginhoux et al. (2010) demonstrated that microglia (tissue-resident macrophages in the adult CNS) originate directly from E7.5 yolk sac progenitors. Progenitors for microglia were further defined as the CD45$^-$c-Kit$^+$ population in the brain (Kierdorf et al., 2013). Using reporter mice labeled with inducible colony stimulating factor 1 receptor *(Csf1r)-Cre*, Gomez Perdiguero et al. (2015) also reported that tissue-resident macrophages are predominantly derived from yolk-sac progenitors that express CSF1R$^+$ at E8.5. They also found that a large number of tissue-resident macrophages, including macrophages in liver, brain, epidermis and lung, originate via Tie-2-expressing cells before E10.5 (Gomez Perdiguero et al., 2015). Macrophages generated directly from these progenitors possess high proliferative potential, enabling them to expand and maintain their pools in the majority of tissues over time in the embryo (Takahashi et al., 1989; Palis, 2016). In contrast, the brain is the only tissue that maintains macrophages generated during primitive hematopoiesis throughout adulthood (Alliot et al., 1991; Alliot et al., 1999; Ginhoux et al., 2010).

Although it has been recognized for some time that macrophages are generated during primitive hematopoiesis, the underlying mechanisms that direct their development are still largely unknown. Nonetheless, it is well accepted that myelopoiesis during primitive hematopoiesis is not dependent on regulation by c-Myb, a transcription factor required for the development of HSC (Sumner et al., 2000; Schulz et al., 2012). Indeed, *c-Myb*$^{-/-}$ mouse embryos exhibit depletion of most other myeloid cells at E16.5 except yolk sac macrophages (Schulz et al., 2012).

Since microglia are the only type of primitive macrophages that persist throughout adulthood, more studies have been focused on the mechanisms of microglia development. Kierdorf et al. (2013) found that hematopoietic transcription factors PU.1 (also known as Spi1) and Interferon regulatory factor-8 (IRF8) are required for the development of microglia. They also observed that primitive c-Kit$^+$ precursors developed and matured into c-Kit$^-$CD45$^+$CX$_3$CR1$^+$ progenitor cells with downregulation of CD31 and concomitant upregulation of adult macrophage marker F4/80, as well as macrophage colony stimulating factor receptor (MCSF-R). The c-Kit$^-$CD45$^+$CX$_3$CR1$^+$ progenitor cells are proliferative, able to infiltrate the developing brain and subsequently develop into microglia.

Development of microglia also requires transforming growth factor β (TGF-β) signaling. Adult microglia cultured *in vitro* without the addition of TGF-β1 fail to express microglia signature genes and no microglia are detected in the brain of adult TGF-β1-deficient mice (Butovsky et al., 2014). Another study utilized mice expressing a *Vav1*-driven inducible Cre to delete *Tgfbr2* (gene encoding transforming growth factor β receptor 2)

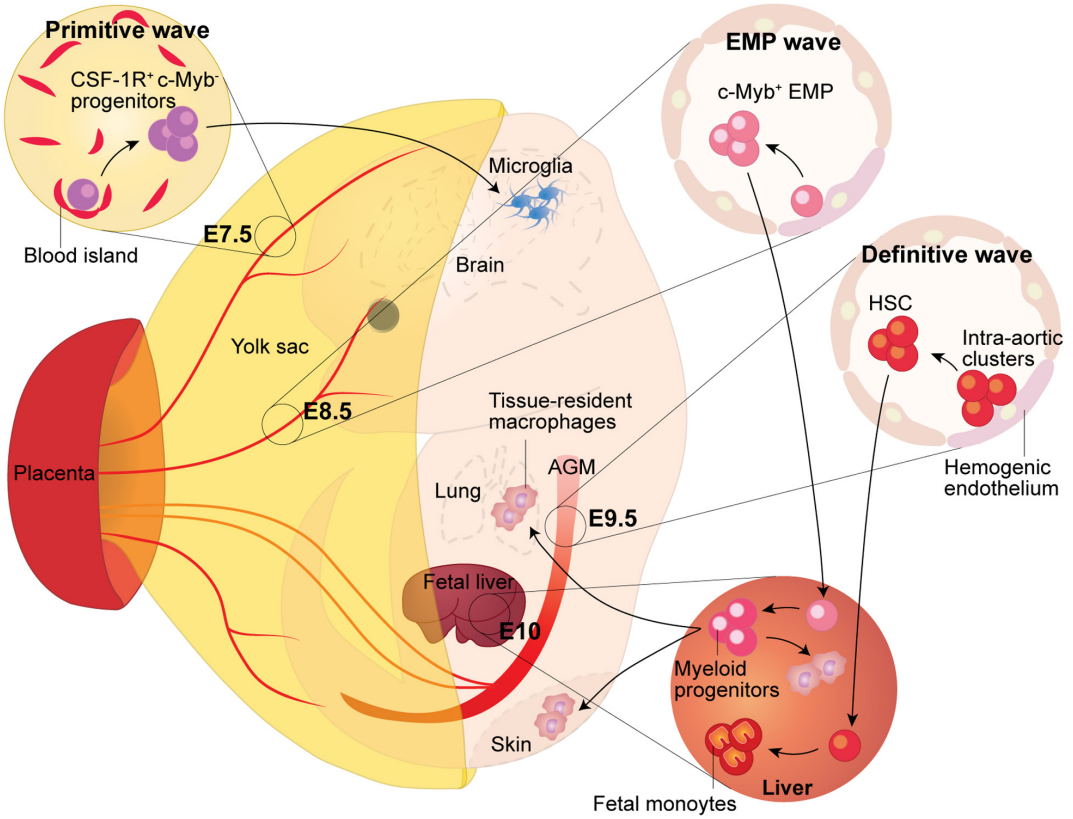

FIGURE 1 | Fetal Macrophage Development. Murine macrophages originate from three successive waves of hematopoiesis. The first wave, termed primitive hematopoiesis, occurs in the blood islands of the extra-embryonic yolk sac at E7.5. It produces primitive erythroblasts and megakaryocytes, as well as CSF-1R$^+$ c-Myb$^-$ progenitors, which give rise to adult microglia in the brain. The second wave arises from the hemogenic endothelium formed at E8.5 in the yolk sac. Because the second wave generates the c-Myb$^+$ hematopoietic progenitors named eythro-myeloid precursors (EMP), thus it is termed the EMP wave. The EMP either give rise to yolk sac macrophages locally or migrate into the fetal liver through the blood circulation, where they expand and differentiate into tissue-resident macrophages, which then migrate to, and colonize, different tissues. The third wave arises from the hemogenic endothelium in the aorta-gonad-mesonephros (AGM) region, which gives rise to fetal hematopoietic stem and progenitor cells beginning at E9.5. Subsequently, these precursors colonize the fetal liver where they establish definitive hematopoiesis and will eventually seed the bone marrow and lead to the generation of adult hematopoietic stem and progenitor cells.

in hematopoietic cells. In this model, at E11.5, microglia were significantly reduced in the brain during development (Utz et al., 2020).

Yolk Sac Hemogenic Endothelium Give Rise to EMP

The second wave of macrophage generation arise from the EMP in the yolk sac around E8.25 (Hoeffel et al., 2015; **Figure 1**). EMP are derived from hemogenic endothelium in the yolk sac vasculature, a transient, specialized vascular endothelium with definitive hematopoietic potential (Gritz and Hirschi, 2016). EMP are highly proliferative, expanding predominantly in the yolk sac. Although EMP are detected in the embryonic bloodstream through E12.5, they start to seed the developing fetal liver by E10.5 (Palis et al., 2001; Frame et al., 2014; McGrath et al., 2015b). In circulation-deficient mouse embryos, these macrophages are retained in the yolk sac, suggesting they require blood circulation to migrate to other embryonic tissues (Ginhoux et al., 2010; Hoeffel et al., 2012).

Erythro-myeloid progenitors in the fetal liver produce the first enucleated erythrocytes and give rise to bipotent granulocyte/macrophage progenitors and mast cells (Palis et al., 1999; Bertrand et al., 2005; McGrath et al., 2015b). Recently, McGrath et al. (2015a) revisited the lineage potential of the EMP and found they give rise to erythrocytes, mast cells, basophils, neutrophils and macrophages in culture. However, EMP are short-term progenitors; transplantation of E10.5 EMP into either normal or immune-compromised adult mouse recipients produce erythroid cells, with very limited myeloid cells or platelets (McGrath et al., 2015a). Due to lack of long-term potential in adults, the EMP wave of hematopoiesis is also called "transient definitive wave" (Hoeffel and Ginhoux, 2018).

Due to temporal and spatial overlap of EMP-mediated hematopoiesis with primitive and HSC-derived myelinogenesis, it is important to identify unique cell surface markers for EMP to better distinguish them from myeloid cells generated during the other two processes. McGrath et al. found that, in E9.5 mouse yolk sac, c-Kit$^+$CD41$^+$ EMP share markers with hemogenic endothelial cells and developing HSC (i.e., CD31, CD45, and

CD61). However, EMP express CD16/32 and not Sca-1; thus, distinguishing them from HSC. In addition, although AA4.1 (also known as CD93) is highly expressed by fetal liver HSC, it is expressed by only 15% of E9.5 EMP; the majority of AA4.1$^+$ cells in E9.5 yolk sacs are c-Kit$^-$, vascular endothelial (VE)-cadherin$^+$ endothelial cells (McGrath et al., 2015a). Hoeffel et al. (2015) further verified that c-Kit$^+$CD41$^+$CD16/32$^+$CD93$^-$ EMP can give rise to almost all tissue-resident macrophages found in adults via monocytic intermediates.

In other studies, using a fate mapping approach, CSF1R-expressing cells in the mouse embryo were found to give rise to tissue-resident macrophages in adult tissues (Schulz et al., 2012); however, the generation of EMP does not require CSF1R (Ginhoux et al., 2010; Hoeffel et al., 2012). In addition, toll-like receptor 2 (TLR2) was found to be essential for E8.5 c-Kit$^+$ yolk sac EMP development and function, and can be used as a cell surface marker to distinguish them from cells generated during primitive hematopoiesis (Balounová et al., 2019).

The fact that *c-Myb*$^{-/-}$ mouse fetal livers are devoid of the EMP-containing c-Kit$^+$ population suggests that development of EMP may be c-Myb dependent (Schulz et al., 2012). Indeed, c-Myb is expressed in EMP in the E9.5 yolk sac, as well as in the fetal liver (Hoeffel et al., 2015). Like HSC development, EMP development requires Runx1. Runx1-deficient mouse embryos die with lack of fetal liver hematopoiesis around E12.5. Runx1-deficient mouse embryonic stem cells (ESC) demonstrate no colony-forming activity in culture. Moreover, injecting Runx1-deficient mouse ESC into blastocysts of wild type recipients reveals no myeloid lineage potential from the Runx1-deficient cells (Okuda et al., 1996). The binding partner of Runx1, core binding factor β (Cbfβ) is also indispensable in EMP development. In *Cbf*β$^{-/-}$ mice, forced expression of Cbfβ driven by *Tie-2* promoter rescues the number of EMP in E12.5 fetal liver (Miller et al., 2002). The role of Runx1 and its binding partner in EMP development are also involved in the endothelial-to-hematopoietic transition (EHT) and HSC generation (Palis, 2016). However, unlike the HSC-associated EHT, development of EMP does not require blood flow or Notch1 (Hadland et al., 2004; Frame et al., 2016).

Several studies have also demonstrated that fetal monocytes contribute to adult macrophages in different tissues. In one study, Hoeffel et al. (2015) depleted yolk sac macrophages in mouse embryonic tissues, but left monocytes intact, by deleting CSF1R, which is essential to development and maturation of macrophages (Hamilton, 2008). They found that fetal monocytes differentiate into macrophages in the skin, lung, liver, kidney, and gut before birth. In homeostatic conditions, activation of *CSF1R* or *Runx1* promoter-driven reporters at E8.5-9.5 leads to labeling of E10.5 EMP. Reporter activation at E13.5 leads to labeling of non-brain fetal macrophages, fetal liver monocytes, granulocytes and adult fetal-derived macrophages except microglia. In other lineage tracing studies utilizing the promoter of *S100a4*, a gene actively expressed in monocytes, activating the reporter from E14.5 onward labels macrophages in all tissues except microglia (Hoeffel et al., 2015). Since no phenotypic marker is known to distinguish macrophages derived from distinct hematopoietic sources, these lineage-tracing studies delineating the origin of fetal monocyte-derived macrophages are highly dependent on the time window of the labeling and tracing studies, relative to the onset of distinct hematopoietic waves. Thus, the temporal overlap of different hematopoietic processes during embryonic development complicates these analyses.

Another issue is that HSC also produce fetal monocytes that generate a small number of resident macrophages in the perinatal period. Studies were done to try to delineate differences in the fates of fetal monocyte-derived macrophages generated from EMP-mediated vs. HSC-mediated waves of hematopoiesis. Guilliams et al. isolated CD45.1$^+$ fetal macrophages and CD45.2$^+$ monocytes from E17 lungs, and transferred them in a 1:1 ratio into CD45.1$^+$CD45.2$^+$ healthy recipient newborn mice. They found that, among recipient lung alveolar macrophages, significantly more are derived from fetal monocytes, indicating they are the main precursors for lung tissue-resident macrophages (Guilliams et al., 2013).

Collectively, these studies suggest that EMP give rise to most fetal-derived tissue-resident macrophages with the exception of brain microglia. Although fetal HSC-derived monocytes may also contribute to tissue-resident macrophages, their overall contribution is not clear.

HSC Generate Macrophages During Definitive Hematopoiesis

HSC-dependent definitive hematopoiesis begins at approximately E9.5 in the mouse intra-embryonic AGM region (Medvinsky and Dzierzak, 1996; **Figure 1**), as well as in vitelline and umbilical arteries (Zovein et al., 2010). Like EMP, HSC are also derived from hemogenic endothelium, which undergoes EHT (Gritz and Hirschi, 2016). Despite the fact that they have the same cellular origin, regulation of HSC development differs significantly from the emergence of EMP (for detailed review, see Wu and Hirschi, 2020). HSC emerge in clusters budding from the endothelial lining of the aortic wall in the AGM region, and then migrate into the developing fetal liver, where they undergo maturation and massive self-expansion. By E16.5, the HSC migrate to and colonize the developing fetal bone marrow, where they remain throughout adulthood, and generate all needed blood cell lineages (Dzierzak and Speck, 2008; Coskun et al., 2014).

It was previously thought that circulating monocytes originated from bone marrow constitute the only precursors for all tissue-resident macrophages in the adult (van Furth et al., 1972). However, as mentioned above, this paradigm was challenged by recent findings that most tissue-resident macrophages have a yolk sac origin. Although adult monocytes can contribute to tissue-macrophage populations, HSC-derived tissue-resident macrophages only marginally replace the yolk-sac-derived macrophages in steady-state conditions in 1-year old mouse tissues and organs, notably the brain, liver and epidermis (Gomez Perdiguero et al., 2015).

Interestingly, EMP-derived macrophages can be replaced by HSC-derived macrophages in some tissues early in the postnatal period. For example, in mouse intestine, EMP-derived macrophages colonize the intestinal mucosa, but do not persist

into adulthood. During the weaning period, they are diluted by tissue-resident macrophages that were generated by HSC-derived Sca-1^+ monocytes. This process is dependent on the chemokine receptor CCR2 and commensal microbiota, and continues throughout adult life (Bain et al., 2014). A similar process was also observed in skin, spleen and heart, in which fetal monocyte-derived macrophages are replaced by adult monocyte-derived macrophages progressively over time (Tamoutounour et al., 2013; Molawi et al., 2014; Hoeffel et al., 2015). Importantly, yolk sac- and HSC-derived macrophages can coexist in the same tissue and maintain a balance in the steady state, except under inflammatory conditions, when large numbers of HSC-derived macrophages invade tissues, such as heart (Yap et al., 2019) and liver (Blériot et al., 2020). However, the contributions of the developmentally distinct macrophage populations to tissue homeostasis and inflammation remain to be investigated (Davies et al., 2013).

ROLES OF TISSUE-RESIDENT MACROPHAGES IN EMBRYOGENESIS

Angiogenesis and Neurogenesis

Tissue-resident macrophages have been reported to regulate neurogenesis. For example, microglia were found to modulate neuron outgrowth and positioning in a CX_3CR1-dependent manner during murine brain development (Squarzoni et al., 2014). In addition, in the developing mouse testis, fetal macrophages derived during primitive hematopoiesis can mediate tissue vascularization (Defalco et al., 2014). Interestingly, a recent finding by Plein et al. suggests another relationship between blood and endothelial lineages. Using inducible *Csf1r* promoter-driven-Cre $ROSA^{YFP}$ reporter mice, they found that circulating EMP contribute to endothelial cells lining blood vessels in multiple tissues, including the liver, brain, heart, lung, and yolk sac, and persist throughout adulthood. Unlike the classic differentiation of angioblasts to endothelium, these EMP are thought to revert to their initial endothelial fate and intersperse within existing vessels (Plein et al., 2018). However, other recent studies by Feng et al. (2020) argue that there is no evidence for EMP-derived vascular endothelial cells in the above-mentioned organs. Thus, the contribution of EMP to endothelial cells needs further investigation.

Osteogenesis

Erythro-myeloid progenitors also give rise to the majority of osteoclasts in mouse neonatal bones. Using lineage tracing and single-cell RNA-sequencing techniques, Yahara et al. (2020) found that CX_3CR1^+ EMP-derived macrophages fuse with local HSC-derived macrophages to form multinucleated long-lasting osteoclasts, which contribute to postnatal bone remodeling in homeostatic and inflammatory conditions.

Erythropoiesis

Macrophages in the blood islands play an important role in supporting erythropoiesis in both homeostatic and stress conditions. They have been shown to attach directly to mouse erythroblast cells and promote their proliferation in culture (Rhodes et al., 2008). Fetal liver-derived macrophages also adhere to mouse primitive erythroblasts and promote their enucleation (McGrath et al., 2008). A similar phenomenon was also observed in human placentas, where human primitive erythroblasts enucleate when in contact with macrophages (Van Handel et al., 2010). Other studies also show that erythroblastic island macrophages play an important role in the clearance of pyrenocytes in homeostatic conditions, and macrophage depletion significantly impairs erythropoietic recovery from hemolytic anemia, acute blood loss and myeloablation (Chow et al., 2013; Ramos et al., 2013).

Clearance of Cell Debris

Another important role of macrophages in development is clearance of cell debris and maintenance of tissue homeostasis. For example, it has been reported that macrophages digest the apoptotic cells in the digits of the remodeling limb bud during mouse limb morphogenesis (Hopkinson-Woolley et al., 1994). Macrophages can also remove germ cells and somatic cells that fail to incorporate into cords during testis development (Defalco et al., 2014). Aging red blood cells are also found to be cleared by splenic macrophages (de Back et al., 2014).

POSTNATAL FUNCTIONS OF EMBRYONIC/FETAL-DERIVED MACROPHAGES

One of the main functions of postnatal tissue-resident macrophages is to sense the perturbation of the microenvironment and induce inflammation. For example, in experimental inflammation conditions, depletion of resident macrophages results in diminished chemokine production, leading to a dramatic attenuation of neutrophil influx, which can be rescued by adoptive transfer of resident macrophages (Cailhier et al., 2005). However, due to the variable nature of the perturbation, the different types and distribution of the expressed recognition receptors, the exact role of tissue-resident macrophages in inflammation induction varies (Davies et al., 2013). Tissue-resident macrophages in postnatal tissue also function in repair processes and wound healing. In addition, microglia stimulate recruitment of anti-inflammatory macrophages from the periphery that promote resolution of tissue injury (Shechter et al., 2013).

SUMMARY

The successive waves of embryonic and fetal macrophage generation are complicated. To date, it is not yet understood how the diverse sources impact the function of tissue-resident macrophages. Thus, further investigation of the impact of hematopoietic origin, and the timing and sites of generation, tissue colonization and maturation are needed to better understand the diverse phenotypes and functions of

tissue-resident macrophages. Indeed, recent advances in single-cell RNA-sequencing are helping to provide more comprehensive characterization of early macrophage development in both mouse and human embryogenesis (Bian et al., 2020; Yahara et al., 2020). In addition, local environment-specific signals are likely to act coordinately with the inherent ontogenetic factors to influence the phenotypes and behavior of tissue macrophages. Thus, defining the cell-autonomous and non-autonomous regulators of tissue-resident macrophages will be necessary to fully understand their functions and, ultimately, how to manipulate their behavior *in vivo*.

AUTHOR CONTRIBUTIONS

Both authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Prenatal Origin of Pediatric Leukemia: Lessons From Hematopoietic Development

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Several lines of evidence suggest that childhood leukemia, the most common cancer in young age, originates during *in utero* development. However, our knowledge of the cellular origin of this large and heterogeneous group of malignancies is still incomplete. The identification and characterization of their cell of origin is of crucial importance in order to define the processes that initiate and sustain disease progression, to refine faithful animal models and to identify novel therapeutic approaches. During embryogenesis, hematopoiesis takes place at different anatomical sites in sequential waves, and occurs in both a hematopoietic stem cell (HSC)-dependent and a HSC-independent fashion. Despite the recently described relevance and complexity of HSC-independent hematopoiesis, few studies have so far investigated its potential involvement in leukemogenesis. Here, we review the current knowledge on prenatal origin of leukemias in the context of recent insights in developmental hematopoiesis.

Keywords: pediatric leukemia, cell of origin, hematopoiesis, hematopoietic stem cells, eryhtro-myeloid progenitors

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INTRODUCTION

Leukemia is the most frequent childhood malignancy and it is characterized by a heterogeneous manifestation (Steliarova-Foucher et al., 2017). Despite improved clinical outcome in recent years, the incidence rate is increasing (Howlader et al., 2020). Therefore, there is an urgent need to develop precision medicine strategies for specific targeting of pediatric leukemias.

Even though the cellular origin of the childhood disease remains unclear, several lines of evidence suggest that its origin is distinct from that of the adult counterpart (Bolouri et al., 2018; Malard and Mohty, 2020). The hypothesis of a prenatal origin of pediatric leukemias was initially proposed in the mid-sixties (MacMahon and Levy, 1964) and various evidences are now supporting this view. Although hematopoietic stem cells (HSCs) sustain the production of most blood cells in adults, the ontogeny of vertebrate hematopoiesis is characterized by the presence of HSC-independent hematopoietic cells that originate sequentially during embryo development and in some cases persist to adulthood (Ghosn et al., 2019). These observations raise the hypothesis that HSC-independent progenitors arising at the embryonic level could be subject to genetic hits leading to childhood leukemia.

Herewith, we will focus on the established evidence of the *in utero* origin of the disease, and discuss about recent advances in the understanding of embryonic hematopoiesis, which is crucial for the identification of the still elusive origin and features of pre-leukemic clones. We will highlight the ontogeny impact on cell transformation focusing on both HSC-independent and HSC-dependent progenitors and report the recent insights into unique embryonic hematopoietic cell populations potentially providing a permissive environment for cell transformation.

CHILDHOOD ACUTE LEUKEMIAS

Acute leukemias are characterized by uncontrolled proliferation of undifferentiated cells, called blasts, which impair normal hematopoiesis, in the bone marrow (BM) and peripheral blood (PB), with secondary infiltration of other tissues. According to morphology and cytochemistry, they are classified into acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL). Less frequently, they can show an intermediate phenotype, with features of both diseases, and are defined as mixed lineage leukemias. During childhood, lymphoid phenotypes are predominant over myeloid ones, with a highest age-specific incidence between 2 and 5 years. On the contrary, the incidence of childhood AML is highest in patients younger than 1 year (infants) (Howlader et al., 2020). 5-year survival rate has increased over time and it currently reaches 90% and over 60% for childhood ALL and AML, respectively (Gatta et al., 2014; Malard and Mohty, 2020). Despite presenting with unique clinical and biological features, the outcome of infant AML patients is similar to that of older children (Masetti et al., 2015), whereas infants with ALL tend to manifest a more aggressive course of the disease with an event-free survival lower than 50% (Pieters et al., 2007).

Cytogenetic and molecular lesions diverge in infant, childhood, and adult acute leukemias in terms of type and recurrence. Chromosomal aberrations and especially translocations involving the gene *KMT2A* (*MLL*) are the most common genetic lesions in both AML and ALL infant leukemias, but are less frequent in children and adults (Malard and Mohty, 2020; Rice and Roy, 2020). Other frequent translocations in young children (<3 years) with AML involve *CBFA2T3* and *MNX1*, while *RUNX1*, *CBFB*, and *RARA* peak in older children (Bolouri et al., 2018). In infants with ALL, *ETV6-RUNX1* (*TEL-AML1*), and *TCF3-PBX1* rearrangements are prevalent, as well as a high-hyperdiploid karyotype (Hein et al., 2020; Malard and Mohty, 2020). Conversely, the *BCR-ABL* translocation is more frequent in adults with ALL (Malard and Mohty, 2020). Focal mutations in the *N/KRAS*, *KIT*, and *CBL* genes are more frequent in children than adults with AML, whereas *IDH1*, *IDH2*, *RUNX1*, *NPM1, DNMT3A*, and *TP53* mutations are almost exclusively found in the latter category (Papaemmanuil et al., 2016; Bolouri et al., 2018). On a similar line, the genomic landscapes of adult and childhood ALL differ (Liu et al., 2016; Gröbner et al., 2018).

Together, these observations led to speculate that childhood and adult leukemias are biologically distinct and might diverge not only in their molecular landscape but also in their cellular origin. Nevertheless, the underlying mechanisms for these differences and the precise entity of the cell(s) of origin of childhood acute leukemia are still unknown.

EVIDENCES FOR A PRENATAL ORIGIN OF CHILDHOOD LEUKEMIAS

The hypothesis of a prenatal origin of childhood leukemias derived from studies on pairs of monozygotic monochorionic twins with both members affected by the disease, with the first case reported in 1882 (Greaves et al., 2003). A proposed explanation of leukemia concordance (shared disease features) in twins is that preleukemic cells arising in one twin fetus can diffuse *via* vascular anastomosis of a monochorionic placenta to the other twin (Clarkson and Boyse, 1971). The key piece of evidence which allowed to conclusively demonstrate prenatal initiation of leukemia was provided by the identification of unique clonal markers of leukemic cells, such as chromosome translocations, which can facilitate tracking of preleukemic clones. Interestingly, chromosome breakpoints always occur in a unique intronic region of the genes involved in the rearrangements and differ from patient to patient, although the fusion proteins generated are functionally equivalent. The evaluation of breakpoints in twins with concordant leukemia allowed to demonstrate that these children share the same breakpoints and consequently leukemia originated prenatally (Greaves, 1999). Preleukemic clones generated *in utero* have been described to evolve to overt leukemia either few days after birth or as long as 14 years later (Ford et al., 1993; Wiemels et al., 1999; Maia et al., 2004).

Rearrangements that involve the histone lysine methyltransferase 2A, *MLL*, at chromosome 11q23, are recurrent events in childhood leukemia, with highest incidence in infants (Wiemels et al., 2002). These rearrangements occur in approximately 50 and 70–80% of infant AML and ALL, respectively, and their frequency decreases with age (Hilden et al., 2006; Pieters et al., 2007; Harrison et al., 2010). Up to 135 *MLL* partner genes have been identified so far, among which *AFF1* (*AF4*), *MLLT3* (*AF9*), *MLLT10* (*AF10*), *MLLT1* (*ENL*), and *ELL* are the most prevalent (Meyer et al., 2018). Especially in infant ALL, and in approximately 60% of AML pediatric cases, *MLL* rearrangements seem sufficient to induce leukemic transformation on their own, since they usually not co-occur with additional mutations (Balgobind et al., 2011; Andersson et al., 2015). Retrospective analysis of blood spots taken at birth (Guthrie cards), which allow for the detection of around 1–20 leukemic cells in a single spot, have shown the acquisition of *MLL* translocations *in utero* (Gale et al., 1997), as already suggested by concordance studies on twins (Ford et al., 1993). Furthermore, the *in utero* appearance of cytogenetic lesions typical of leukemia has been supported by the detection of a *MLL*-fusion gene in fetal tissue and BM from abortions (Uckun et al., 1998). Another frequent gene fusion detected in pediatric leukemia involves the genes *ETV6*, on chromosome 12, and *RUNX1*, on chromosome 21. *ETV6-RUNX1* occurs in approximately 25% of B lineage pediatric ALL (Bernard et al., 1996). Monozygotic twins, which both developed ALL before their fifth birthday, have been described to share the

same *ETV6-RUNX1* sequence (Ford et al., 1998). Additionally, after the evaluation of Guthrie cards of newly diagnosed ALL patients with *ETV6-RUNX1* fusion, both twins and singletons of 2–5 years, have been revealed to share unique or clonotypic sequence of the translocation (Wiemels et al., 1999). The *AML1-ETO* translocation (*RUNX1-RUNX1T1*) derives from the rearrangement of chromosomes 8 and 21 and has been shown to be the most common rearrangement in both children and adults with AML, suggesting its appearance as a postnatal event. Nevertheless, the prenatal occurrence of the translocation is supported by its detection in blood spots collected at birth, which were still available at the time of AML diagnosis (Wiemels et al., 2002). Altogether, the early onset of the disease, the high concordance rate between twin pairs (5–50% within the range of 0–15 years and less than 1% for adults) (MacMahon and Levy, 1964; Buekley et al., 1996; Greaves et al., 2003; Greaves, 2005), and the presence of the first mutation hit already at birth strongly suggest a prenatal origin of leukemia. More recent studies showed that bone marrow mesenchymal stem cells (MSC) derived from infants with acute leukemia harboring *ETV6-RUNX1*, *E2A-PBX1*, and *MLL*-rearrangements express the fusion genes, suggesting that rearrangements can also occur in early embryonic progenitors before hemogenic specification (Menendez et al., 2009; Shalapour et al., 2010). These and other evidences suggest that the first mutation event can take place at different levels along prenatal hematopoietic development.

INSIGHTS INTO EMBRYONIC HEMATOPOIESIS

A fine understanding of hematopoietic ontogeny is critical to determine the processes that initiate and sustain the progression of hematopoietic disorders. Increasing knowledge in the field of developmental hematopoiesis unraveled a complex organization of the hematopoietic system during gestation, and showed that embryonic/fetal and adult hematopoiesis differ in many aspects. In this regard, mouse, zebrafish, and chicken models have provided essential information on the dynamics of emergence of hematopoietic stem and progenitor cells in vertebrates (Dzierzak and Bigas, 2018). In adults, HSCs are located in the bone marrow niche and reside at the top of the hematopoietic hierarchy. In embryos, instead, several tissues harbor hematopoietic activity and HSCs appearance is preceded by the emergence of other progenitors endowed with various potency.

In vertebrates, embryonic hematopoietic development occurs in sequential waves (**Figure 1**). In the mouse, at embryonic day (E) 7.25 the extra-embryonic yolk sac (YS) represents the site of origin of the first hematopoietic wave which gives rise to primitive nucleated erythroid cells, macrophages (which do not transit through a monocyte intermediate), and megakaryocytes (Palis et al., 1999, 2001; Tober et al., 2007). A second wave originates from E8.25 in the YS and possibly other sites. Several progenitors with various potential are generated in this wave, including erythromyeloid progenitors (EMPs), lymphoid primed multipotent progenitors (LMPPs), and progenitors with multi-lineage mesodermal potential (Azzoni et al., 2014; Palis, 2016; Ghosn et al., 2019). All of these cells arise from hemogenic endothelium through a endothelial-to-hematopoietic transition (EHT) (Swiers et al., 2016; Ottersbach, 2019). EMPs differ in their surface marker profile from primitive hematopoietic cells and early definitive progenitors with HSC potential (**Table 1**). They possess myeloid potential and sustain erythropoiesis, megakaryocytes, and myeloid cell production, including macrophages and neutrophils (McGrath et al., 2015a). As more recently shown, they also have some lymphoid potential as they give rise to cytotoxic natural killer cells (NK) (Dege et al., 2020). Between E10.5 and E11.5, EMPs seed the fetal liver (FL) (Gomez Perdiguero et al., 2015; McGrath et al., 2015a). Similar to HSCs, EMPs are regulated by the c-Kit signaling pathway (Azzoni et al., 2018). Although EMPs were initially considered transient, it is now well accepted that they constitute a source of tissue-resident macrophages which persist and self-renew throughout adulthood, independently of HSCs (Schulz et al., 2012; Epelman et al., 2014; Gomez Perdiguero et al., 2015; Mass et al., 2016). Later than EMPs, but still before HSCs and FL hematopoiesis, LMPPs arise in the YS at around E9.5 (**Figure 1** and **Table 1**). In the developing embryo they contribute to lymphopoiesis and myelopoiesis, and are devoid of the potential to generate erythrocytes, basophils, eosinophils, and tissue resident macrophages (Böiers et al., 2013). The HSC-independent wave is required for embryo survival (Chen M. J. et al., 2011). Given this relevance, it is possible that HSC-independent progenitors are subject to genetic hits leading to childhood leukemia.

HSCs can be detected starting from E10.5 (**Figure 1** and **Table 1**) in the aorta-gonad-mesonephros (AGM) region, budding from the ventral endothelium of the dorsal aorta through EHT (Müller et al., 1994; Medvinsky and Dzierzak, 1996; de Bruijn et al., 2000, 2002; Bertrand et al., 2010; Boisset et al., 2010; Kissa and Herbomel, 2010). Vitelline/umbilical arteries, embryonic head, and placenta represent additional embryonic vascular sites in which HSC activity takes place (de Bruijn et al., 2000; Ottersbach and Dzierzak, 2005; Robin et al., 2010; Ivanovs et al., 2011; Li et al., 2012). Until E12.5, very few transplantable HSCs arise in the embryo, although pro/pre-HSCs are already present from E9.5 (Rybtsov et al., 2016; **Table 1**). HSC maturation and expansion takes place in the FL from E12; subsequently, HSCs colonize the bone marrow where they reside throughout adult life (Kumaravelu et al., 2002; Rybtsov et al., 2016). Although it is well acknowledged that mammalian hematopoiesis is highly conserved and shares many similarities with the mouse (Enzan, 1964; Tavian et al., 2001; Ivanovs et al., 2011; Julien et al., 2016; Bian et al., 2020), the mechanisms that describe human hematopoiesis are less clear and need to be further investigated (Easterbrook et al., 2019).

MODELS FOR THE STUDY OF THE ORIGIN OF CHILDHOOD LEUKEMIAS

A comprehensive understanding of the cellular origins of childhood leukemia is fundamental for the establishment of faithful animal models. However, despite intensive investigation of the intrinsic and extrinsic factors regulating HSPC biology and

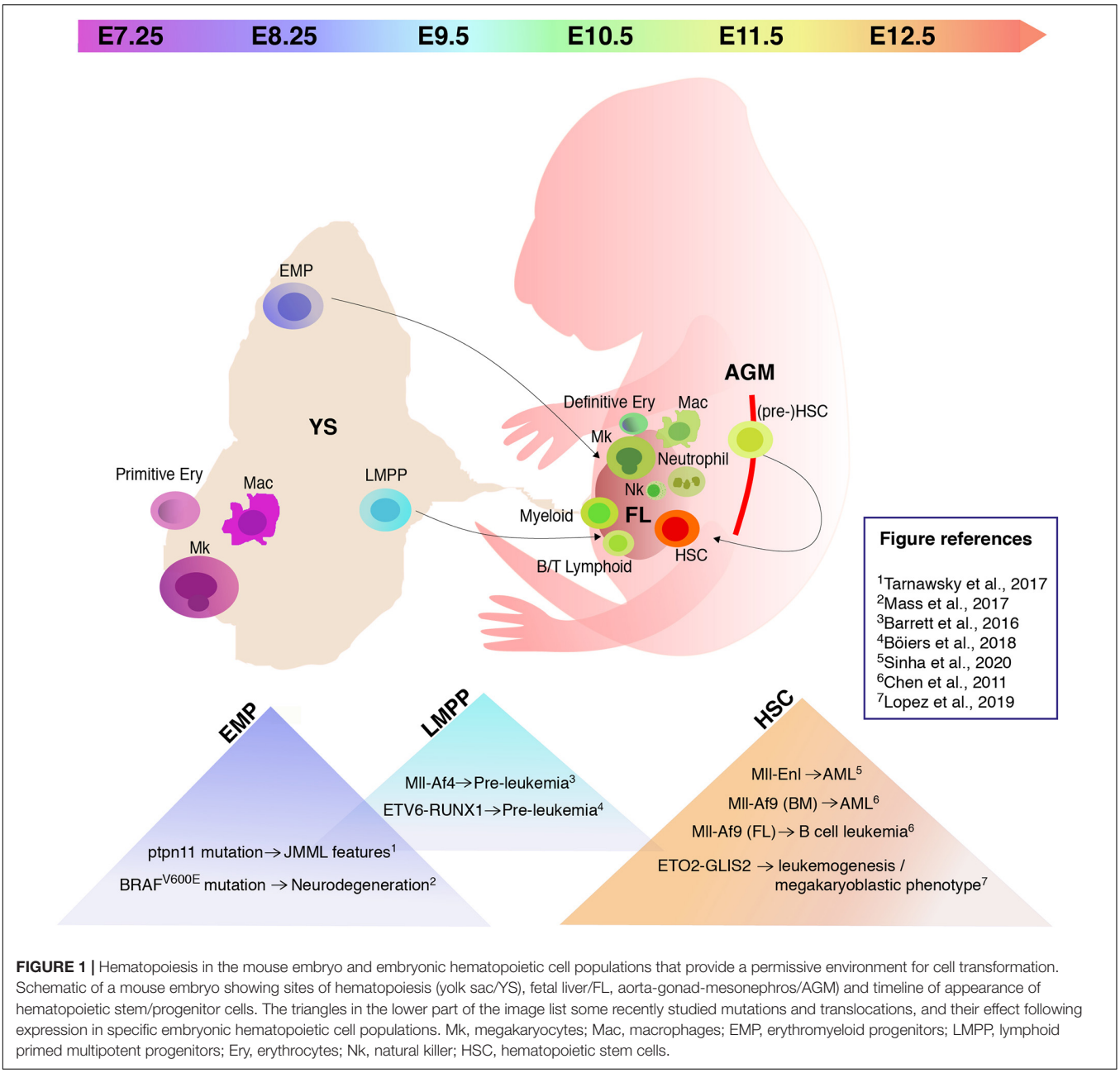

FIGURE 1 | Hematopoiesis in the mouse embryo and embryonic hematopoietic cell populations that provide a permissive environment for cell transformation. Schematic of a mouse embryo showing sites of hematopoiesis (yolk sac/YS), fetal liver/FL, aorta-gonad-mesonephros/AGM) and timeline of appearance of hematopoietic stem/progenitor cells. The triangles in the lower part of the image list some recently studied mutations and translocations, and their effect following expression in specific embryonic hematopoietic cell populations. Mk, megakaryocytes; Mac, macrophages; EMP, erythromyeloid progenitors; LMPP, lymphoid primed multipotent progenitors; Ery, erythrocytes; Nk, natural killer; HSC, hematopoietic stem cells.

their relationship to leukemogenesis, this has not been achieved so far. Several studies showed that gene fusions recurrent in pediatric leukemias can lead to divergent outcomes in terms of disease aggressiveness, latency, phenotype, and transcriptional features, according to their time of appearance during ontogeny (Chen W. et al., 2011; Horton et al., 2013; Man et al., 2016; Chaudhury et al., 2018; Lopez et al., 2019; Sinha et al., 2020). Differences in lineage specification and disease latency have been clearly shown after the induction of *Mll-Af9* in FL and adult BM HSCs. In these two models the translocation gave rise to B cell leukemia with a prolonged latency and AML, respectively (Chen W. et al., 2011). Similarly, transduction of *MLL-AF9* and *MLL-AF4* in human neonatal cord blood (CB) HSPCs mainly resulted in ALL (Horton et al., 2013; Lin et al., 2016). In contrast, *MLL-AF9* transduced human adult BM HSPCs gave rise to non-leukemic myeloid-biased engraftment and *MLL-Af4* transduced mouse adult BM cells led to AML (Horton et al., 2013; Lin et al., 2016). Consistent with these findings it has been demonstrated that B-cell committed progenitors harbor transforming potential in ALL, (Castor et al., 2005; Kong et al., 2008), but there are also evidences hinting at a cell of origin at an earlier developmental stage (le Viseur et al., 2008). The inducible expression of the recurrent gene fusion product *ETO2-GLIS2*, associated with acute megakaryoblastic leukemia, triggered leukemogenesis in both FL HSPCs and adult BM HSPCs, but gave rise to a megakaryoblastic phenotype with a shorter latency and caused more evident

TABLE 1 | List of the main hematopoietic stem and progenitor cell populations in the early mouse embryo, their time and site of first emergence and surface marker expression profile.

Cell type	Wave	First emergence	Surface marker expression	References
Primitive erythroid cells	HSC-independent	E7.25 YS	TER119$^{low/+}$ Kit^{low} CD41^{low}	Palis et al., 1999; Ferkowicz et al., 2003
Primitive megakaryocytes	HSC-independent	E7.25 YS	CD41+GP1bβ+	Xu et al., 2001; Tober et al., 2007
Primitive macrophages	HSC-independent	E7.25 YS	CD45$^+$ F4/80$^+$ CD16/32$^+$	McGrath et al., 2015a
EMPs	HSC-independent	E8.25 YS	CD41$^+$ Kit$^+$ CD16/32$^+$ CD31$^+$ Sca1$^-$ Flt3$^-$	McGrath et al., 2015a; Frame et al., 2016
LMPPs	HSC-independent	E9.5 YS	Lin$^-$ CD45$^+$ Kit$^+$ Rag1$^+$ Flt3$^+$ IL7Rα$^+$	Böiers et al., 2013
Pro-HSCs	HSC-dependent	E9.5 caudal part	Lin$^-$ VE-Cadherin$^+$ CD45$^-$ CD43$^-$ CD41^{low}	Rybtsov et al., 2014
Pre-HSC type I	HSC-dependent	E10.5 AGM	Lin$^-$ VE-Cadherin$^+$ CD45$^-$ CD43$^+$ CD41$^+$ CD201$^+$	Rybtsov et al., 2011, 2014; Zhou et al., 2016
Pre-HSC type II	HSC-dependent	E10.5 AGM	Lin$^-$ VE-Cadherin$^+$ CD45$^+$ CD43$^+$ CD201$^+$	Taoudi et al., 2008; Rybtsov et al., 2011, 2014; Zhou et al., 2016
FL HSCs	HSC-dependent	E12.5 fetal liver	Lin$^-$ Sca1$^+$ Kit$^+$ CD45$^+$ CD48$^-$ CD150$^+$ CD11b^{low} Flt3$^+$ CD201$^+$	Kim et al., 2006; Benz et al., 2012; Zhou et al., 2016

transcriptional changes only in the former (Lopez et al., 2019). The induction of the *Mll-Enl* translocation in FL HSPCs at E12.5 led to an overt, more aggressive AML form than the one triggered in adults, and to a transplantable disease in secondary recipients (Sinha et al., 2020). Overall, these studies suggested that childhood leukemias originating from the FL possess unique features that differentiate them from the ones resulting from the same genetic lesions occurring in the adult. However, they did not precisely address the cellular origin of the disease, as the FL is not a site of *de novo* hematopoietic generation. Cells seeding the FL during embryogenesis can have multiple origins, most of which are HSC-independent, and could already carry genetic lesions at the time of FL seeding. Besides the cell subtype in which the genetic hit takes place, the developmental stage of the hematopoietic niche also elicits an important contribution in regulating leukemic lineage commitment (Chaudhury et al., 2018; Rowe et al., 2019). Indeed, it has been recently shown how a *MLL*-rearrangement can differentially give rise to either a mixed lineage or a myeloid leukemia according to the developmental age of the microenvironment in a setting where the cell of origin is the same (Rowe et al., 2019). In addition to the fact that the susceptibility for transformation and the resulting phenotype change during ontogeny, differences in human and mouse embryonic hematopoietic development could also affect the faithful modeling of the human pediatric leukemia.

AN HSC-INDEPENDENT ORIGIN OF PEDIATRIC LEUKEMIA?

The potential link between HSC-independent hematopoiesis and leukemogenesis remains so far largely unexplored. Only few studies have investigated which of the unique embryonic hematopoietic cell populations provide a permissive environment for cell transformation. As EMPs sustain fetal myelopoiesis and their progeny persists in the adult (Gomez Perdiguero et al., 2015; McGrath et al., 2015b), it has been recently suggested that EMPs may represent cells of origin of diseases associated with fetal development. Furthermore, in the context of the Mll-Af9 translocation, it has been shown how the proliferation rate is a limiting factor for malignant transformation (Chen et al., 2019). Thus, the rapid proliferation of EMPs (McGrath et al., 2015a) suggests that these cells may be susceptible to oncogenic transformation. A proof of principle of the notion that EMPs could be cells of origin for post-natal diseases derives from a recent study that investigated whether the neurodegeneration observed in patients with histiocytosis could be caused by somatic mutations in the EMP lineage. In particular, the mosaic expression of the BRAF^{V600E} mutation in EMPs at E8.5 was shown to cause expansion of microglia and neurodegeneration in adult mice (Mass et al., 2017). The involvement of HSC-independent hematopoietic progenitors has been assessed in the context of juvenile myelomonocytic leukemia (JMML). EMPs looked like good candidates, given the high relapse rates after HSCs transplantation (Locatelli et al., 2013) and the plausible *in utero* initiation of the disease (Matsuda et al., 2010). For this purpose, the ptpn11 gain-of-function JMML-initiating mutation had been introduced into EMPs. Although mice demonstrated features of JMML and mutant EMPs engrafted spleens of neonatal recipients, the disease was not transplantable (Tarnawsky et al., 2017).

Even though no studies have so far evaluated the susceptibility of EMPs to acute leukemias-specific genetic hits, some have specifically targeted acute leukemia-related translocations to LMPPs. LMPPs have recently emerged as potential cells of origin of B cell acute lymphoblastic leukemia (B-ALL) (Barrett et al., 2016; Böiers et al., 2018). Conditional activation of the *Mll-AF4* translocation in murine embryonic hematopoietic cells before the predominance of HSC-dependent hematopoiesis (E12.5–E14.5) resulted in a pre-leukemic phenotype. Even if mice showed B-cell lymphomas after a long latency, the model was unable to fully recapitulate the disease seen in patients harboring the same

genetic alteration (Barrett et al., 2016). A possible counterpart for mouse LMPPs has also been identified in human FL as a IL-7R$^+$ progenitor which maintains both myeloid and lymphoid potential. Evidence of the susceptibility of human fetal B cells progenitors to dysregulation by *ETV6-RUNX1* was provided by the introduction of the translocation in human induced pluripotent stem cells (iPSC). This led to a pre-leukemic initiation with expansion of the CD19$^-$ IL-7R$^+$ population, suggesting IL-7R progenitors as candidate cells of origin for *ETV6-RUNX1* preleukemia (Böiers et al., 2018). Moreover, the introduction of *Runx1* and *Ezh2* mutations in early thymic progenitors (ETPs), which are closely related to LMPPs, could model ETP leukemia features in mice and lead to acute lympho-myeloid leukemia progression upon introduction of *Flt3-ITD* (Booth et al., 2018). To summarize, recent work has shown that (i) HSC-independent progenitor cells can be subject to genetic hits occurring prenatally, which can lead at least to a pre-leukemic state; (ii) HSC-independent progenitors are susceptible to pre-leukemic initiation during a limited time frame, and (iii) multiple genetic hits can be required before an overt manifestation of the disease.

CONCLUSION AND FUTURE PERSPECTIVES

Prenatal leukemic development in humans is a multifactorial process. Because of the complexity of the events leading to childhood acute leukemia and the difficulty of studying *in utero* stages, the identification and characterization of the cell(s) of origin is still a challenge. Although recent studies shed light on the potential of HSC-independent hematopoietic progenitor cells to act as the cell of origin for pediatric leukemia, there is urgent need to further investigate this aspect and in particular how leukemia-associated genetic hits may impact early stages of disease development *in utero*. This knowledge would be critical to better understand the etiology and pathogenesis of the disease, which would enable the refinement of animal models, the identification of new therapeutic approaches and to define preventive measures.

AUTHOR CONTRIBUTIONS

AC and EA conceived the topic, reviewed the literature, made the figure, and wrote the manuscript. GC, SB, AB, and RM revised and edited the manuscript. All authors read and approved the final version of the manuscript.

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REVIEW
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In utero Therapy for the Treatment of Sickle Cell Disease: Taking Advantage of the Fetal Immune System

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Sickle Cell Disease (SCD) is an autosomal recessive disorder resulting from a β-globin gene missense mutation and is among the most prevalent severe monogenic disorders worldwide. Haematopoietic stem cell transplantation remains the only curative option for the disease, as most management options focus solely on symptom control. Progress in prenatal diagnosis and fetal therapeutic intervention raises the possibility of *in utero* treatment. SCD can be diagnosed prenatally in high-risk patients using chorionic villus sampling. Among the possible prenatal treatments, *in utero* stem cell transplantation (IUSCT) shows the most promise. IUSCT is a non-myeloablative, non-immunosuppressive alternative conferring various unique advantages and may also offer safer postnatal management. Fetal immunologic immaturity could allow engraftment of allogeneic cells before fetal immune system maturation, donor-specific tolerance and lifelong chimerism. In this review, we will discuss SCD, screening and current treatments. We will present the therapeutic rationale for IUSCT, examine the early experimental work and initial human experience, as well as consider primary barriers of clinically implementing IUSCT and the promising approaches to address them.

Keywords: prenatal therapy, *in utero* transplantation, sickle cell disease, tregs, congenital blood disorders, haematopoietic stem cell transplantation (HSCT)

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INTRODUCTION

Sickle Cell Disease (SCD) is an autosomal recessive disorder resulting from a β-globin gene missense mutation (**Figure 1**). The "sickle" hemoglobin HbS is prone to polymerisation, thereby changing erythrocyte morphology, and inducing subsequent haemolytic anemia and vaso-occlusive crises (VOCs). The primary management strategy of SCD remains focused on symptom control, and despite significant progress in understanding the condition, haematopoietic stem cell transplantation (HSCT) remains the only curative option (Lucarelli et al., 2012). Advancement in prenatal diagnosis and fetal therapeutic intervention has increased the possibility of *in utero* management, drastically changing the paradigm for SCD treatment before birth. Indeed, this life-long, debilitating condition can be diagnosed prenatally, using traditional invasive procedures such as chorionic villus sampling in high-risk heterozygous patients or fetal DNA quantification

from maternal blood (Daniel et al., 2019). Among the possible prenatal treatments, *in utero* stem cell transplantation (IUSCT) shows the most promise (Jeanblanc et al., 2014). IUSCT is a non-immunosuppressive alternative conferring various fetally focused advantages over postnatal stem cell management. Fetal immunologic immaturity may allow engraftment of allogeneic cells before fetal immune system maturation, enhancing donor-specific tolerance and lifelong chimerism (Flake, 2004). Despite host cell competition within the fetal and maternal immune systems, as well as practical aspects of IUSCT, progress is being made—preclinical studies are underway to overcome these barriers and achieve successful clinical implementation.

Haemoglobinopathies

Sickle cell disease is part of a group of diseases called haemoglobinopathies. The severity of these diseases can vary from fatal to asymptomatic and result from structural abnormalities of the globin protein, thereby affecting erythrocytes and oxygen transport. SCD is one of the most common inherited diseases caused by a single base-pair point mutation. There is an estimated 5% global prevalence of healthy gene carriers of SCD or thalassemia as reported by the World Health Organization (Inusa et al., 2019; Sickle Cell Disease|WHO Regional Office for Africa, 2020).

Hemoglobin Development

Hemoglobin is formed by four different globin subunits; the combination of erythrocytes and reticulocytes varies depending on the age of the individual (Inusa et al., 2019). Up to 6 weeks post-birth, fetal hemoglobin (HbF) is formed by two alpha and two gamma globin chains, coded by gene loci on chromosomes 16 and 11, respectively (Sankaran and Nathan, 2010). At 6-weeks post-partum, erythrocyte progenitors begin to produce adult (HbA) hemoglobin (Sankaran and Nathan, 2010; Diepstraten and Hart, 2019). Unlike HbF, HbA is made of two alpha and two beta globin chains and typically forms 90–95% of the total hemoglobin in adult erythrocytes, although this is subject to variation (Wood et al., 1975; Kato et al., 2018).

CAUSE OF DISEASE

Sickle Cell Disease

SCD is the name given to a group of disorders, which contain at least one hemoglobin S allele (HbS). The second pathogenic variant can be another hemoglobin S allele or other Hb variants such as HbC (HBB GLU6LYS). The allele is caused when the Adenine base replaces Thymine in a missense mutation at the 6th position of the β-globin chain, resulting in a GAG to GTG codon change (**Figure 1**) (Ingram, 1957, 2004; Lettre and Bauer, 2016). SCD can be inherited as homozygous variants of hemoglobin S allele (HbSS) (Neel, 1949; Inusa et al., 2019; Mohammed-Nafi'u et al., 2020), resulting in a severe form of the disease labeled sickle cell anemia (SCA) or by compound heterozygous inheritance (HbSC). Co-inheritance of beta-thalassemia, resulting in low globin protein production, can result in HbS/B+ or HbS/Bo genotypes (Steinberg and Sebastiani, 2012; Inusa et al., 2019). Environmental factors, such as healthcare access, socioeconomic status, nutrition, and even humidity, are also known to affect disease severity (Jones et al., 2005; Tewari et al., 2015; Kato et al., 2018).

Incidence, Burden, and Screening

Individuals of African, Indian or Arabian ancestry have a disproportionately higher prevalence of SCD. There is a theoretical advantage provided by heterozygous SCD inheritance, as carriers of SCD are 90% less likely to suffer from severe malaria (Allison, 1954; Weatherall, 2011; Luzzatto, 2012). In Africa, SCD is estimated to account for 9–15% of all deaths of children under five (Weatherall, 2010). In comparison, even with the severe form of the disease, SCA patients have a life expectancy in the UK of 67 years, with 94% childhood survival rates for uncomplicated SCD (Quinn et al., 2010; Gardner et al., 2016). Migration has distributed SCD worldwide, highlighting healthcare inequalities, where the transition from acute to chronic settings remains limited to high-income countries (Piel et al., 2013b; Lindenau et al., 2016).

In 2006, the World Health Organization recognized SCD as a global problem with significant economic implications with the incidence expected to rise above 400,000 births per year in 2050 (Piel et al., 2013a; Thein and Thein, 2016). In the UK, prenatal screening for SCD is performed in high-risk pregnancies at around 11–13 weeks, through chorionic villus sampling. A potential new non-invasive technique, which acquires fetal DNA from maternal circulation, could be the key to a safer, and potentially earlier diagnosis at 10 weeks of gestation (Lewis et al., 2014; Twiss et al., 2014; Daniel et al., 2019).

Pathophysiology

SCD and its complications are a result of erythrocyte and reticulocyte morphology changes, seen in **Figure 1** (Inusa et al., 2019; Carden et al., 2020). The mutation causes Glutamic acid to be replaced by the amino acid Valine, which changes the shape and physical properties of hemoglobin (Inusa et al., 2019). The SCD erythrocytes are then subject to intracellular HbS polymerisation (Pawliuk et al., 2001; Vekilov, 2007). Whilst healthy hemoglobin reversibly rearranges during deoxygenation, sickle hemoglobin, on the other hand, forms helical structures (Kuypers, 2014; Sundd et al., 2019). The rigid, insoluble strands form fibers which align in parallel, creating intracellular crystals (Inusa et al., 2019). At first, sickling is reversible, yet repetitive cycles of oxygenation and deoxygenation eventually lead to the characteristic irreversibly sickle cell (Noguchi and Schechter, 1985; Nash et al., 1988; Harrington et al., 1997; Goodman, 2004).

HbS polymerisation results in rigid, fragile sickle cells with up to a 75% reduction in lifespan (Kaul et al., 1983; Evans and Mohandas, 1987; Quinn et al., 2016). While normal erythrocytes live 120 days, sickle erythrocytes typically last only 10–20

Abbreviations: ACK2, C-kit Receptor; BM, Bone Marrow; DAMPs, Damage-Associated Molecular Patterns; HbA, Adult Hemoglobin; HbF, Fetal Hemoglobin; HbS, Sickle Hemoglobin; HSC, Hematopoietic Stem Cells; HSCT, Haematopoietic Stem Cell Transplantation; IUSCT, *In Utero* Stem Cell Transplantation; MHC, Major Histocompatibility Complex; SCA, Sickle Cell Anemia; SCD, Sickle Cell Disease; SDF-1, Stromal Derived Factor 1; Tregs, Regulatory T cells; VOC, Vaso-occlusive Crises.

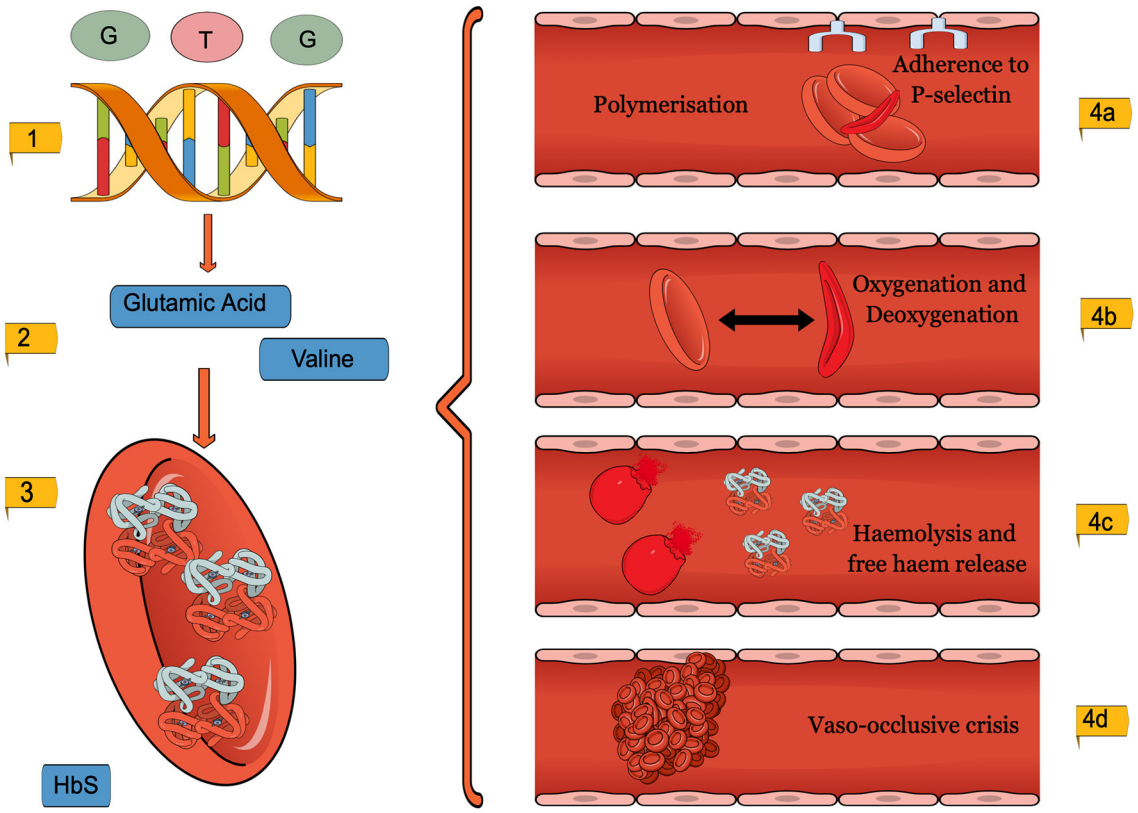

FIGURE 1 | A diagram to show the pathophysiology of SCD. (1). GAG to GTG missense mutation (2). Amino acid substitution from Glutamic acid to Valine. (3). Morphology changes in the SCD erythrocytes (4). The adverse consequences, micro and macrovascular complications of SCD erythrocytes. This figure was created using MindtheGraph.com under a Creative Commons license.

days due to the processes of extravascular phagocytosis and intravascular haemolysis (Crosby, 1955; Sebastiani et al., 2007).

Oxidative stress is both cause and effect of increased erythrocyte haemolysis (Kato et al., 2018). It is triggered by HbS polymerisation and cumulative impacts of reactive oxygen species and free radicals, produced by intracellular hemoglobin metabolism (Jagadeeswaran and Rivers, 2017; Alayash, 2018). Haemolysis leads to higher levels of nitric oxide consumption, disruption of arginine metabolism, depletion of endogenous glutathione and increased expression of oxidases such as xanthine dehydrogenase (Aslan and Freeman, 2007; Wood and Granger, 2007). High levels of oxidative stress also increase damage to cell membranes. The oxidation of cytoskeletal proteins and membrane lipids prematurely ages sickle cells, further increasing haemolysis (Rogers et al., 2013).

Haemolysis releases hemoglobin and haem into the bloodstream (Kato et al., 2018). The presence of free hemoglobin and haem exacerbates extracellular oxidative stress, leading to a higher attraction between HbS molecules, agglutination and polymer formation (Kuross et al., 1988; Uzunova et al., 2010; Alayash, 2018). Free plasma hemoglobin activates both the innate immune system, through Damage-Associated Molecular Patterns (DAMPs) and platelets, through P-selectin cell surface expression (Kato et al., 2018).

SCD erythrocytes contain dysfunctional membrane channels, such as K-Cl cotransporters or Gardos channels, which contribute to cell dehydration, further increasing the HbS concentration and polymerisation (Frenette and Atweh, 2007; Gardner, 2018). Altered morphology increases membrane micro vesiculation and the release of erythrocyte microparticles, containing cell surface markers, which affect coagulation, inflammation and endothelial adhesion (Piccin et al., 2007; Westerman et al., 2008; Hebbel and Key, 2016). The resulting endothelial dysfunction is thought to involve other adhesion molecules, such as integrins, blood group antigens and white cell proteins (Takehara et al., 1991; Telen, 2005; Chen et al., 2016; Koehl et al., 2017).

Complications

SCD causes a wide range of complications in various of the body's systems, shown in **Figure 2**, including the cardiovascular, reproductive, pulmonary, renal, hepatobiliary, and can even cause neurocognitive dysfunction (Kato et al., 2018; Inusa et al., 2019). Beyond physical complications, SCD significantly affects the patients' quality of life, leading to psychosocial implications in infancy, adulthood, and pregnancy (Oteng-Ntim et al., 2015).

The clinical hallmark and primary cause of SCD hospitalisations are vaso-occlusive crises (VOCs) (Rees et al.,

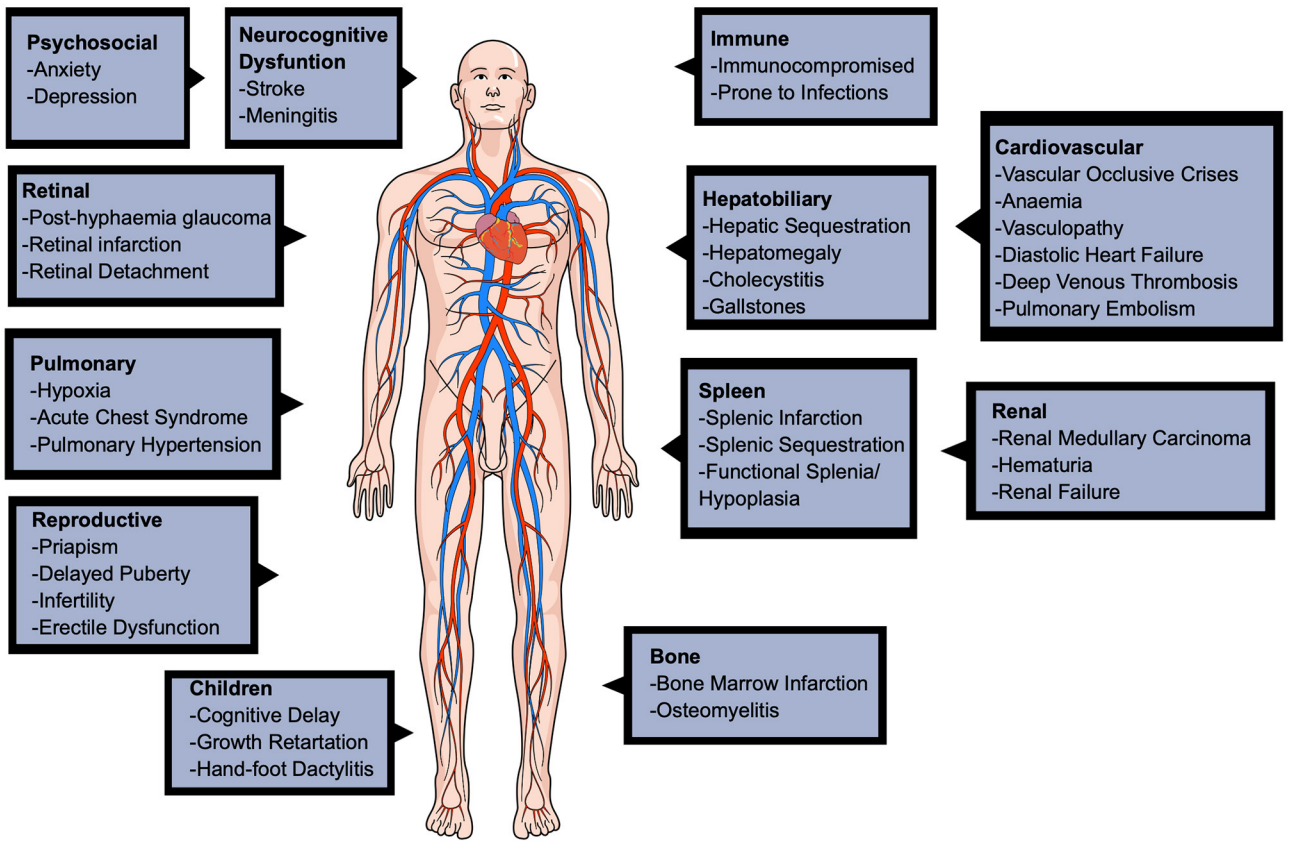

FIGURE 2 | A diagram to show the various complications of sickle cell disease. The complications are seen in many major organ systems, such as cardiovascular, pulmonary, renal, hepatobiliary, reproductive and even neurological. This figure was created using MindtheGraph.com under a Creative Commons license.

2010; Manwani and Frenette, 2013). VOCs are sudden, episodic, severe pains in the extremities, chest and back that significantly impact the patients' quality of life (de Montalembert, 2002). The painful episodes are unpredictable, spontaneous and of variable duration, generally triggered by a range of stressors such as infection, fever, dehydration, acidosis, and temperature or humidity changes. VOCs are a result of microvascular occlusions, leading to ischaemia, oedema, pain, through stimulation of nociceptive nerve fibers and papillary necrosis, eventually leading to multi-organ damage. In infants, VOCs present as irritability and inability to weight-bear, walk or crawl (Inusa et al., 2019).

DISEASE-MODIFYING DRUGS

Hydroxyurea

Hydroxyurea acts as an inhibitor of ribonucleotide reductase, thereby inactivating enzymes involved in cellular division (Inusa et al., 2019). The result is an intermittent cytotoxic suppression of erythroid progenitors and cell stress signaling, due to cell cycle arrest and death at S phase (Agrawal et al., 2014). The resulting increased recruitment of erythroid progenitor cells (which can produce red blood cells) is associated with an overall higher HbF synthesis (Wood et al., 1976; Agrawal et al., 2014). Higher levels of HbF increase SCD lifespan, reduce the incidence of acute chest syndrome and occurrence of VOCs, stroke and chronic organ damage (Ballas et al., 1989; Strouse and Heeney, 2012; Diepstraten and Hart, 2019; Lagunju et al., 2019).

Hydroxyurea has been shown to improve both clinical and hematological complications, with particular benefit to high-risk children (Platt et al., 1984; Ballas et al., 1989; Mvalo et al., 2019). However, effectiveness varies among individuals. Due to its mechanism of action and teratogenic effect, it is contraindicated in acute liver diseases and pregnancy (Depass and Weaver, 1982; Agrawal et al., 2014; Husain et al., 2017). Adverse effects, although transient and dose-dependent, include severe anemia (1%), transient gastrointestinal symptoms, abdominal pain, vomiting, diarrhea, skin, palm and nail hyperpigmentation and short-lived hematological toxicity, with potential leukopenia, thrombocytopenia, and pancytopenia (Ballas et al., 1989; Wang et al., 2011).

Although 63% of SCA patients in high-income countries receive hydroxyurea, due to lack of treatment guidelines, reduced clinical infrastructure and medication inaccessibility, this percentage can be as low as zero in under-resourced settings (McGann et al., 2016; Power-Hays and Ware, 2020). Treatment is also burdened by poor medication adherence, high patient discontinuation, or qualitative HbF level assessments every 3–6 months (Agrawal et al., 2014; Shah et al., 2019).

L-Glutamine

Glutamine, in addition to 5 other amino acids, is a "conditionally essential" amino acid for the body, meaning its synthesis is limited under specific physiological conditions (Inusa et al., 2019). It is thought to contribute to the production of nicotinamide adenine dinucleotide (NAD), a ubiquitous redox cofactor in red blood cells (RBC), which is reduced in SCD erythrocytes. The reduced redox potential of SCD erythrocytes increases oxidative stress, thus potentiating SCD pathophysiology (Niihara et al., 2018). In 2017, L-glutamine was approved for use in adults and children above five (Quinn, 2018; Sadaf and Quinn, 2020). Dosing is determined through weight-based formulation and given by oral administration, either in capsule, powder, or tablets (Mok and Hankard, 2011). However, hepatic and renal function monitoring is recommended (Niihara et al., 2018; Cox et al., 2020). Moreover, L-glutamine is up to 20 times more expensive than hydroxyurea and lacks long-term follow-up studies (Quinn, 2018). It requires dosage twice a day and therefore poses further feasibility, accessibility and adherence issues compared with hydroxyurea (Langley, 2020).

New Therapeutic Approaches

Increased knowledge regarding SCD pathophysiology has led to new therapeutic strategies. These focus on fetal hemoglobin induction, HbS polymerisation and oxidation inhibition and reduced endothelial activation (Inusa et al., 2019). However, current SCD management using hydroxyurea, L-glutamine or other disease-modifying drugs is only aimed at delaying, preventing, and mitigating complications. The introduction of curative therapies, such as IUSCT has given new hope, not only for the management of SCD but for its eradication.

IUSCT FOR SCD

Rationale for IUSCT

IUSCT is an appealing approach to SCD treatment as it tends to avoid the many complications associated with current prescribed management. The only viable curative treatment option for SCD is HSCT. There are various ontogenetic advantages unique to fetal development, which provide convincing arguments as to why HSCT would be far more effective if administered in the fetus rather than postnatally. Firstly, the undeveloped fetal immune system allows for the introduction of an antigen without immunosuppression and results in antigen-specific immune tolerance (Witt et al., 2018). IUSCT evades the postnatal HSCT complications such as myeloablative preconditioning, graft-vs.-host disease, and graft rejection (Witt et al., 2018). In humans, this immunological immaturity tends to be present until the end of the first trimester and so provides a succinct "window of opportunity." Another potential biological advantage is that the early gestational period is the only time in life during which large-scale migration of hematopoietic stem cells (HSCs) to tissue compartments occurs; definitive haematopoiesis begins in the yolk sac and aorta-gonad-mesonephric region, migrates to the fetal liver, and finally resides in the bone marrow (BM) (Christensen et al., 2004). Although it was previously thought that the development of these new niches would facilitate donor cell engraftment, it is now recognized that the fetal haematopoietic system is incredibly competitive with excess circulating HSCs. However, if one could understand and utilize the natural mechanisms that regulate migration and engraftment of stem cells, it may be possible to drive the engraftment of donor cells.

In addition, the physiological conditions for the systemic distribution of donor cells are superior in the fetus, due to the presence of circulatory shunts. This allows donor cells to bypass the pulmonary circulation via the ductus arteriosus and foramen ovale and avoid sequestration in the lung microcirculation. Instead, donor cells are delivered directly into the systemic circulation and to the peripheral organs (Ekblad-Nordberg et al., 2019). Furthermore, the small size of the fetus (∼35 g at 12-weeks' gestation) confers a clear advantage over transplantation in a pediatric/adult patient as it allows the cell dose per weight of the recipient to be maximized, favoring successful engraftment (Almeida-Porada et al., 2016). This approach may partly facilitate overcoming the competitive advantage of host haematopoiesis.

Perhaps the most persuasive motivation for IUSCT is the possibility of pre-empting the clinical manifestations of SCD. Treating SCD early enough to prevent disease onset in adult erythropoiesis eliminates the need for lifelong, non-curative treatment and significantly improves the quality of life for both the patient and their family.

The Fetal Immune System

Early in development, the fetal immune system undergoes the process of self-education, principally in the fetal thymus: pre-lymphocytes that recognize "self" major histocompatibility complex (MHC) antigens are positively selected for, while those with high-affinity recognition of self-antigens are eliminated (Takahama, 2006). This results in central tolerance and should leave T cells that are reactive to foreign antigens unaffected. Theoretically, the introduction of donor cells into the fetal thymic microenvironment by IUSCT should lead to the removal of alloreactive T cells and, consequently, donor-specific immunologic tolerance. In order to achieve immunologic tolerance to donor antigens, the transplant should be performed before the emergence of mature T cells in the fetal thymus and peripheral circulation (∼12–14 weeks gestation in humans) (Takahama, 2006). Thymic selection is highly effective, but not infallible; self-reactive T cells can evade deletion and circulate peripherally. However, most of these self-reactive T cells do not cause any problems as they are suppressed by fetal regulatory T cells (Tregs) (Burt, 2013).

IUSCT Experimental Studies

The notion of using the fetus as the recipient of stem cell transplantation traces back to the landmark finding that exposure to foreign antigens can result in immune tolerance. Owen (1945), observed lifelong haematopoietic cell chimerism and sibling-specific tolerance in monochorionic dizygotic bovine twins that shared cross-placental circulation (Merianos et al., 2008). Natural haematopoietic chimerism has now been observed in numerous species, including humans and primates (Picus et al., 1985; Rinkevich, 2001).

Efforts to reproduce "natural chimerism" and treat genetic disorders in the laboratory have had varying degrees of success. The first experimental IUSCT success employed transplacental injection of adult allogeneic BM haematopoietic cells at E11, into c-kit deficient mouse fetuses, which reversed the genetic anemia produced by the c-kit defect. Interestingly, they found that the donor HSCs engrafted and successfully replaced the defective erythroid lineage. This was demonstrated by using strain-specific hemoglobin markers. The degree of correction was proportional to the degree of anemia. No engraftment was observed in non-anemic, control recipients. There was tolerance toward the transplanted HSCs with no graft-vs.-host disease observed (Fleischman and Mintz, 1979). This key finding suggests that when normal cells have a competitive advantage, greater donor cell engraftment occurs. This was also the first study to identify host cell competition as a barrier to donor cell engraftment; this is essential to consider for clinical implementation of IUSCT and will be discussed later.

Murine models of SCD have been used to demonstrate stable allogeneic engraftment following IUSCT of donor BM or fetal liver cells (Hayashi et al., 2003). However, the quantity of chimerism achieved did not correct the SCD phenotype. Despite greater levels of donor Hb (13–35%) with minimal donor mononuclear cell engraftment (1.1–4%), a substantial amount of sickling cells persisted in circulation (Hayashi et al., 2003). This finding aligns with previous studies that suggest thresholds of 70 and 40% donor myeloid engraftment are necessary to eradicate peripheral erythrocyte sickling and anemia, respectively (Iannone et al., 2001). In beta thalassemia previous experience with mixed chimerism, indicates that the disease can be significantly ameliorated by levels of BM engraftment of 15–20% (Andreani et al., 1996, 2000; Hayashi et al., 2003). Even if low engraftment is achieved (<10%), the potential of these HSCs achieving complete RBC chimerism is high, as seen in postnatal studies (Fouzia et al., 2018). Extending these studies further, Peranteau et al. administered donor BM cells intraperitoneally to murine fetal SCD model on day 14 of gestation. Postnatally, the recipients had boost transplantation with the same donor BM cells (Peranteau et al., 2015). They showed that IUSCT alone allowed for the development of donor-specific tolerance and long-lasting low levels of allogeneic engraftment. After the postnatal intervention, engraftment was enhanced to high-level chimerism and near-complete Hb replacement with normal donor Hb (>90%), ultimately correcting the SCD phenotype (Peranteau et al., 2015).

IUSCT in Clinical Practice

The initial success of IUSCT in experimental animal models was highly promising for clinical application. As yet, IUSCT has been performed on numerous human patients for various genetic disorders, notably osteogenesis imperfecta and alpha-thalassaemia (Le Blanc et al., 2005; Götherström et al., 2014; Mackenzie, 2020). These cases demonstrated that, when using the current methodology, IUSCT is not able to reach clinically significant, therapeutic levels of engraftment unless immunodeficiency or donor cell-selective advantage was present (Merianos et al., 2008). The first successful IUSCT was achieved for "bare lymphocyte syndrome," an immunodeficiency disorder (Touraine et al., 1989). Following this breakthrough, successful correction of severe combined immunodeficiency (SCID) was reported by several groups; stable split chimerism was achieved, with the reconstitution of the T-cell lineage only, as previously observed in murine experiments (Blazar et al., 1998). With regards to SCD, a female fetus prenatally diagnosed with SCA received donor fetal liver cells at 13 weeks of gestation (equivalent to E16 in murine pregnancy), intraperitoneally. Disappointingly, cord blood at birth and peripheral blood at 8 months of age did not demonstrate engraftment of transplanted donor cells (Westgren et al., 1996). It is, however, essential to highlight that this was accomplished over two decades ago and technical expertise has improved immensely. In addition, animal studies have uncovered many barriers to engraftment, which we will discuss.

At present, the future clinical application of IUSCT will likely ensue via two possible routes:

- A single IUSCT resulting in levels of engraftment adequate to treat SCD
- IUSCT that induces donor-specific tolerance, allowing for a second same-donor (non-myeloablative, non-immunosuppressive) transplant postnatally to boost engraftment to clinically relevant levels.

It is difficult to draw conclusions based on human clinical experience due to a number of variables; a variety of donor cell sources and transplantation methodologies have been tested, including differences in gestational age at the time of transplantation, route and amount of donor cells introduced into the fetus. These inherent inconsistencies have made it impossible to determine the factors responsible for the observed poor engraftment in human clinical cases. It is therefore vital to perform well-planned preclinical research in large animal models that somewhat resemble human fetal biology to optimize IUSCT protocols before human trials. Indeed, in 2015, MacKenzie et al. produced a consensus statement describing guidelines for IUSCT clinical trials (MacKenzie et al., 2015).

Barriers to IUSCT

Although compelling in concept, several barriers to IUSCT have been identified through subsequent clinical and animal studies.

Host Competition

Perhaps the most significant barrier to donor cell engraftment following IUSCT is host cell competition. The fetus maintains strong, highly proliferative fetal compartments; therefore, IUSCT success relies on the hypothesis that donor cells can compete effectively to achieve significant levels of chimerism. The existing fetal niche has been implicated as a barrier to engraftment after it was documented that SCID recipients experienced successful engraftment of lymphoid specific cell lines and c-kit deficient mice underwent reconstitution of both erythroid and lymphoid cell lines after IUSCT (Fleischman and Mintz, 1979; Blazar and Taylor, 1995). When donor BM cells have a competitive advantage, engraftment of just a few cells can lead to reconstitution, as was seen in c-kit deficient mice

where 1–2 normal HSCs fully reconstituted the haematopoietic compartment after IUSCT (Mintz et al., 1984). Nevertheless, the reverse is also true; when host cells have an advantage, it is unlikely that donor cells proliferate and engraft, irrespective of the quantity introduced into the fetus. Mechanistically, fetal stem cells show superior long-term repopulating potential compared with adult equivalents. This can be ascribed to their rapid cycling kinetics (Mazo et al., 2011; Witt et al., 2018). Murine IUSCT models have highlighted that after intravascular delivery of significantly large doses of donor total BM cells (2×10^{11} cells/kg fetal weight), 100% of mice maintained long-term donor cell multilineage chimerism. However, the average level of chimerism was <10% (Peranteau et al., 2006a). This suggests that host cell competition limits the level of chimerism, but not the frequency at which it occurs.

Manipulation of the fetal environment and IUSCT methodology can confer a competitive advantage to donor BM cells. One way is to deplete host niches and "create space" for donor cell expansion. It has been assumed that "space" would be available due to the rapid development of the fetal haematopoietic compartment, although this has been increasingly challenged. Indeed, Stewart et al. showed that exposure to low-dose irradiation in mice led to high levels of donor chimerism, and 10–40 million donor total BM cells produced chimerism of 40–100% (Stewart et al., 1998). Additionally, they introduced donor cells that were either non-irradiated or irradiated with the same dose as the host. Transplantation of irradiated cells reduced engraftment seven-fold, supporting the notion that competition between donor and host cells determines whether engraftment is successful. Space could still be an important factor; conditioning regimes, which can be delivered directly to the umbilical vein (avoiding maternal circulation), using ultrasound guidance might work by providing space and is worthy of consideration (Derderian et al., 2014).

Strategies to decrease competition and improve donor engraftment *in utero* have also been investigated. Derderian et al. showed that selective *in utero* depletion of host HSCs using an antibody against the c-kit receptor (ACK2), injected directly into fetal mice on E13.5–14.5, resulted in depletion of host HSCs within the BM with minimal toxicity (Derderian et al., 2014). The antibody was cleared from serum before neonatal congenic HSCT and chimerism was higher in these pups than controls. The maternal circulating erythrocyte and leukocyte counts remained within normal levels. In addition, low levels of allogenic chimerism—induced by IUSCT—were enhanced to near-complete donor engraftment in mice by postnatal minimally myeloablative total body irradiation of the pups followed by same-donor transplantation (Peranteau et al., 2002). Furthermore, after Busulfan-conditioned transplantations, mice with <1% and >1% chimerism showed a marked improvement to 60 and 100% engraftment, respectively (Ashizuka et al., 2006).

Improving the competitiveness of the donor cells is another strategy. *In vitro* studies indicate that this can be done by decreasing cleavage of stromal-derived factor-1 (SDF-1) by CD26 inhibition. CD26 is a chemokine expressed on HSCs (Christopherson et al., 2004). CD26 cleaves the amino-terminal dipeptide from SDF-1CXCL12, producing an inactive form which prevents chemotaxis. CD26 inhibitors work by enhancing the chemotaxis of HSCs to SDF-1/CXCL12. Treatment of donor HSCs along with a CD26 inhibitor (dipeptidyl peptidase) enhanced homing and long-term engraftment of allogeneic donor cells following IUSCT (Peranteau et al., 2006b). This suggests that CD26 inhibition may be a suitable adjunct. Additionally, co-transplantation with stroma can selectively influence the expression of homing receptors and ultimately improve engraftment, as hematopoietic activity increases considerably after stromal establishment (Flake and Zanjani, 1999). Indeed, co-transplanted stromal cells expressing CD146, CXCL12, and VEGF led to improved engraftment in sheep (Mokhtari et al., 2016).

Immunological Barriers

The only successful clinical findings have been achieved in immunodeficiency disorders, indirectly suggesting an immunological barrier to IUSCT. Historically, the overriding contradiction to this was the lack of support for a congenic advantage to engraftment over allogeneic cells. In fact, Howson-Jan et al. found a higher incidence of engraftment using allogenic (5.2%) compared with congenic (0.7%) donor cells (Howson-Jan et al., 1993). This was a transient result since all the recipients had non-detectable donor cells at 6 weeks postnatally. However, Carrier et al. (1995) reported higher microchimerism rate in congenic (25%) as opposed to allogeneic (7%) recipients after IUSCT, but there were no differences in the incidence of organ engraftment (Carrier et al., 1995). However, the insignificant chimerism and low frequency of engraftment in these studies make interpretation problematic.

The seminal paper that demonstrated the superiority of congenic transplantation, and hence suggested the presence of an immunological barrier, came from Peranteau et al. (2006a). At E14, mouse fetuses were intravascularly transplanted with high doses of donor cells of allogeneic or congenic BM cells. Despite comparable homing and initial engraftment, by 5 weeks of age, engraftment was lost in 70% of the allogeneic recipients, while all congenic mice maintained stable long-term multilineage chimerism (Peranteau et al., 2006a). Adaptive cellular and humoral alloresponses were quantitatively higher in non-chimeric than chimeric animals, suggesting that the fetal immune response was responsible for restricting donor cell engraftment. Because all congenic and allogeneic animals maintained measurable chimerism levels 2 weeks after IUHCT, it seemed that engraftment failure was a postnatal occurrence, supporting the presence of a previously unrecognized adaptive immune barrier. Studies from Merianos et al. were crucial in explaining these findings. The group observed that, if transplanted murine pups were placed with surrogate mothers that had not received IUSCT, 100% of recipients maintained chimerism (Merianos and Tiblad, 2009). This suggests that IUSCT triggered maternal alloimmunisation, resulting in the transfer of alloantibodies to the pup via breast milk, inducing an adaptive immune response and loss of chimerism. Although the exact mechanism is unknown, these observations suggest that maternal sensitization during IUHCT in humans could pose a

considerable difficulty, as transplacental alloantibody transport cannot be avoided.

In addition to alloantibodies, maternal cell microchimerism, due to maternal-fetal cell trafficking, may also be a reason for rejection in IUSCT. Bidirectional cellular trafficking occurs in normal pregnancy, with maternal microchimerism representing a small percentage of total circulating fetal immune cells (Burt, 2013). The significance of maternal microchimerism in IUSCT was initially explored by Nijagal et al. who demonstrated that murine fetuses contain a substantial amount of maternal cells at baseline, which increase considerably after IUSCT of allogenic cells (Nijagal et al., 2011a,b). Interestingly, cell composition suggested that cell trafficking is an active and selective process rather than the mechanical result of the fetal injection. A significant number of maternal T cells have been found in the fetus; a maternal T cell response was proposed as the reason for lower early engraftment rates in allogeneic IUSCT (Merianos et al., 2008). In support of this, engraftment was substantially increased in T cell-deficient mothers, but not if the graft matched maternal MHC, suggesting that transplacental trafficking of maternal allospecific T cells pose a considerable obstacle. Interestingly, no maternal cells were found within the recipient after the fetal period, making it challenging to explain engraftment loss by this mechanism.

There is ample experimental and clinical evidence in human fetuses to suggest that exposure to maternal antigens by cellular trafficking leads to the establishment of fetal Tregs (Burt, 2013). These prevent fetal T cells acting against maternal antigens. The constant, small volume of immigrating maternal cells is wholly distinct from the large bolus supplied in IUSCT that disrupts normal cell number ratios. Nonetheless, it may be a mechanism of instigating tolerance (Mold et al., 2008).

Rejection by maternal immune cells does not provide a comprehensive explanation for the patterns of graft rejection seen in animal IUSCT models. This is apparent when considering that engraftment may occur in alternating littermates exposed to the same maternal environment (Durkin et al., 2008). Additionally, the fact that the maternal immune system has been intact in all human cases who have undergone IUSCT suggests that the maternal immune response cannot be a critical contributing factor in IUSCT failure. Indeed, phase 1 clinical trials at the UCSF Fetal Treatment Center are taking advantage of the possible maternal-fetal tolerance during pregnancy and are using (Mackenzie, 2020). Instead, the role of innate immunity has been brought into question after recent studies suggested the potential of fetal NK cell-mediated immune response to allogeneic donor cells (Alhajjat et al., 2015).

Durkin et al. found that engraftment correlated with a level of initial chimerism of >1.8% (chimerism threshold) and with a donor-tolerant NK response (Durkin et al., 2008). Moreover, when NK cells were depleted from sub-threshold fetal chimeras, rejection did not occur as expected. Instead, the recipients maintained stable engraftment. When NK cells were allowed to recover and increase in numbers, the engraftment was lost (Durkin et al., 2008; Alhajjat et al., 2015). These findings were vital in confirming the involvement of the innate immune system

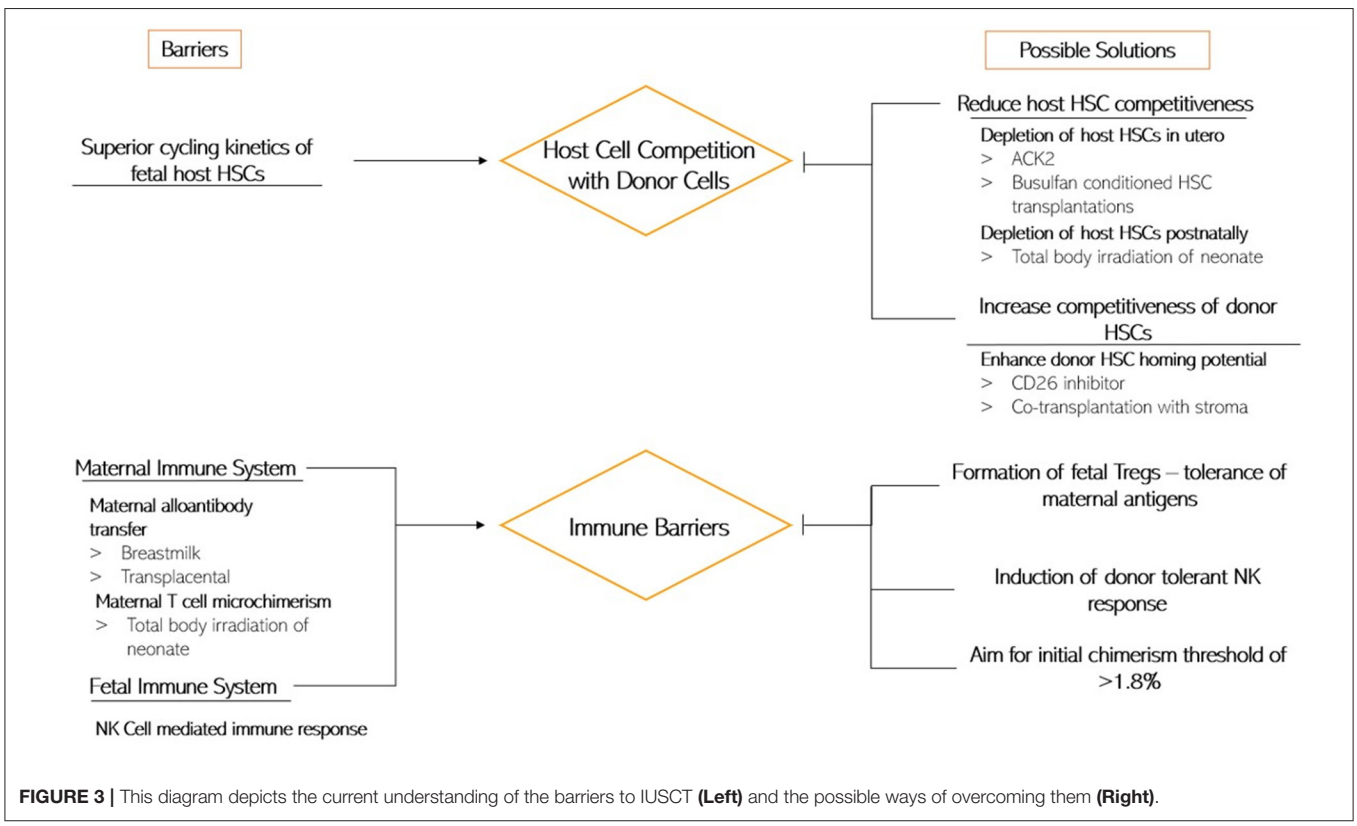

FIGURE 3 | This diagram depicts the current understanding of the barriers to IUSCT **(Left)** and the possible ways of overcoming them **(Right)**.

in IUSCT engraftment rejection. By identifying the chimerism threshold, this study suggested that NK cell education is essential for success. Alhajjat et al. recently showed a mechanistic link between the induction of prenatal NK cell tolerance and trogocytosis (Alhajjat et al., 2013). Trogocytosis is a process whereby lymphocytes, conjugated to antigen-presenting cells, extract surface molecules from these cells and express them on their surface. They explain how engraftment levels of 1–2% could result in adequate exposure of donor antigens to a sufficient number of NK cells to reliably induce donor-specific tolerance (Alhajjat et al., 2013).

As discussed, IUSCT has numerous fetal and maternal obstacles to overcome before it can become clinically valuable. **Figure 3** summarizes the current understanding of the impediments to an application.

Another option, which bypasses some of the obstacles IUSCT has, is to deliver direct gene therapy to the fetal haematopoietic niche. For example, we could deliver gene therapy to the fetal liver, which is one of the haematopoietic organs in the early fetus. This was demonstrated by the *in-utero* injection of a lentiviral vector in a mouse model of beta-thalassaemia. This method was efficient and phenotypically corrected the heterozygous mouse model of disease (Shangaris et al., 2019). Direct gene therapy to the fetus sounds promising, even though it would raise other ethical and safety concerns which are beyond the scope of this review (Shangaris, 2020).

IUSCT Protocol

IUSCT is a novel course of treatment for SCD; thus patient selection and treatment protocol must be comprehensively defined. Several aspects of IUSCT have shown potential areas for improvement. As mentioned previously, the timing of this procedure is paramount to promote donor cell tolerance. In humans, the most appropriate timing for IUSCT would be during the 12th week of gestation, before the HSC colonization of the BM (Mazo et al., 2011). In addition, decisions on the source of stem cells are important. Technical improvements have highlighted that the intravascular route may be a promising new approach. Vrecenak et al. used the canine model to demonstrate that the previous method of intraperitoneal injection of donor cells was inefficient and consistently resulted in donor chimerism levels of <3%. In contrast, intravascular injections resulted in increased levels of sustained donor chimerism (>10%) (Vrecenak et al., 2014). Long-term follow-up studies have confirmed that intravascular injections are more efficient in homing to the fetal liver and intercostal vascular bundles, whereas most cells introduced via the peritoneum remained in the abdomen and were unable to migrate to haematopoietic sites. However, ovine studies have produced contradictory results, stating that the intravascular route confers no benefit (Tanaka et al., 2010). Perhaps, the safer option—intraperitoneal—may be more suitable for clinical implementation as intravascular is technically more difficult, especially at <14 weeks gestation. Infusion of donor cells into the coelomic cavity may present a future approach as it has exhibited great potential in fetal sheep models. The coelomic cavity is now believed to be an important transfer interface for the embryo, present very early in gestation (Cumano and Godin, 2007).

Ethical Considerations

IUSCT is ultimately performed on two patients: the mother and her fetus. Ethical considerations are essential for IUSCT studies and clinical implementation. If one is to consider the fetus as a patient, possible interventions must be of tolerably low risk to the mother.

Also, as mentioned in Kregel et al. communication and sound understanding is crucial in preparing families for IUSCT (Kreger et al., 2016). Firstly, the prenatal diagnosis of SCD must be correct and reliable and needs to be explained clearly to the parents. They must be well-supported to make an informed decision about further management options. Reasonable alternatives must be offered and explained clearly. The therapeutic outcome of IUSCT is uncertain, and this must be made known, with realistic expectations clarified. It is also important to note that the availability of treatment *in utero* may impact the decision to continue or terminate the pregnancy. Therefore, provision of counseling to families considering IUSCT is vital.

CONCLUSION

SCD poses a global problem with few treatment alternatives. IUSCT is non-immunosuppressive transplant approach allowing for donor cell engraftment and donor-specific tolerance in the fetus. It has the potential for treatment early on in gestation, before fetal immune system maturation. Despite promising experimental and clinical progress, there are several remaining obstacles to overcome before IUSCT is widely accepted clinically. With a greater understanding of the requirements of engraftment and the mechanisms of tolerance, strategies can be developed to achieve induction and maintenance of high or complete donor chimerism. Barriers to successful engraftment such as host cell competition, the immune systems of the fetus and mother and the methodology of IUSCT, warrant further investigation, to find new, efficacious ways of tackling them.

AUTHOR CONTRIBUTIONS

AC, LM, and PS conceptualized the topic and structure of the review. AC and LM drafted and revised the manuscript. PS, JS, EO-N, and GL revised and edited the draft paper. AC, LM, PS, JS, EO-N, and GL approved the final manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Monocyte/Macrophage Lineage Cells From Fetal Erythromyeloid Progenitors Orchestrate Bone Remodeling and Repair

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A third of the population sustains a bone fracture, and the pace of fracture healing slows with age. The slower pace of repair is responsible for the increased morbidity in older individuals who sustain a fracture. Bone healing progresses through overlapping phases, initiated by cells of the monocyte/macrophage lineage. The repair process ends with remodeling. This last phase is controlled by osteoclasts, which are bone-specific multinucleated cells also of the monocyte/macrophage lineage. The slower rate of healing in aging can be rejuvenated by macrophages from young animals, and secreted proteins from macrophage regulate undifferentiated mesenchymal cells to become bone-forming osteoblasts. Macrophages can derive from fetal erythromyeloid progenitors or from adult hematopoietic progenitors. Recent studies show that fetal erythromyeloid progenitors are responsible for the osteoclasts that form the space in bone for hematopoiesis and the fetal osteoclast precursors reside in the spleen postnatally, traveling through the blood to participate in fracture repair. Differences in secreted proteins between macrophages from old and young animals regulate the efficiency of osteoblast differentiation from undifferentiated mesenchymal precursor cells. Interestingly, during the remodeling phase osteoclasts can form from the fusion between monocyte/macrophage lineage cells from the fetal and postnatal precursor populations. Data from single cell RNA sequencing identifies specific markers for populations derived from the different precursor populations, a finding that can be used in future studies. Here, we review the diversity of macrophages and osteoclasts, and discuss recent finding about their developmental origin and functions, which provides novel insights into their roles in bone homeostasis and repair.

Keywords: macrophage, osteoclast, yolk sac, erythromyeloid progenitors, fracture, remodeling

FRACTURE REPAIR

A third of all individuals will fracture a bone. As we age there is an increased chance of sustaining a fracture, and the pace of repair slows. This increases the chance that a fracture goes on to a delayed or non-union. The risk of a non-union increases with age approaching 5% in some fracture types in patients over 60. Non-union is associated with decreased mobility, and this results in significant morbidity and even mortality in older individuals (Nicoll, 1964; Sarmiento et al., 1989; Kyro et al., 1993; van Staa et al., 2001; Meyer et al., 2003; Lu et al., 2005; Ekholm et al., 2006; Gruber et al., 2006; Kanakaris and Giannoudis, 2007).

Fractures heal by either primary or secondary mechanisms. Primary healing is less common, occurring when a fracture is rigidly fixed through certain types of surgery, characterized by new cortical bone laid down without any intermediate. In the more common secondary healing, immature and disorganized bone forms between the fragments, which is termed the callus (McKibbin, 1978; Perren, 1979; Arnold, 1987; Einhorn, 1998). Secondary healing progresses through three phases. In the initial phase, bleeding from the damaged tissues causes a hematoma at the fracture site, and hematopoietic cells such as macrophages reach the fracture site (Ozaki et al., 2000). The blood supply is temporarily disrupted for a few millimeters of the bone on either side of the fracture, producing local necrosis and hypoxia. This process results in the release of proteins that promote differentiation of mesenchymal cells into bone-forming cells (Onishi et al., 1998; Schmitt et al., 1999; Champagne et al., 2002; Cho et al., 2002; Kloen et al., 2002). In the proliferative phase, undifferentiated mesenchymal cells aggregate at the site of injury, proliferate, and differentiate (Arnold, 1987). This process involves both intramembranous and endochondral ossification. Intramembranous ossification involves the formation of bone directly from undifferentiated mesenchymal cells (Rozalia et al., 2005). During endochondral ossification, mesenchymal cells differentiate into chondrocytes, producing a cartilaginous matrix, which is then replaced by bone. In the last phase, extensive remodeling occurs, until the damaged skeletal element regains its original shape (McKibbin, 1978; Einhorn, 1998; Rozalia et al., 2005).

When fracture healing is impaired, osteoblast differentiation is inhibited, and undifferentiated mesenchymal tissue remains at the fracture site. This results in a delayed union, or non-union. A variety of factors, including motion at the fracture site and the age of the patient impair healing. Non-union is less common in younger individuals (Girgis and Pritchard, 1958; DeAngelis, 1975; Kwong and Harris, 2008). The pace of fracture repair slows after skeletal maturity, with 3 month old juvenile mice (equivalent to a older teenager or young adult) healing almost twice as fast as 20-month old mice (equivalent to a mid 60 year old) (Tonna, 1964; Meyer et al., 2006). *In-vitro* studies show that a smaller proportion of undifferentiated mesenchymal cells differentiate to osteoblasts in older animals, and this block to differentiation delays fracture healing (Meyer et al., 2001; Calori et al., 2007; Strube et al., 2008; Clement et al., 2011).

MACROPHAGE AND MONOCYTE CELLS

Macrophages were initially defined in the Early nineteenth century by Metchnikoff, a finding that contributed to his Nobel prize with Paul Ehrlich (Teti et al., 2016). These heterogenous myeloid derivatives participate in nearly every biological role from development, injury/repair processes, and homeostasis. Since their discovery, macrophages have been found to localize and inhabit many locations throughout the body (Hume and Gordon, 1983; Hume et al., 1984; Tidball and Villalta, 2010; Libby et al., 2013, 2014; Odegaard and Chawla, 2013; Ma et al., 2018). In adult mammalian organisms, bone marrow progenitor cells influenced by macrophage colony stimulating factor (M-CSF) can differentiate into monocytes and enter circulation, later entering tissue as macrophages (Akashi et al., 2000; Hettinger et al., 2013). Functionally, macrophages specialize in sentinel like functions; phagocytosing cell debris, actively promoting tissue growth, and interact closely with dendritic cells for antigen presentation (Italiani and Boraschi, 2014). However, their plasticity and variable gene expression has made these cells types difficult to study. Long term sustainability of macrophage populations is suggested to be as a result of myeloid cells, and while not yet known, potentially early embryonic precursor (Kaur et al., 2018; Yahara et al., 2020). This review will cover how these components contributes to repair, regeneration, and bone homeostasis.

There is heterogeneity in monocyte population in peripheral blood (Passlick et al., 1989). The Nomenclature Committee of the International Union of Immunologic Societies defined three major human monocyte populations (Ziegler-Heitbrock et al., 2010). The major population (∼90% of blood circulating monocytes) is referred to as "Classical monocytes," expressing high levels of cluster of differentiation 14 (CD14). Intermediate monocytes are approximately 10% of this population expressing high levels of both CD14 and CD16. A "non-classical" subset is classified by high CD16 expression and lower CD14 expression. In mice, classical monocytes are featured by the surface marker combination lymphocyte antigen 6 complex $(Ly6C)^{high}$ CX3C chemokine receptor 1 $(CX3CR1)^{int}$ C-C Motif Chemokine Receptor 2 $(CCR2)^{+}CD62L^{+}CD43^{low}$, while "non-classical" monocytes are distinguished by the $Ly6C^{low}CX3CR1^{high}CCR2^{low}CD62L^{-}CD43^{+}$. Classical monocytes have a lifespan of about 1 day, while non-classical monocytes live about 2 days in mice and 7 days in humans (Yona et al., 2013; Patel et al., 2017).

$Ly6C^{high}CX3CR1^{int}$ classical monocytes, previously called inflammatory monocytes, are a transient population of cells with a wide variety of differentiation potential. Classical monocytes shift into the circulation from the bone marrow during the steady-state to replenish the tissue-resident macrophages. However, the epidermis (Chorro et al., 2009), the central nervous system (Ajami et al., 2007; Mildner et al., 2007; Ginhoux et al., 2010), and the alveolar space (Guilliams et al., 2013; Hashimoto et al., 2013) are characterized by the limited or no monocyte engraftment, because of the high self-renewal potential of the local tissue-resident macrophages or the restricted blood access to their locations. $Ly6C^{low}CX3CR1^{high}$

non-classical monocytes, previously called patrolling monocytes, can adhere and crawl along capillary endothelial cells to monitor vascular integrity (Auffray et al., 2007). In the steady-state, $Ly6C^{high}CX3CR1^{int}$ classical monocytes are able to re-enter the bone marrow and convert to $Ly6C^{low}CX3CR1^{high}$ cells (Varol et al., 2009).

ROLE OF MACROPHAGES IN FRACTURE REPAIR

Macrophage responsiveness to external environmental stimuli is essential for keeping pathogens at bay and ameliorating damaged tissue (Shapouri-Moghaddam et al., 2018). While macrophages had been described existing as either classical (inflammatory response) or alternative activation (resolving response), cumulative evidence has points toward macrophages inhabiting a spectrum of plasticity between the two polar extremes of "M1" and "M2". Classically activated macrophages (M1) are stimulated through a variety of processes. Bacterial or viral infections may initiate cell-cell mediated responses, which help to produce activating molecules like interferon, lipopolysaccharide, or toll-like receptor. Early studies found that M1 like macrophages are able to enhance woven bone formation through MSC (mesenchymal stromal cell) interactions. Alternatively, activated macrophages (M2) assist in anti-inflammatory signals and help promote processes like angiogenesis and revascularization of new mineral deposits. These cells are abundant during bone healing processes (Biswas et al., 2012; Murray et al., 2014; Sivaraj and Adams, 2016; Murray, 2017; Atri et al., 2018 ; Nathan et al., 2019).

Macrophages in bone are referred to as osteomacs. During development, these macrophages associate closely with osteoblasts during new tissue formation (Winkler et al., 2010). After an injury, macrophages play roles orchestrating an immune response, causing inflammation, and eventually contributing resolution factors that finalize wound healing (Pajarinen et al., 2019). This has been observed in many organs (Naito, 1993; T'Jonck et al., 2018; Xie et al., 2019). During bone fracture, an immune response is rapidly activated with macrophages being one of the first cells at the fracture site (Mosser and Edwards, 2008). These macrophages are activated toward an M1 phenotype recruiting other effector cells to the site of injury. Macrophages also work to clear debris as revascularization tissue forms creating granulated tissue (Thomas and Puleo, 2011; Baht et al., 2018). After the initial phase of inflammation, macrophages begin to help form a soft cartilaginous callus. After depleting macrophages at the time of femoral fracture, there is a complete lack of callus formation. Similarly, depletion during the anabolic stages of repair also showed impaired callus formation and an inability to properly restore bone integrity (Raggatt et al., 2014). Studies by Vi et al. expanded the understanding of macrophage-bone communication during osteoblast differentiation. By depleting macrophage cells during development, it was found that animals had a substantially reduced bone mineral density and maintenance of mesenchymal progenitors was compromised (Vi et al., 2015).

In intramembranous repair, macrophages have been identified to closely associate with the fracture site throughout regeneration (Sinder et al., 2015). Similar to endochondral bone formation, macrophage fas-induced apoptosis (MAFIA) mice deficient in macrophages had bone which lost healing robustness (Alexander et al., 2011). A recent study using both MAFIA mice and clodronate liposome depletion models saw a loss in woven bone integrity. Alexander et al. had also shown that macrophages serve a temporal role throughout bone repair. Evidence had suggested that macrophage depletion at the time of injury resulted in a worse phenotype compared to earlier time points (Lin and O'Connor, 2017).

Macrophage function changes with age. A blunted response to stimuli and general hyperinflammation is often observed with aging (Strube et al., 2008; Tarantino et al., 2011). Early studies showed that the polarization of macrophages and their plasticity is blunted with age (Loi et al., 2016). Data from the study of old rats show that M2 macrophages lose their anti-inflammatory abilities (Löffler et al., 2019). Examination of *Ccr2* deficient mice show that the angiogenic capacity of M2 macrophages is important in the remodeling process (Xing et al., 2010). This angiogenic capacity is associated with the ability to dissociate collagen matrices (Moldovan et al., 2000). The pace of fracture healing slows with age. Using heterochronic parabiosis (Vi et al., 2018), it was shown that macrophages from young mice showed rejuvenating effects on fracture repair when circulated into old mice. Furthermore, macrophage cells from old mice slowed the pace of repair in young animals.

It has been thought that stimulating M2 macrophages while inhibiting M1 polarization will improve regeneration. However, macrophages recruited sites of injury exhibit a plastic state and can self-modulate toward M2 phenotypes. Indeed, as reviewed by Pajarinen et al. M1, macrophages can serve both a beneficial role and inhibitory role during regeneration (Pajarinen et al., 2019), supporting the notion that the specific M1 or M2 state regulates regenerative capacity is an oversimplification.

ONTOGENY OF MACROPHAGES

Macrophages have several embryonic origins. They can derive from yolk sac (YS) posterior plate mesoderm directly, an erythro-myeloid progenitor (EMP) that derives from the YS hemogenic endothelium, or from a hematopoietic stem cell. Their embryonic development occurs in what is often termed "waves" (McGrath et al., 2015a; Munro and Hughes, 2017). Their embryonic development occurs in what is often termed "waves" (McGrath et al., 2015a; Munro and Hughes, 2017). In mice, primitive hematopoiesis starts as a first wave around embryonic day 7 (E7) in the blood island of the YS and directly seed embryonic tissues (Moore and Metcalf, 1970; Palis et al., 1999; Ginhoux and Guilliams, 2016; Hoeffel and Ginhoux, 2018; Lee et al., 2018). The YS hemogenic endothelium gives rise to EMPs that can differentiate into fetal macrophages. EMPs appear around E7-7.5 in the yolk-sac (Ginhoux et al., 2010; Italiani and Boraschi, 2017) and can differentiate into colony stimulating factor 1 receptor $(CSF1R)^{+}$ yolk-sac macrophages at E8.5 (Gomez Perdiguero

et al., 2015; Hoeffel and Ginhoux, 2018). While EMPs were initially described to arise from the yolk sac endothelium, just prior to vascular remodeling, they can also emerge directly from endothelial cells. Early c-Myb-independent EMPs that give rise to primitive yolk sac macrophages without passing through monocyte intermediates and a late c-Myb-dependent EMPs that seed the fetal liver to produce fetal monocytes (Hoeffel et al., 2015). This first-wave, in which EMPs/primitive myeloid precursors development occurs independent of the transcriptional activator c-Myb, is instead dependent on PU.1 (Schulz et al., 2012; Gomez Perdiguero and Geissmann, 2013; Ginhoux and Guilliams, 2016). Myb-independent EMPs can differentiate into CX3CR1 positive YS macrophages at E8.5, also called premacrophage (pMac), resulting in a source of tissue-resident macrophages (Mass et al., 2016). Mouse EMPs are generated from Tie2+ yolk sac ancestors when the heart tube begins to contract just prior to vascular remodeling (Frame et al., 2013; Gomez Perdiguero et al., 2015). EMPs are also present in other hemogenic tissues, such as the placenta and umbilical cord, but at a much lower frequency than the yolk sac (Dzierzak and Speck, 2008). Refinement of markers will help better define the various progenitor calls and how they can differentiate For instance, Expression of kit, aa4.1, cd41, cd45 may mark lympho-myeloid progenitors in the yolk sac (Yamane et al., 2009).

A second wave of EMPs develop from E8.25 adopt monocyte stage in the fetal liver. At E9, EMPs that differentiate through this process a Runx1-dependent endothelial-to-hematopoietic transition (Yzaguirre et al., 2017). Endothelial EMPs elongate and integrate in the endothelium and further asymmetrically divide. One of the daughter cells remains in the vessel wall, whereas the other enters circulation. During this process, blood flow facilitates the transition of EMPs from the endothelium into circulation through a nitric oxide-dependent mechanism but is not required for differentiation (Kasaai et al., 2017). EMPs migrate to the fetal liver where they can differentiate into multiple hematopoietic cell types including fetal liver monocytes (Hoeffel et al., 2015). At E10.5, EMPs are found in the fetal liver, which indicates that EMPs migrate to the fetal liver after entering circulation and starts to rapid differentiation here (McGrath et al., 2015b).

The fetal liver niche provides critical cues or an environment for CSF1R+ EMPs to give rise to pMacs (pre-macrophage cells). Although previous studies indicated that only adult HSCs are dependent on c-Myb expression, recent studies show that late EMP in the fetal liver requires c-Myb to contribute to the fetal monocytes (Hoeffel et al., 2015; Hoeffel and Ginhoux, 2018). Fate-mapping analysis of EMP differentiation indicated that pMacs lose Kit expression and increase CD45 expression (Gomez Perdiguero et al., 2015). Although the location where CSF1R+ EMPs acquire CX3CR1 expression is not clear, upregulated CX3CR1 is found in fetal liver pMacs. $CX3CR1^{+}$ pMacs then rapidly proliferate and gain access to the bloodstream to migrate toward the embryo. Colonization of the head, caudal region, and limbs is delayed in CX3CR1-deficient embryos, indicating tissue colonization by pMacs is dependent on CX3CR1 (Mass et al., 2016). Intravital microscopy revealed that trafficking of EMPs and pMacs from the yolk sac to liver primordium and other organ rudiments peaks around E10.5, dramatically decreases toward E12.5 and is no longer evident from E14.5 onwards (Stremmel et al., 2018). As pMacs invade the embryo organ rudiments, Elvira Mass et al. revealed a core macrophage transcriptional program by scRNA-seq and bulk RNA-seq that genes differentially expressed during differentiation from pMacs to fetal and postnatal organ-specific tissue-resident macrophage before acquiring F4/80 expression (Mass et al., 2016). In the third wave, hematopoietic stem cell precursors (pro-HSCs) emerge in the aortogonado-mesonephros region at E10.5 and differentiate to embryonic HSCs at E12.5, which shift later to the bone marrow (Ginhoux and Guilliams, 2016). Bone marrow HSCs eventually establish the circulating monocyte-derived macrophages (Italiani and Boraschi, 2017). The adult bone marrow HSCs-derived monocyte and macrophages are fundamentally different cell populations from embryonically established tissue-resident macrophages.

While it was initially thought that tissue-resident macrophage populations are replenished by monocytes from the blood, more recent lineage tracing data shows that many adult resident macrophages are instead established during development and maintain themselves in the postnatal tissue by proliferation (Epelman et al., 2014; Gomez Perdiguero et al., 2015). EMPs are the first definitive hematopoietic stem cell (HSC)-independent cells and are the source of these adult/postnatal tissue-resident macrophages.

In human embryos, the yolk sac serves as the initial site of hematopoiesis and gives rise to macrophage progenitors. Early studies found that human myeloid precursors emerge in the human yolk sac and migrate to the fetal liver during 28–35 days post-conception (Migliaccio et al., 1986). Given that at this time neither the human fetal liver possess repopulating ability nor HSC start colonization, the cells seeding the early fetal liver are likely to include yolk sac-derived EMPs (Ivanovs et al., 2017). Recent studies confirmed the expression of CX3CR1 in human yolk sac-derived pMacs as in mice (Stremmel et al., 2018). Although the yolk sac EMPs wave has not been formally characterized during human development, the analogies discovered between mice and human hint the conservation in their process of EMP differentiation into tissue-resident macrophage. However, previous studies uncovered that epidermal growth factor module-containing mucin-like receptor 2 (EMR2, CD312) is upregulated during differentiation and maturation of human macrophages, while mice lacks *Emr2* gene (Kwakkenbos et al., 2006; Chang et al., 2007). Thus, the differences between mice and human EMP differentiation into tissue-resident macrophage may exist and are areas for future exploration.

OSTEOCLASTS AND HEMATOPOIETIC STEM CELL DIFFERENTIATION

Osteoclasts are derived from the monocyte/macrophage lineage and are responsible for the resorption of bone tissues (Udagawa et al., 1990; Takahashi et al., 1994). They undergo cell-cell fusion

to form multinucleated cells under the influence of the receptor activator of the nuclear factor-κB ligand (RANKL) (Lacey et al., 1998; Yasuda et al., 1998). Osteoclasts can originate from the HSCs in the bone marrow. HSCs have the capacity to self-renew and differentiate into each hematopoietic cell type (Spangrude et al., 1988). While the traditional concept of linear development of HSCs down a "hematopoietic differentiation tree" has been being challenged by recent studies, this general framework is useful in understanding how macrophage cells and osteoclasts develop from HSCs(Laurenti and Gottgens, 2018). Self-renewing HSCs give rise to multipotent progenitors (MPPs) that in turn generate the lineage-restricted precursors (Kawamoto et al., 2010; Seita and Weissman, 2010). The precursors then bifurcate into oligopotent progenitors, common myeloid progenitors (CMPs), and common lymphoid progenitors (CLPs). The CMPs develop into megakaryocyte/erythrocyte progenitors, and the granulocyte (GR)/macrophage progenitors (GMPs). Further, the GMPs differentiate into a common macrophage/osteoclast/dendritic cell progenitor (MODP) that later produces osteoclasts under the influence of RANKL and colony-stimulating factor-1 (CSF-1) (Arai et al., 1999; Miyamoto et al., 2001). However, emerging single-cell transcriptome technologies, and studies of stem cell differentiation, are challenging the traditional view of the hematopoietic hierarchy. These studies have found additional levels of plasticity, and unipotent stem cells (Notta et al., 2016; Laurenti and Gottgens, 2018; Jacobsen and Nerlov, 2019). As additional studies confirm and build on these findings, our view of HSC differentiation will be refined.

The hematopoietic transcription factor, PU.1 (encoded by the *Spi-1* gene), regulates the initial step of myeloid differentiation; it also regulates the CSF1R and receptor activator of nuclear factor-κ B (RANK) gene expression in myeloid progenitors (Tondravi et al., 1997; Kwon et al., 2005; Ishiyama et al., 2015). Followed by the subsequent activation of RANK on the surface of osteoclast precursors, the RANKL-RANK signaling recruits tumor necrosis factor receptor-associated factor 6 (TRAF-6) to modulate a variety of signaling cascades (Kobayashi et al., 2001), such as the canonical and non-canonical nuclear factor-kappa B (NF-kB) pathway, calcium signaling (Sato et al., 2006), and mitogen-activated protein kinase (MAPK) pathway that includes protein kinases, such as extracellular signal-regulated kinase (Miyazaki et al., 2000), Janus N-terminal kinase, p38, and phosphatidylinositol 3-kinase PI3K. This leads to the activation of many transcription factors related to the osteoclast formation, including activator protein 1 (Matsuo et al., 2000) and nuclear factor of activated T cell cytoplasmic 1 (NFATc1) (Takayanagi et al., 2002). Cell surface receptors, such as triggering receptor expressed in myeloid cells-2 (TREM2) and osteoclast-associated receptor also transmit intracellular signals and trigger the phosphorylation of spleen tyrosine kinase, resulting in the Ca2+ mobilization and activation of NFATc1 (Koga et al., 2004). Thus, these factors stimulate the transcriptional activation of osteoclast-specific genes, such as tartrate resistant acid phosphate (TRAP, encoded by the *Acp5* gene), cathepsin K, and matrix metalloproteinase-9, and coordinate the differentiation and maturation of mononuclear osteoclast precursors into bone-resorbing mature osteoclasts.

OSTEOCLASTS FROM EMBRYONIC MYELOID PROGENITORS

Osteoclasts were initially thought to derive from the circulating monocyte lineage progenitor cells in the bone marrow. However, recent lineage tracing studies show that osteoclast precursors arise from the yolk sac (Yahara et al., 2020) questioned the osteoclast ontogeny (Gomez Perdiguero et al., 2015; Mass et al., 2016) (**Figure 1**). Jacome-Galarza et al. found that the mice lacking RANK or CSF-1R in both EMPs and HSCs lineage developed severe bone disease and failure of tooth eruption at a young age. However, the mice lacking RANK or CSF-1R in only HSCs lineage showed normal eruption of tooth and no phenotypic defect in the bone at a young age (4 weeks), but increased bone mass after 16 weeks of age. These results suggest that the EMP-derived osteoclasts are essential for bone development and tooth eruption, and they are gradually replaced by the HSC-derived progenitor cells (Jacome-Galarza et al., 2019). We recently reported that macrophages originating from the EMPs in the yolk sac produce neonatal osteoclasts that can provide a space for the postnatal bone marrow and gives rise to a population of long-lived osteoclast precursors. This population contributes to bone remodeling at steady state and fracture healing. Furthermore, the data from cell-fate analyses of EMP and HSC lineages indicated the possibility of cell-cell fusion between these two lineages. The yolk sac-derived macrophages can migrate through the bloodstream to bone after an injury (Yahara et al., 2020). These osteoclasts are also involved in the bone remodeling of other pathologic conditions, such as rheumatoid arthritis and metastatic bone disease.

CHEMOATTRACTION OF MYELOID CELLS DURING FRACTURE REPAIR

Proteins secreted during early inflammation are critical for the successful healing of bone fractures, as they initiate a cascade leading to skeletal repair. Chemoattractants, such as chemokines and cytokines, attract inflammatory cells to the site of tissue damage. Neutrophils are the primary cells that arrive at the site of injury, in response to the localized secretion of damage-associated molecular patterns (DAMPs) (Zhang et al., 2010) that facilitate the release of hydrogen peroxide (H2O2) and guide the further recruitment of neutrophils. DAMPs, including the high-mobility group box 1 (HMGB1) proteins, N-formyl peptides, S100 proteins, and heat shock proteins, are mainly recognized by the nucleotide oligomerization domain (NOD)-like receptors (NLRs) and toll-like receptors (TLRs) (Vourc'h et al., 2018; Relja and Land, 2020). Further, the neutrophils and other immune cells induce monocyte chemotaxis via the secretion of several CXC chemokine ligands (CXCL1, CXCL2, CXCL3, CXCL8, CXCL10, and CXCL12) (Kitaori et al., 2009; Kolar et al., 2011; Myers et al., 2015; Förster et al., 2016; Hoff et al., 2016; Furman et al., 2018; Burska et al., 2020) and CC chemokine ligands (CCL2, CCL3, CCL4, and CCL5)(Xing et al., 2010; Wu et al., 2013; Ishikawa et al., 2014; Hoff et al., 2016) during fracture healing.

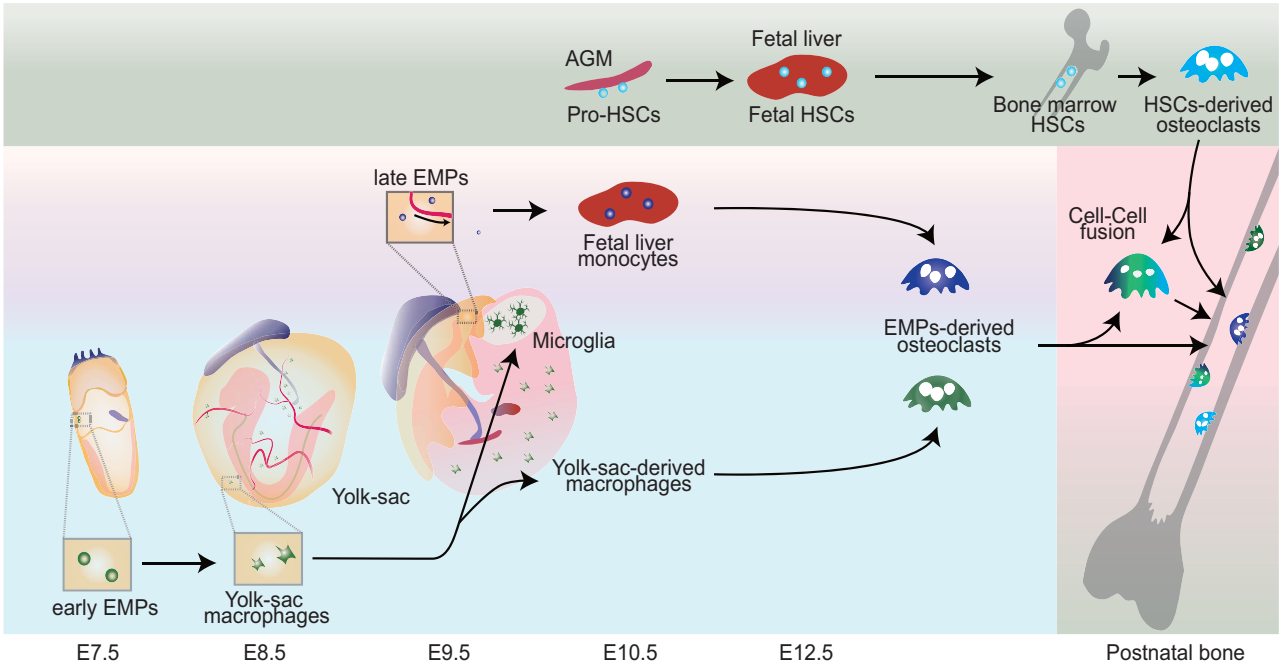

FIGURE 1 | Schematic representation showing the origin of osteoclasts. Early erythromyeloid progenitors (EMPs) appear around E7-7.5 in the yolk-sac and differentiate into yolk sac macrophages without passing through monocyte intermediates. CX3C chemokine receptor 1 (CX3CR1) positive pre-macrophages produce a significant source of yolk-sac-derived macrophages. Late EMPs emerge in the yolk-sac at E9 and migrate to the fetal liver to produce fetal liver monocyte. Hematopoietic stem cell precursors (pro-HSCs) emerge at E10.5 and migrate to the fetal liver around E12 and turns to fetal HSCs, which later shift to the bone marrow. Bone marrow HSCs eventually can establish HSCs-derived osteoclasts. Fetal liver monocytes and yolk sac derived macrophages can differentiate into osteoclast (EMPs-derived osteoclasts) in the neonatal bone with possible cell-cell fusion with HSCs-derived Osteoclasts.

CCL2, also known as monocyte chemotactic protein-1 (MCP-1), and its receptor CCR2, are involved in the recruitment of neutrophils (Johnston et al., 1999), monocytes/macrophages (Tsou et al., 2007; Xing et al., 2010; Arakaki et al., 2016; Biguetti et al., 2018), and mesenchymal progenitor cells (Ishikawa et al., 2014) during inflammation and fracture repair. Classical Ly6C^{high} monocytes, also called as pro-inflammatory monocytes, represent about 2–5% of the circulating white blood cells in a steady state and express high levels of CCR2, but low levels of CX3CR1 (Geissmann et al., 2003). Once inflammation or injury occurs, the Ly6C^{high} and CCR2^{high} monocytes rapidly infiltrate the site of injury via CCR2-CCL2 signaling that attracts other inflammatory monocytes from the bone marrow or blood circulation to the site and promotes their differentiation to macrophages. It has been reported in previous studies that the expression level of CCR2 and its ligand, CCL2, significantly increased during the acute phase of fracture healing (Xing et al., 2010; Ishikawa et al., 2014). In addition, mice lacking the *CCR2* gene showed impaired recruitment of monocytes and macrophages to the site of injury, as well as delay in callus remodeling, cartilage maturation, and endochondral ossification (Xing et al., 2010). Thus, the impaired recruitment of macrophage in the initial step of fracture healing may be able to affect the attraction and differentiation of osteo-chondro progenitors, resulting in the delayed fracture healing. Both CCR2 and CCL2-null mice exhibited an increase in bone mass due to insufficient osteoclast formation and bone resorption (Binder et al., 2009; Sul et al., 2012). However, there was no significant difference in the number of osteoclasts at the fracture callus in *CCR2*$^{-/-}$ mice as compared to that in the wild type mice (Xing et al., 2010). The possible explanations of this phenomenon could be that other molecules may be involved in orchestrating the attraction of osteoclast precursors to the site of fracture during bone healing, or the osteoclasts that participate in the fracture callus remodeling may not be the same population as found during physiological conditions. The molecular mechanisms important for macrophage cell migration and how they are triggered by chemoattractant is an area in which continued investigation is needed.

ROLE OF OSTEOCLASTS IN CALLUS REMODELING

Secondary fracture repair progresses through endochondral ossification (Gerstenfeld et al., 2003; Zhang et al., 2016). Chondrocytes derived from the skeletal stem cells or mesenchymal progenitors (Nakahara et al., 1990; Chan et al., 2013, 2015, 2018; Worthley et al., 2015; Yue et al., 2016; Mizuhashi et al., 2018) differentiate into hypertrophic

chondrocytes and mineralize the cartilage matrix, resulting in the initial soft cartilaginous callus. While it was previously thought that hypertrophic chondrocytes undergo apoptosis, recent studies using the genetic lineage tracing mice model revealed that the chondrocytes could directly differentiate into osteoblast lineage cells during both the physiological growth phase and fracture repair (Ono et al., 2014; Yang et al., 2014a,b; Zhou et al., 2014). Bone regeneration is orchestrated by the invading-osteoblasts along with vasculature that is derived from the mesenchymal progenitors of the periosteum (Nakahara et al., 1990; Murao et al., 2013; Duchamp de Lageneste et al., 2018), bone marrow mesenchymal stromal cells (Sekiya et al., 2002), and/or the cells from terminally differentiated chondrocytes (Zhou et al., 2014; Hu et al., 2017). Soft callus remodeling is a process of gradual removal of the cartilage/fibrocartilage and its systematic replacement with woven bone. The woven bone is subsequently transformed into a lamellar bone, also called as a hard callus, in the final step of fracture healing.

Several animal models were used to examine the role of osteoclasts during callus remodeling (**Table 1**). The disruption of osteoclastogenesis by pharmacological substances, such as RANKL inhibitors (RANK-Fc and denosumab), osteoprotegerin (OPG), bisphosphonates (alendronate and zoledronic acid), clodronate liposomes, and cathepsin K (CTSK) inhibitors, differentially affected the callus remodeling (Flick et al., 2003; Ulrich-Vinther and Andreassen, 2005; Gerstenfeld et al., 2009; Soung do et al., 2013; Pennypacker et al., 2016; Lin and O'Connor, 2017). Treatment with alendronate and zoledronic acid did not reduce the number of osteoclasts in the callus and serum tartrate-resistant acid phosphatase (TRACP) 5b level, but increased its volume and the cartilaginous callus, indicating delayed callus remodeling (Gerstenfeld et al., 2009; Soung do et al., 2013). Similarly, the treatment with RANK-Fc, denosumab, and OPG delayed the cartilage resorption and remodeling due to impaired differentiation of osteoclasts in the fracture callus (Flick et al., 2003; Ulrich-Vinther and Andreassen, 2005; Gerstenfeld et al., 2009). Both alendronate and denosumab retarded the following: fracture callus remodeling, elimination of cartilage (Soung do et al., 2013), improvement in mechanical strength, and bone mineral content (BMC), as compared to those in the control groups, during fracture repair (Gerstenfeld et al., 2009). Although the pharmacological inhibition of osteoclastogenesis delays the callus remodeling and forms a large woven bone callus, it may help to provide mechanical support at the fracture site, rather than a remodeled lamellar callus. Genetic ablation of RANK and CSF1 showed osteopetrotic (op) phenotype and affected the fracture callus remodeling and healing. The RANK knockout (KO) and op/op mice lacked osteoclasts but showed radiographic and histological evidence of callus formation (Flick et al., 2003). This data indicated that osteoclasts are not essential for fracture callus formation. However, the bone healing rate was reduced in the RANK KO mice as compared to that in the osteopetrotic op/op mice. The OPG KO mice developed an increased number of osteoclasts in the fracture callus. The accelerated resorption of cartilaginous callus in OPG KO mice promoted fracture healing (Ota et al., 2009). In addition, the pharmacological and genetic ablation of osteoclast progenitor cells (macrophage and monocyte) also delayed fracture healing. The clodronate liposome treatment did not prevent the callus formation, but it reduced the number of osteoclasts and delayed the callus cartilage remodeling (Lin and O'Connor, 2017). Ablation of the lysozyme-M-positive cells using LyzM-Cre in diphtheria toxin subunit A-expressing (DTA) mice suppressed both cartilage and bony callus formation while accelerating fibrosis, thus, resulting in delayed fracture healing (Vi et al., 2015). Interestingly, constitutive macrophage deficiency did not affect the number of TRAP+ osteoclasts in the fracture callus. The possible reason for this observation is that osteoclasts of different sources may have contributed to the callus remodeling during fracture repair. Pharmacological inactivation or genetic ablation of CTSK led to an increase in the number of osteoclasts in the fracture callus, high bone formation and strength, and an increase in the bone mineral density (Ota et al., 2009; Soung do et al., 2013; Gentile, 2014; Pennypacker et al., 2016). CTSK inhibitors have a potential to inhibit the bone resorption ability of osteoclasts without changing the ability of bone synthesis (Soung do et al., 2013; Pennypacker et al., 2016). Therefore, odanacatib (ODN), which is a selective oral inhibitor of CTSK, has been considered as a strong candidate for the treatment of osteoporosis and a potent inhibitor of osteoclastic activity. Although ODN diminished the risk of fractures, it was related to elevated cardiovascular risks, such as strokes, especially in osteoporotic postmenopausal women. Thus, on analyzing the overall benefits and risks associated with the ODN drug, the investigators of the study decided to discontinue the use of ODN for the treatment of osteoporosis (McClung et al., 2019).

Traditionally, hard bony callus remodeling has been thought to be the final step in fracture repair. However, Takeyama et al. using the medaka fin ray fracture model, recently demonstrated that two types of functional osteoclasts are activated in the different phases of fracture healing (Takeyama et al., 2014). Immediately after a fracture, the early-induced osteoclasts, which are of small size and have high morbidity with low TRAP activity, localize on the edge of the bone fragments. However, the late-induced osteoclasts start to appear at the inner surface of the callus with high TRAP-activity and large morphology. We found that fms-like tyrosine kinase 3 (FLT3)-positive progenitors of the HSCs lineage could migrate to the injury site and differentiate into TRAP+ and Vpp3+ osteoclasts, thereby contributing to the early phase bone resorption during bone repair in mice. The $CX3CR1^{+}$ EMP-derived osteoclast precursors can migrate to the site of injury through blood circulation and differentiate into multinucleated osteoclasts that take part in the later phase of callus remodeling (Yahara et al., 2020). Additionally, Novak et al. showed that the circulating $CX3CR1^{high}$ osteoclast precursor cells could migrate through blood circulation to the fracture callus and differentiate into TRAP-positive mature osteoclasts in the later phase of callus remodeling (Novak et al., 2020). In summary, each subset of osteoclasts has a distinct morphology, feature, and origin, which suggests that they have specialized and phase-specific functions.

TABLE 1 | Animal models to examine the disruption of osteoclastogenesis during fracture healing.

Pharmacological agents	**Species**	**Age**	**Sex**	**Locus**	**Method**	**Post-operative fixation**	**OC number**	**Callus formation**	**BMD or BMC**	**Mechanical testing**	**Bone healing**	**References**
Alendronate	Mouse	8-17 W	M	Femur	Three-point bending	Intramedullary pin	↑	↑	↑	↑	N/A	Gerstenfeld et al., 2009
Denosumab	Mouse	8-17 W	M	Femur	Three-point bending	Intramedullary pin	↓	↑	↑	↑	N/A	Gerstenfeld et al., 2009
Osteoprotegerin (OPG)	Rat	3 M	F	Tibia	Three-point bending	Intramedullary pin	↓	→	↑	→	N/A	Ulrich-Vinther and Andreassen, 2005
RANK:Fc (high doze)	Mouse	12 W	N/A	Tibia	Three-point bending	Intramedullary pin	↓	↑	N/A	→	→	Flick et al., 2003
Clodronate liposome	Mouse	10-12 W	F	Femur	Three-point bending	Intramedullary pin	↓	↑	N/A	→	↓	Lin and O'Connor, 2017
CTSK-I (L-235)	Mouse	7-8 W	M	Femur	Three-point bending	Intramedullary pin	↑	↑	↑	N/A	N/A	Soung do et al., 2013
Alendronate	Mouse	7-8 W	M	Femur	Three-point bending	Intramedullary pin	→	↑	↑	N/A	N/A	Soung do et al., 2013
Odanacatib	Rabbit	9 M	F	Ulnar	Low-speed bone saw	Splint	↑	→	↑	↑	↑	Pennypacker et al., 2016
Genetic mouse model												
RANK KO	Mouse	12 W	N/A	Tibia	Cutting with scissors	Unstabilized	↓	→	N/A	N/A	↓	Flick et al., 2003
Op/Op	Mouse	12 W	N/A	Tibia	Cutting with scissors	Unstabilized	↓	N/A	N/A	N/A	→	Flick et al., 2003
Lyz-Cre; DTA	Mouse	12 W	N/A	Tibia	Cutting with scissors	Intramedullary pin	→	↓	N/A	N/A	↓	Vi et al., 2015
CTSK KO	Mouse	8-10 W	F	Femur	Three-point bending	Intramedullary pin	↑	→	↑	↑	↑	Gentile, 2014

RANK, receptor activator of nuclear factor-κB; CTSK-I, cathepsin K-inhibitor; KO,knockout; op/op, colony-stimulating factor 1(CSF-1)-less osteopetrotic mouse; W, week-old; M, male; F, female; OC, osteoclast; BMD, bone mineral density; BMC, bone mineral content; N/A, not applicable.

MOBILIZATION OF OSTEOCLAST PRECURSORS FROM EXTRAMEDULLARY ORGANS: THE SPLEEN AS A RESERVOIR FOR EMBRYONIC CELLS

Besides the bone marrow, cells from other extramedullary organs can also form mature osteoclasts *in vitro* (Boyle et al., 2003; Lianping and Edward, 2005). However, the contribution of the extramedullary reservoir to the osteoclast pool for bone homeostasis and repair has not been fully understood. Since osteoclasts as well as macrophages and monocytes have common precursor cells, several studies have shown that the extramedullary reservoir of macrophages and monocytes also plays an essential role in tissue inflammation and repair. Hoyer et al. observed that localized damage stimulated tissue macrophages in distant organs, which aided in the recovery from systemic complications after myocardial infarction, stroke, and sepsis (Hoyer et al., 2019). Wang et al. found that GATA6+ macrophages migrated directly from the peritoneal cavity in response to liver injury and contributed to the tissue repair (Wang and Kubes, 2016). Swirski et al. reported that splenic monocytes that reside in the subcapsular region of the red pulp increase their motility and accumulate at the site of ischemic myocardial injury (Swirski et al., 2009). Furthermore, Sabatel et al. reported that lung interstitial macrophages arise from the splenic monocytes by interlukin-10 (IL-10) signaling in a CCR2-independent manner during allergic airway inflammation (Sabatel et al., 2017). These data clearly showed that acute inflammation and injury caused the mobilization of monocytes and macrophage from the extramedullary organs to the site of injury.

In fracture healing, the spleen is a reservoir of osteoclast precursors. The spleen is a central lymphoid organ that has multiple functions, including the removal of cellular debris, hematopoiesis, recycling of red blood cells, and activation of the immune system during infection and inflammation. Osteoclast precursors that reside in the spleen can migrate to the bone cavity and change into mature osteoclasts (Kotani et al., 2013). Splenectomy inhibited macrophage recruitment and reduced the number of osteoclasts at the site of fracture in a rat model. Patients with fractures who received splenectomy had a significantly lower number of blood monocytes and reduced bone density than patients with fractures who did not undergo splenectomy (Xiao et al., 2017, 2018).

The spleen sustains the embryonic macrophage population derived from the EMPs in the red pulp. The red pulp macrophages are produced, at least in part, during embryonic development and are subsequently maintained throughout adulthood (Schulz et al., 2012; Hashimoto et al., 2013; Yona et al., 2013; Epelman et al., 2014). These EMP-derived macrophages can travel through the bloodstream and differentiate into osteoclasts to participate in bone remodeling during fracture repair (Yahara et al., 2020). However, the mechanism of orchestration and mobilization of EMP-derived macrophages from the spleen to the fracture site have not been elucidated. Nakamichi et al. found that IL-34 signaling induced the mobilization of osteoclast precursors from the spleen of osteopetrotic op/op mice (Nakamichi et al., 2012). Further, in a recent *in vivo* embryogenesis study of zebrafish, a CRISPR/Cas9-based reverse genetic screening also identified IL-34 as a regulator of the distribution of tissue macrophages; IL-34 can mobilize yolk sac macrophages to other embryonic tissues (Kuil et al., 2019). The contribution of circulating osteoclast precursors to *in vivo* osteoclasts pool in steady state and disease is an area of controversy. Jacome-Galarza et al. demonstrated that circulating blood monocytic cells are a major source of osteoclasts in steady condition. On the other hand, Novak et al. argued that the bone tissue is relatively protected from engraftment of circulating osteoclast precursors under steady conditions (Novak et al., 2020). There are several studies which show that the engraftment and maturation of circulating osteoclasts into mature osteoclasts is increased in fracture repair (Novak et al., 2020) and during bone resorption(Kotani et al., 2013). Thus, additional studies are needed to reveal the mechanisms that orchestrate the mobilization of circulating osteoclasts precursors from the extramedullary organs to bone in homeostasis and disease.

CELL FUSION AND MULTINUCLEATION OF OSTEOCLASTS

Osteoclasts are formed by the cell-cell fusion of mononuclear osteoclast precursors (Jansen et al., 2012). Cell-cell fusion and their multinucleation are essential for osteoclast maturation and maintenance of bone homeostasis (Li et al., 2018). The fusion of mononuclear osteoclast precursors is carried out by extra and intracellular dynamics of the interaction of various molecules. Mononuclear osteoclast precursors migrate to the bone tissue by chemotaxis through blood circulation or directly from the bone marrow. RANKL and vascular endothelial growth factor promote chemotaxis through an extracellular signal-regulated kinase 1/2-dependent pathway (Henriksen et al., 2003). Transforming growth factor β, which is released from the extracellular matrix during bone resorption, also has the potential to accelerate osteoclast recruitment through phosphatidylinositol-3 kinase (PI3K) and MAPK signaling pathways (Pilkington et al., 2001). Stromal cell derived factor-1 (SDF-1), also known as CXCL12, is critical for cell migration (Yu et al., 2003; Kollet et al., 2006; Gronthos et al., 2007). PI3K activates the transcription of SDF-1, resulting in the migration of osteoclast precursors via C-X-C chemokine receptor type 4 (CXCR4) (Adapala et al., 2019). Sphingosine-1-phosphate, a lipid mediator enriched in blood and lymph, can induce and regulate the homing of osteoclast precursors to bone (Ishii et al., 2009; Kikuta et al., 2013). Indeed, the monocyte-specific conditional sphingosine-1-phosphate knockout mice exhibited osteoporotic phenotype due to increased osteoclast activity and attachment to the bone surface (Ishii et al., 2009).

For cell-cell fusion, the migrated monocular osteoclast precursors must be in close proximity to each other and adhere (Pereira et al., 2018). The αvβ3 integrin is expressed

in osteoclasts and has been implicated in cell migration as well as the formation of the sealing zone (Nakamura et al., 1999; McHugh et al., 2000). E-cadherin is a cell surface glycoprotein responsible for cell-cell adhesion and IL-4-driven macrophage fusion (Mbalaviele et al., 1995; Van den Bossche et al., 2009). During membrane fusion and multinucleation, dendritic cell-specific transmembrane protein (DC-STAMP) and osteoclast stimulatory transmembrane protein (OC-STAMP) are essential regulators of the osteoclast cell-cell fusion (Yagi et al., 2005; Miyamoto et al., 2012; Khan et al., 2013). CD44, CD47, syncytin-1, Pin1 (peptidyl-prolyl cis-trans isomerase NIMA-interacting 1), and the tetraspanins (CD9 and CD81) are also involved in osteoclast fusion and multinucleation (Sterling et al., 1998; Takeda et al., 2003; Cui et al., 2006; Søe et al., 2011; Islam et al., 2014; Møller et al., 2017). Moller et al. showed that CD47 accelerated cell fusion involving mononucleated osteoclast precursors (Møller et al., 2017). On the other hand, Synchitin-1 facilitated the fusion of multinucleated osteoclasts but regulated a reduced number of fusions between mononucleated osteoclast precursors. Lee et al. reported that mice deficient in the d2 isoform of vacuolar (H+) ATPase (v-ATPase) V0 domain (ATP6V0D2) had disrupted osteoclast fusion and increased bone formation (Lee et al., 2006). Thus, osteoclast fusion is not a random process, but there is a strict mechanism for the selection of fusion partners based on the heterogeneity of the osteoclast precursors and surrounding environmental cues.

CELL FUSION BETWEEN EMPs- AND HSCs-DERIVED OSTEOCLAST PRECURSORS

Jacome-Galarza et al. proposed a model for the development and maintenance of osteoclast fusion. Osteoclasts of EMP origin are essential for normal bone development, and their postnatal maintenance is sustained by cell-cell fusion, resulting in the fusion with HSC-derived cells and acquisition of their nuclei, instead of proliferation of the osteoclast precursors (Jacome-Galarza et al., 2019). They showed that mature osteoclasts are long-lived in adults bone depending on interactive fusion between individual HSC-derived circulating cells with locally existing osteoclasts. On the other hand, we found that EMP-derived precursors are long-lived and maintained in extramedullary organs such as in the spleen. In steady state, HSC/EMP-derived osteoclast precursors can fuse in a developing bone. Once an injury happens, unknown factors mobilize EMP-derived osteoclast precursors from the spleen. These precursors travel via blood circulation to participate in fracture repair, by cell-cell fusion between EMP- and local HSC-derived osteoclasts, resulting in multinucleated osteoclasts (Yahara et al., 2020). However, detailed mechanisms of attraction of EMP-derived precursors and cell-cell fusion between EMP- and HSC-derived osteoclast precursors have not yet been elucidated. The life span and maintenance of osteoclast precursors/mature osteoclasts *in vivo* is still under debate.

UNIQUE EXPRESSION PROFILES OF EMPs- AND HSCs-DERIVED MACROPHAGES AND OSTEOCLAST PRECURSORS

EMPs and HSCs provide postnatal macrophages and osteoclast precursors, and they differentiate into mature osteoclasts (Jacome-Galarza et al., 2019; Yahara et al., 2020). However, the functional differences between EMP- and HSC-derived macrophages and osteoclasts are not fully understood. Mass et al. identified the expression of the cytokine receptor RANK [coded by the TNF Receptor Superfamily Member 11a (TNFRSF11A) gene] in pMacs by RNA-seq (Mass et al., 2016). They found that *Rank-Cre; Rosa26eYFP* mice efficiently labeled EMP-derived tissue-resident macrophages but not bone marrow derived-HSCs and their progeny. Interestingly, epithelial cells are also related to the potential epithelial origin of some EMP progenitors (Plein et al., 2018). Because microglia are macrophage-related cells of the central nervous system and originated from embryonic EMPs (Konishi et al., 2020), the majority of postnatal brain microglia expressed eYFP in *Rank-Cre; Rosa26eYFP* mice. It is well-recognized that RANK is the receptor for RANKL and RANK-RANKL signaling essential for osteoclast differentiation and activation. Therefore, osteoclasts and other EMP-derived tissue-resident macrophages such as in brain (microglia), liver (Kupffer cells), and epidermis (Langerhans cells) rise a possibility that they are from same origin. Analysis of the expression profiles of eYFP-negative bone marrow-derived and eYFP-positive EMP-derived macrophages in the postnatal tissue of *Rank-Cre; Rosa26eYFP* mice showed that macrophages from eYFP-positive EMPs expressed high levels of Lyve-1, Stab1, and Gas6, which have been related to homeostatic and anti-inflammatory immune functions. On the other hand, eYFP-negative HSC-derived macrophages characterizes higher expression of Ccr2, which is a marker of HSCs-derived monocyte/macrophage (Weinberger et al., 2020). Use of these markers may be useful in future research to understand the contributions of these populations in homeostasis, tissue repair, and pathology.

CONCLUSION AND PERSPECTIVES

EMP-derived embryonic macrophages persist during adult life and are long-lived cells that can self-renew locally, independent of the HSCs-derived peripheral monocytes. Macrophages derived from the definitive hematopoietic progenitors in the bone marrow are short-lived and are replenished during steady and pathological conditions by monocyte in a CCR2-dependent manner. Monocyte-derived macrophages show distinct gene modifications and profiles compared to the embryonically established macrophages depend on their local tissue environments. However, the principal mechanisms causing the differences in the transcriptomic, epigenomic and their functional signatures between HSCs- and EMPs-derived macrophages and osteoclast remain to be elucidated. Insight into the will inform the optimal functions and distinct roles of EMP-derived osteoclasts in bone homeostasis and repair.

The data reviewed here strongly supports a role for macrophage cells orchestrating fracture repair. Furthermore, heterochronic parabiosis shows that young macrophage cells rejuvenate fracture repair (Vi et al., 2018), and that there is a population of YS derived cells labeled by *Cx3cr1* during embryogenesis that reside in the red pulp of the spleen and are mobilized to bone when injured (Yahara et al., 2020). It is likely that this embryonic population is critical in effective repair, and as this population changes with age, the pace and quality of fracture healing declines. Interestingly, the embryonically derived cell population in the spleen also populates the kidney (Ide et al., 2020), suggesting roles in other processes besides fracture repair. Understanding the role of this embryonic cell population in repair and regeneration will likely have important implications in a variety of reparative and pathologic processes.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Contributions of Embryonic HSC-Independent Hematopoiesis to Organogenesis and the Adult Hematopoietic System

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During ontogeny, the establishment of the hematopoietic system takes place in several phases, separated both in time and location. The process is initiated extra-embryonically in the yolk sac (YS) and concludes in the main arteries of the embryo with the formation of hematopoietic stem cells (HSC). Initially, it was thought that HSC-independent hematopoietic YS cells were transient, and only required to bridge the gap to HSC activity. However, in recent years it has become clear that these cells also contribute to embryonic organogenesis, including the emergence of HSCs. Furthermore, some of these early HSC-independent YS cells persist into adulthood as distinct hematopoietic populations. These previously unrecognized abilities of embryonic HSC-independent hematopoietic cells constitute a new field of interest. Here, we aim to provide a succinct overview of the current knowledge regarding the contribution of YS-derived hematopoietic cells to the development of the embryo and the adult hematopoietic system.

Keywords: yolk sac, lineage tracing, embryonic hematopoiesis, organogenesis, macrophage, hematopoietic waves, HSC-independent hematopoiesis

INTRODUCTION

In mammals, the hematopoietic system is established during embryogenesis in three consecutive overlapping waves (Dzierzak and Bigas, 2018). In mice, the first wave, also termed primitive hematopoiesis, is initiated around embryonic day (E)7 and produces unipotent precursors that give rise to either primitive-erythrocytes, -megakaryocytes, or -macrophages (Palis et al., 1999; Tober et al., 2007). The primitive macrophage precursors have also been named primitive myeloid precursors (pMP). Furthermore, it has also been reported that the first wave may give rise to c-Myb independent erythroid–myeloid progenitors (early EMP) (Hoeffel et al., 2015). However, so far, macrophage (microglia) but not erythrocyte potential has been experimental confirmed for these so-called early EMP (Wittamer and Bertrand, 2020). Henceforth, we will refer to wave 1 myeloid cells as pMP/early EMP. The second wave of hematopoiesis marks the onset of definitive (erythroid) hematopoiesis and sees the emergence of both EMPs around E8.25 (late-EMPs) (McGrath et al., 2015; Palis, 2016), and lymphoid–myeloid progenitors (LMPs) (Adolfsson et al., 2005; Boiers et al., 2013) around E9.5. Around E10.5 the third wave generates both hematopoietic stem and progenitor cells (HSPC) (**Figure 1A**). The hematopoietic stem cells (HSCs) subsequently play a central role in

maintaining the hematopoietic system for the lifetime of the organism (Medvinsky et al., 1993; Muller et al., 1994). Hematopoietic cells of all waves are generated from the mesoderm, which is known to give rise to both endothelial and hematopoietic lineages (Davidson and Zon, 2000; Dzierzak and Bigas, 2018). For the definitive hematopoietic waves (waves 2 and 3), it is now well established that hematopoiesis occurs via an endothelial-to-hematopoietic transition (EHT) from a specialized endothelial subpopulation known as hemogenic endothelium (HE) (Jaffredo et al., 1998; Zovein et al., 2008; Chen et al., 2009; Eilken et al., 2009; Lancrin et al., 2009; Boisset et al., 2010; Lacaud and Kouskoff, 2017; Garcia-Alegria et al., 2018; Ottersbach, 2019). The cellular origin of the first wave of primitive hematopoiesis is still disputed. It is unclear whether primitive hematopoiesis emerges directly from mesoderm, a hemangioblast, a HE, or another type of precursor (Amaya, 2013; Myers and Krieg, 2013). However, several recent studies suggest that primitive hematopoiesis (wave 1) is generated through a HE(-like) intermediate that has been termed hemogenic angioblast (Lancrin et al., 2009; Stefanska et al., 2017; Garcia-Alegria et al., 2018). Despite this potential common cellular origin, not all waves originate from the same anatomical site. The first two waves arise extra embryonically in the yolk sac (YS). In contrast, the third wave mainly takes place in the dorsal aorta within the aorta-gonad-mesonephros (AGM) region of the embryo, where HSC arise within so-called intra-aortic hematopoietic clusters (IAHC) (Boisset et al., 2010; Dzierzak and Bigas, 2018; Ottersbach, 2019). The HSC mature and amplify in the fetal liver (FL) before taking up residence in the bone marrow (BM).

Initially, the YS waves of hematopoiesis were thought to provide an essential but transient blood supply for the embryo destined to be replaced by HSC-derived cells. However, there is accumulating evidence that HSC-independent blood cells can make significant contributions to the adult hematopoietic system. Furthermore, it is also becoming evident that in the embryo, the HSC-independent hematopoietic waves can play essential roles beyond erythrocyte-mediated oxygen exchange and early immune surveillance. These roles have been mainly assigned to myeloid-derived populations that have been shown to play crucial roles in embryonic organogenesis.

Here we provide a concise overview of the recent findings obtained in mouse models regarding the contribution of HSC-independent hematopoiesis to embryonic organogenesis and the adult hematopoietic system.

RESOLVING THE ROLE AND ORIGIN OF HEMATOPOIETIC POPULATIONS

The overlapping and transient nature of the different hematopoietic waves makes it challenging to determine their individual contributions towards organogenesis and the adult hematopoietic system. Transgenic mouse knockout (KO) and fate mapping models have been instrumental in shaping our current understanding of the contribution of the different hematopoietic waves towards the hematopoietic system (**Table 1**). KO models (via gene deletion/mutation or lineage-specific activation of diphtheria toxin) provide functional information for specific populations, while lineage tracing models (typically using fluorescent proteins) highlight the contribution of specific populations. However, the perfect model to delineate the hematopoietic waves does not exist, and it is important to consider the target cell type, wave specificity and labeling efficiency of the models used when interpreting results.

Knockout mouse models have demonstrated specific dependencies of (wave-specific) hematopoietic populations on distinct transcription factors and signaling pathways. *Csf1r* (Colony Stimulating Factor 1 Receptor, cytokine receptor) KO mainly disrupts the early EMP differentiation (wave 1) and to a lesser extent the late-EMP differentiation (wave 2) (Dai et al., 2002). Although these mice are viable, they display drastically reduced levels of microglia and YS macrophages (Ginhoux et al., 2010; Hoeffel et al., 2012). *Csf1* (Marks and Lane, 1976) (Colony Stimulating Factor 1, cytokine) null mice display a similar but milder phenotype, with varying degrees of microglia and YS macrophages depletion, due to partial compensatory effects of the alternative CSF1R ligand IL-34 (Wiktor-Jedrzejczak et al., 1990; Cecchini et al., 1994; Kondo and Duncan, 2009; Greter et al., 2012; Wang et al., 2012; Easley-Neal et al., 2019). *Myb* (Mucenski et al., 1991) (MYB Proto-Oncogene, transcription factor) KO disrupts late-EMP and HSC differentiation (wave 2 and wave 3) and results in anemia and embryonic lethality around E15.5 (Schulz et al., 2012; Hoeffel et al., 2015). A similar phenotype is observed in KitL (KIT Ligand, cytokine) KO mice which die perinatally (Ding et al., 2012). *Nur77* (Lee et al., 1995) (Nuclear Receptor Subfamily 4 Group A Member 1, nuclear receptor) KO is viable but lacks circulating monocytes due to disrupted BM HSC differentiation (Hanna et al., 2011). Disrupting cell migration has also emerged as a useful strategy. *Plvap* (Rantakari et al., 2015) (Plasmalemma Vesicle Associated Protein, membrane protein) and *CCR2* (Boring et al., 1997) (C-C Motif Chemokine Receptor 2, chemokine receptor) KOs are viable but respectively show impaired FL and BM monocyte migration (Rantakari et al., 2016). *Cx3cr1* (Jung et al., 2000) (C-X3-C Motif Chemokine Receptor 1, chemokine receptor) KO is viable but shows impairment of leukocyte migration (Imai et al., 1997; Jacquelin et al., 2013) regardless of their wave of origin. There are several other models which disrupt all waves of hematopoietic development. *Pu.1* (Scott et al., 1994; McKercher et al., 1996) (Spi-1 Proto-Oncogene, transcription factor) KO mice have defective YS myelopoiesis and HSC maintenance and die shortly after birth (Olson et al., 1995; Kim et al., 2004; Kierdorf et al., 2013). Deletion of *Runx1* (Okuda et al., 1996; Wang et al., 1996a; North et al., 1999) (RUNX Family Transcription Factor 1, transcription factor) or its essential co-factor *Cbf*β (Sasaki et al., 1996; Wang et al., 1996b; Niki et al., 1997) (Core-Binding Factor Subunit Beta, transcription factor) is embryonically lethal and results in the complete absence of hematopoiesis apart from primitive erythroid cells. These KOs are useful when coupled with targeted approaches. For example, the *Cbf*β KO model can be used to deplete EMP or HSC by

TABLE 1 | Mouse model for studying the contribution of different hematopoietic waves to hematopoietic system.

Knockout models	Function	Affected wave	Major impact on hematopoietic waves	References
Csf1r (Dai et al., 2002)	Early/Late-EMP differentiation	1, 2	Lack of early EMP and reduced late-EMP contribution to TRM	Ginhoux et al., 2010; Hoeffel et al., 2012
Csf1 (null mutation) (Marks and Lane, 1976)	Early EMP differentiation	1	Reduced early EMP contribution to TRM	Cecchini et al., 1994; Kondo and Duncan, 2009; Easley-Neal et al., 2019
Myb (Mucenski et al., 1991)	Late-EMP and HSC differentiation	2, 3	Lack of late-EMP and HSC contribution to TRM	Schulz et al., 2012; Hoeffel et al., 2015
Nur77 (Lee et al., 1995)	BM monocytes differentiation		Reduced BM monocytes/macrophages contribution to TRM	Hanna et al., 2011
Plvap (Rantakari et al., 2015)	FL macrophage migration		Reduced FL monocytes/macrophages contribution to TRM	Rantakari et al., 2016
Ccr2 (Hanna et al., 2011)	BM monocytes/macrophages migration		Reduced BM monocytes/macrophages contribution to TRM	Boring et al., 1997
KitL (Ding et al., 2012)	Late-EMP and HSC maintenance	2, 3	Reduced late-EMP and HSC contribution to TRM	Ajami et al., 2007; Azzoni et al., 2018
Cx3cr1 (Jung et al., 2000)	Mediates monocyte retention in the BM		None	Imai et al., 1997; Jacquelin et al., 2013
Pu.1 (Scott et al., 1994; McKercher et al., 1996)	YS myeloid differentiation and HSC maintenance	1, 2, 3	Lack of EMP and HSC contribution to TRM	Scott et al., 1994; Olson et al., 1995; McKercher et al., 1996; Kim et al., 2004; Iwasaki et al., 2005; Kierdorf et al., 2013
Runx1 (Okuda et al., 1996; Wang et al., 1996a; North et al., 1999)	Master regulator of hematopoiesis, expressed from HE onwards	1, 2, 3	Lack of EMP and HSC	Okuda et al., 1996; Wang et al., 1996a; North et al., 1999
*Cbf*β (Sasaki et al., 1996; Wang et al., 1996b; Niki et al., 1997)	Essential RUNX1 co-factor	1, 2, 3	Lack of EMP and HSC	Sasaki et al., 1996; Wang et al., 1996b; Niki et al., 1997
*Cbf*β (*Tie2-Cbf*β) (Miller et al., 2002)	Essential RUNX1 co-factor	1, 2, 3	Lack of HSC	Chen et al., 2011
*Cbf*β (*Ly6a-Cbf*β) (Chen et al., 2011)			Lack of EMP	Chen et al., 2011

(Continued)

TABLE 1 | Continued

Constitutive fate mapping models	Relevance	Labeling efficiency (assay time point)				References
		LMP (Wave 2)	pMP/Early EMP (Wave 1)	Late-EMP (Wave 2)	HSC (Wave 3)	
S100a4 (Bhowmick et al., 2004) (Transgene)	Active in FL monocytes and not in FL macrophages	Not done	∼20% (5wo)	64.5 ± 6.7% (5wo)	Not done	Hoeffel et al., 2015
		Not done	∼20% (Adult)	∼60% (Adult)	∼100% (Adult)	Hashimoto et al., 2013
Flt3 (Benz et al., 2008) (KI)	Active in HSC progeny	Not done	Not done	Not done	∼80% (NB)	Hoeffel et al., 2015
		Not done	Not done	∼20% (Adult)	∼80% (Adult)	Hashimoto et al., 2013
		Not done	<2% (P8 onwards)	10–30% (P8 onwards)	∼80% (P8 onwards)	Gomez Perdiguero et al., 2015
		Not done	Not done	10–20% (4wo)	∼80% (4wo)	Schulz et al., 2012
Ms4a3 (Liu et al., 2019) (KI)	Active in GMP	Not done	None (NB onwards)	Trace (NB onwards)	BM GMP: 68.7 ± 1.58% (Adult)	Liu et al., 2019
Tnfrs11a (Maeda et al., 2012) (KI)	Essential for osteoclast development. Tracks YS progenitors.	Not done	∼80% (E14.5)	∼90% (6wo)	∼10% (E14.5 onwards)	Mass et al., 2016
Tnfrs11a (Percin et al., 2018) (KI)		Not done	Not done	∼90% (Adult)	None (Adult)	Percin et al., 2018
Rag1 (McCormack et al., 2003) (KI)	Lymphoid-specific activity	∼100% (E14.5)	Trace (E14.5)	<5% (E14.5)	Not done	Boiers et al., 2013

(Continued)

TABLE 1 | Continued

Inducible fate mapping models	Relevance	Pulse timepoint	Labeling efficiency (assay time point)			References
			pMP/Early EMP (Wave 1)	Late-EMP (Wave 2)	HSC (Wave 3)	
Csf1r (Qian et al., 2011) (Transgene)	Mainly a myeloid marker	E8.5	63.2 ± 5.6% (E13.5)	Trace (E13.5)	Not done	Hoeffel et al., 2015
			∼60% (E11.5)	Marked (E12.5)	Not done	Gomez Perdiguero et al., 2015
Cx3cr1 (Yona et al., 2013) (KI)	Mainly a myeloid marker	E9.0	∼40% (E16.0); ∼30% (NB onwards)	Trace (6wo onwards)	Trace (6wo)	Hagemeyer et al., 2016
Runx1 (Samokhvalov et al., 2007) (KI; driven by P2/Runx1b promoter)	Master regulator of hematopoiesis, expressed from HE onwards	E7.25-E7.5	∼30% (E10.5)	<5% (8wo)	<3% (8wo)	Ginhoux et al., 2010
			Not done	Not done	none (9–12mo)	Samokhvalov et al., 2007
			∼30% (E10.5); ∼20% (E13.5)	Not done	Not done	Hoeffel et al., 2012
		E7.5	22.2 ± 0.9% (E13.5)	< 5% (E13.5); <10% (E16.5)	Trace (E13.5)	Hoeffel et al., 2015
			Not done	∼10% (8wo)	∼10% (8wo)	Ginhoux et al., 2010
			Not done	Not done	1–10% (9–12mo)	Samokhvalov et al., 2007
			Not done	∼12.5% (8wo)	∼7.5% (8wo)	Hoeffel et al., 2012
		E8.5	∼15% (E13.5); <5% (E16.5)	∼25% (E13.5); ∼30% (E16.5)	1–3% (E11.5); <5% (E13.5)	Hoeffel et al., 2015
			Not done	∼30% (8wo)	∼30% (8wo)	Ginhoux et al., 2010
			Not done	Not done	1–50% (9–12mo)	Samokhvalov et al., 2007
			Not done	∼30% (8wo)	∼30% (8wo)	Hoeffel et al., 2012
		E9.5	<5% (E13.5)	∼15% (E13.5)	∼30% (E13.5)	Hoeffel et al., 2015
			Not done	∼20% (8wo)	∼30% (8wo)	Ginhoux et al., 2010
			Not done	Not done	50–100% (9–12mo)	Samokhvalov et al., 2007
			Not done	∼25% (8wo)	∼30% (8wo)	Hoeffel et al., 2012
Tie2 (Gomez Perdiguero et al., 2015) (KI)	Endothelial marker (including HE) also expressed in subset of HSC and myeloid cells	E7.5	∼60% (E12.5)	∼40% (6–8wo)	∼40% (E12.5)	Gomez Perdiguero et al., 2015
		E8.5	∼30% (E12.5)	∼20% (6–8wo)	∼75% (E12.5)	
		E9.5	trace (E12.5)	∼10% (6–8wo)	∼80% (E12.5)	
		E10.5	none (E12.5)	∼5% (6–8wo)	∼40% (E12.5)	
Kit (Sheng et al., 2015) (KI)	Express in early HSPC and YS HE cells	E7.5	∼70% (E13.5)	Trace (E13.5)	Trace (6wo)	Sheng et al., 2015
		E8.5	∼70% (E13.5)	∼40% (E13.5)	∼60% (6wo)	
		E9.5	∼50% (6wo)	∼50% (6wo)	∼40% (6wo)	
Cdh5 (Sorensen et al., 2009) (Transgene)	Endothelial marker (including HE)	E7.5	∼80% (E10.5); ∼90% (E14.5)	∼80% (E10.5)	<10% (E14.5)	Gentek et al., 2018a
		E10.5	Trace (E14.5)	Not done	∼80% (E14.5)	

EMP, erythroid–myeloid progenitors; HSC, hematopoietic stem cells; TRM, tissue-resident macrophages; FL, fetal liver; BM, bone marrow; pMP, primitive myeloid precursors; HSPC, hematopoietic stem and progenitors; YS, yolk sac; LMP, lymphomyeloid progenitors; GMP, granulocyte-macrophage progenitors; HE, hemogenic endothelium; KI, knock-in; NB, new born; wo, week old; mo, month old.

combination with respectively *Ly6a-Cbf*β or *Tie2-Cbf*β rescue alleles (Chen et al., 2011).

Constitutive lineage tracing models rely on lineage-specific promoter activity to drive *Cre* recombinase expression, which in turn irreversibly activates or deletes a target gene (Hoess and Abremski, 1984; Sauer and Henderson, 1988). Such models have been established to trace long term lineage contribution of HSC- and YS-derived hematopoietic cells. *Flt3*-Cre, *Ms4a3*-Cre, *S100a4*-Cre predominantly track HSC progeny, albeit with several restrictions. *Flt3*-Cre (Schulz et al., 2012; Hashimoto et al., 2013; Gomez Perdiguero et al., 2015; Hoeffel et al., 2015) and *S100a4*-Cre (Hashimoto et al., 2013; Hoeffel et al., 2015) mark the majority of HSC-derived cells (>80%). However, both also mark some YS-derived hematopoietic cells (**Table 1**). In contrast, *Ms4a3*-Cre does not mark any YS cells from the first or second wave and only marks HSC-derived GMPs (∼70%) (Liu et al., 2019). The *Tnfrs11a*-Cre model is currently best suited to track YS hematopoiesis with little (Maeda et al., 2012; Mass et al., 2016) or no (Percin et al., 2018) HSC labeling. However, this model cannot distinguish the two YS waves of hematopoiesis. Currently, the only option to track LMP progeny is the *Rag1*-Cre fate mapping model (Boiers et al., 2013) which marks all FL B and T cells alongside a small number of myeloid cells.

Inducible tracing models [tamoxifen-inducible Cre-mediated recombination (Metzger et al., 1995; Feil et al., 1997)] add an extra layer of specificity that can overcome certain limitations of the constitutive models. This approach allows not only for reporter activation or gene deletion in specific cell types but also during a defined developmental time window. The latter has allowed for the specific marking of the first hematopoietic wave in the YS (pMP/early EMP) using multiple models (**Table 1**). In this context, a caveat of the *Csf1r*-Mer-iCre-Mer and *Cx3cr1*-CreER based systems is that they only label myeloid progeny (Gomez Perdiguero et al., 2015; Hoeffel et al., 2015; Hagemeyer et al., 2016). In contrast, *Tie2*-Mer-iCre-Mer, *Kit*-Mer-Cre-Mer, *Runx1*-Mer-Cre-Mer, and *Cdh5*-CreERT2 provide less restricted marking. Distinguishing progeny from late-EMP (wave 2) and HSC (wave 3) is still challenging, as illustrated in **Table 1** (Samokhvalov et al., 2007; Ginhoux et al., 2010; Hoeffel et al., 2012, 2015; Gentek et al., 2018a).

HSC-INDEPENDENT HEMATOPOIETIC CELLS CONTRIBUTE TO THE ADULT HEMATOPOIETIC SYSTEM

In the adult hematopoietic system, several hematopoietic populations have been shown to consist of cells with an HSC-independent embryonic origin (**Figure 1B**). This has been best studied for tissue-resident macrophages (TRM), which were traditionally thought to be continuously replenished by BM HSC-derived monocytes. However, this view was challenged by the discovery of radiation-resistant and self-repopulating Langerhans cells (Merad et al., 2002), microglia (Ajami et al., 2007; Ginhoux et al., 2010), and alveolar macrophages (Guilliams et al., 2013; Hashimoto et al., 2013; Jakubzick et al., 2013) in BM transplantation and parabiosis studies. Subsequent lineage tracing studies have convincingly demonstrated that microglia are the progeny of myeloid cells produced during the first wave of hematopoiesis. *Csf1r*-Mer-iCre-Mer, *Cx3cr1*-CreER, *Runx1*-Mer-Cre-Mer, and *Kit*-Mer-Cre-Mer lineage tracing models all support the pMP/early EMP origin of microglia (Gomez Perdiguero et al., 2015; Hoeffel et al., 2015; Sheng et al., 2015; Hagemeyer et al., 2016). This has been further strengthened by the analysis of *KitL* and *c-Myb* KO models in which the number of late-EMP and HSC (waves 2 and 3) is drastically decreased, while the microglia population remains unaffected (Ding et al., 2012; Schulz et al., 2012; Azzoni et al., 2018).

It is now widely accepted that TRM populations do not have a unified common origin. Some tissues retain and maintain YS-derived cells while in others they are replaced or co-exist with BM-HSC-derived cells (for review Ginhoux and Guilliams, 2016; Mass, 2018; Wittamer and Bertrand, 2020). Below, we highlight recent findings concerning the persistence of YS-derived embryonic hematopoietic cells in adults.

HSC-Independent Origin of Adult TRM

Alveolar macrophages (AM) and interstitial macrophages (IM) are two major subsets of lung TRM (Lehnert et al., 1985; Liegeois et al., 2018). Around E10.5, YS pMPs/early EMPs (Tan and Krasnow, 2016) seed in the primordial lung buds. Parabiosis, adoptive transfer, and lineage tracing experiments have shown that a subset of pMP/early EMP-derived IM (Hoeffel et al., 2012, 2015; Guilliams et al., 2013; Gomez Perdiguero et al., 2015; Tan and Krasnow, 2016; Liu et al., 2019) and AM (van de Laar et al., 2016; Li et al., 2020) persist into adulthood. The functional significance of these sub-populations, if any, is currently unknown. However, it has been shown that FL monocyte-derived TRM possess enhanced mitochondrial respiratory and glycolytic capacity (Li et al., 2020) versus their HSC-independent counterparts.

Adipose tissue macrophages (ATM), are the most abundant immune cells in adipose tissues and play a prominent role in regulating inflammation and insulin sensitivity (Russo and Lumeng, 2018). ATM, which can proliferate and self-renew, are partially derived from embryonic HSC-independent cells (E9.0 pulsed *Cx3Cr1*-CreER) (Hassnain Waqas et al., 2017; Waqas et al., 2017). Further studies using the *Ms4a3*-Cre fate-mapping model, which traces BM-HSC monocyte-derived cells (Jaitin et al., 2019; Liu et al., 2019), demonstrated that half of the white adipose tissue ATM is Ms4a3 positive under normal physiological conditions. Interestingly, a high-fat diet increases the proportion of HSC-derived monocytes within ATM population (Jaitin et al., 2019).

The *Ms4a3*-Cre fate-mapping model has also revealed that adult renal macrophages (RM) are of mixed origin (Schulz et al., 2012; Epelman et al., 2014; Gomez Perdiguero et al., 2015; Hagemeyer et al., 2016; Mass et al., 2016; Liu et al., 2019; Munro et al., 2019). Furthermore, YS-derived RM (E9.5 pulsed *Cx3cr1*-CreER) are more proliferative than their HSC-derived counterpart and their contribution to the kidney TRM population expands with age (Ide et al., 2020).

Osteoclasts are multinucleated myeloid cells that resorb bone tissue and are critical for the development, repair, and remodeling

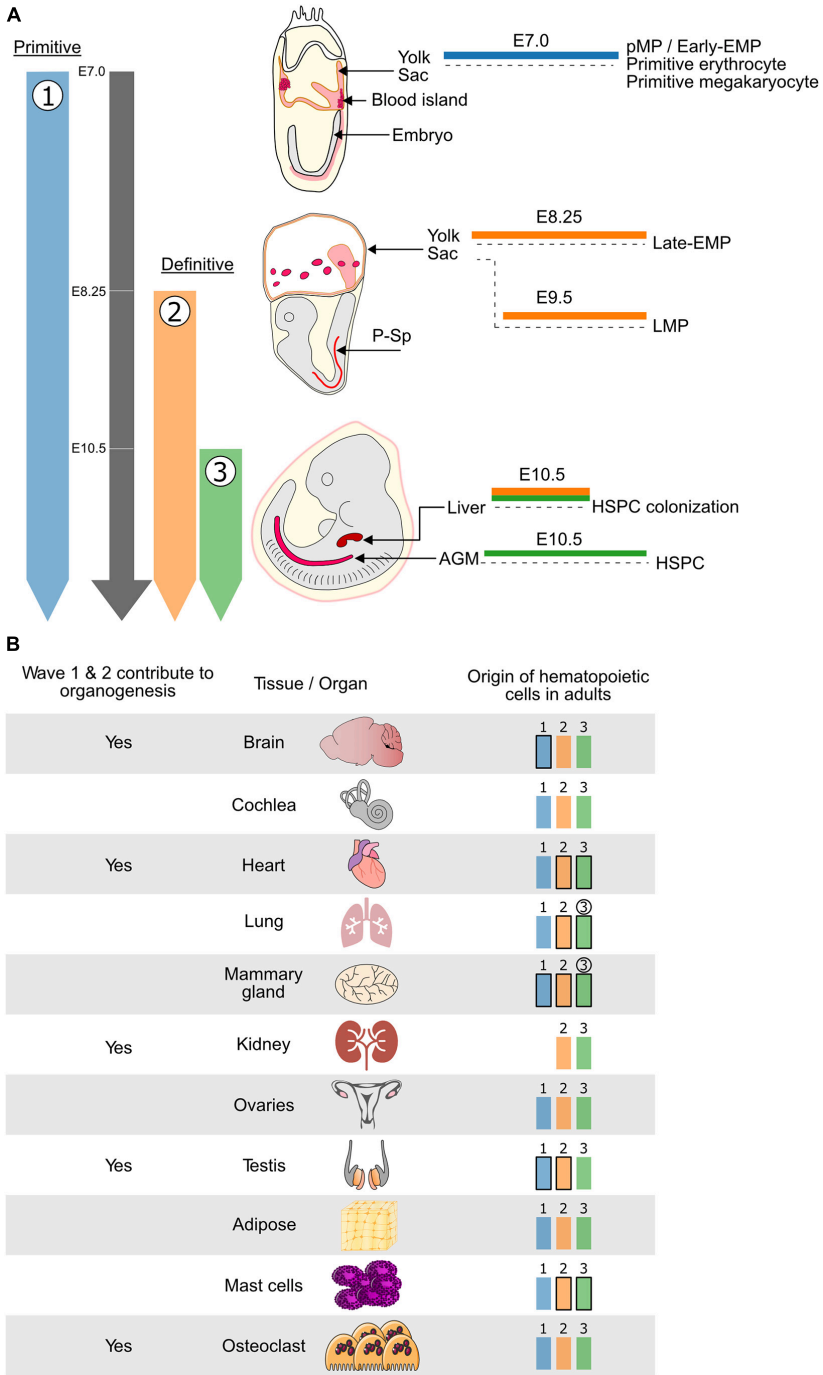

FIGURE 1 | The hematopoietic system is established in developmental waves that differentially contribute to embryonic organogenesis and the adult hematopoietic system. **(A)** Schematic representing the timing and embryonic sites of hematopoietic activity during mouse development. The three waves of hematopoiesis are represented in Blue (wave 1: primitive erythrocytes, megakaryocytes, macrophages and Early EMP), Orange (wave 2: Late EMP and LMP) and Green (wave 3: HSPC). Wave 1 is known as the primitive wave while waves 2 and 3 constitute the definitive waves of hematopoiesis. Waves 1 and 2 which do not generate HSCs originate in the Yolk Sac while wave 3, which generates the HSPCs, is initiated in the AGM region of the embryo proper. Cells from both waves 2 and 3 can colonize the fetal liver where they can mature and expand before moving to their final destination. **(B)** (Left) HSC-independent macrophages which originate from the first two hematopoietic waves have been shown to play important roles during embryonic development of several tissues and organs. (Right) In addition to the wave 3 (Green) HSC-derived hematopoietic cells, the HSC-independent hematopoietic cells generated during wave 1 (Blue) and 2 (Orange) persist, to varying degrees, in adult tissues and organs. Waves that contribute most of the hematopoietic cells are depicted in bold and waves for which the contribution increases overtime are circled. Blue: hematopoietic wave 1 (primitive), Orange: hematopoietic wave 2 (Late-EMP/LMP), Green: hematopoietic wave 3 (HSC). E, embryonic day; Mφ, macrophage; HSC, hematopoietic stem cell; EMP, erythroid–myeloid progenitor; LMP, lymphoid–myeloid progenitor; HSPC, hematopoietic stem and progenitor cell; P-Sp, para-aortic splanchnopleura; AGM, aorta-gonad-mesonephros.

of the skeleton (Udagawa et al., 1990; Takahashi et al., 1994). The HSC-independent origin of osteoclast was first suggested by *ex vivo* co-culture experiments (Thesingh, 1986) and has been recently confirmed *in vivo* using *Csf1r*-Mer-iCre-Mer and *Cx3cr1*-CreER models (Jacome-Galarza et al., 2019; Yahara et al., 2020). Early/late EMP-derived osteoclasts not only persist but also expand in the adult BM (Yahara et al., 2020). Furthermore, a subpopulation of early/late EMP-derived osteoclasts was found to reside in the spleen. Interestingly, this spleen population can fuse with BM-derived monocytes creating long-lived osteoclast syncytia with a mixed origin (Jacome-Galarza et al., 2019; Yahara et al., 2020).

Based on CD206 and MHCII expression, three testis subpopulations of TRM can be distinguished. These populations possess different levels of phagocytic capacity (Lokka et al., 2020). Tracing (E8.5 pulsed *Csf1r*-Mer-iCre-Mer, E13.5 pulsed *Cx3cr1*-CreER) and KO (*Ccr2*, *Nur77*, *Plvap*) studies have shown that all three waves of hematopoiesis contribute to adult testis TRM. Strikingly, antibody-based macrophage depletion experiments have demonstrated that adult BM-derived cells play no part in testis TRM maintenance (Lokka et al., 2020).

Finally characterization of *Csf1* KOs, the *Csf1r*-EGFP constitutive tracing model, and E8.5 pulse-labeled *Csf1r*-Mer-iCre-Mer mice point to an HSC-independent origin for part of the TRM in adult cochlea (Kishimoto et al., 2019), ovaries (Jokela et al., 2020), and mammary glands (Gouon-Evans et al., 2000; Jappinen et al., 2019; Stewart et al., 2019).

Beyond Tissue Resident-Macrophages: Mast- and Lymphoid-Cells

Mast cells (MC) can be classified into two groups. Connective tissue MC (CTMC) populate the skin, tongue, trachea, esophagus, adipose tissues, and peritoneal- and pleural cavities while mucosal MC (MMC) are found in the gut and respiratory mucosa. The exclusive BM HSC-derived (van Furth and Cohn, 1968) origin of MC was first challenged by transplantation assays that showed BM only minimally contributes to MC repopulation in MC-depleted hosts (Kitamura et al., 1977, 1978). Subsequent fate-mapping studies using *Csf1r*-Mer-iCre-Mer and *Runx1*-Mer-Cre-Mer suggested that the majority of MMC are derived from HSC, whereas CTMC are largely derived from the HSC-independent EMP (Li Z. et al., 2018). Interestingly, EMP-derived and HSC-derived MC have distinct transcriptional profiles suggesting distinct biological functions (Gentek et al., 2018a; Li Z. et al., 2018). If the CTMCs derived from HSC-independent cells can persist in significant numbers in the adult is unclear. *Csf1r*-Mer-iCre-Mer and *Runx1*-Mer-Cre-Mer fate-mapping studies suggest that they can, while *Cdh5*-CreERT2 based lineage tracing suggests a mostly fetal HSC origin of adult CTMC (Gentek et al., 2018a). These contradictory findings highlight that data from fate-mapping models should be interpreted with caution and that currently, no model can definitively distinguish the progeny of late-EMP from fetal HSC.

Finally, YS-derived lymphoid cells have also been found to persist into adulthood. Early B and T-cells [B1a (Yoshimoto et al., 2011; Kobayashi et al., 2014) and γδ T (Boiers et al., 2013; Gentek et al., 2018b) cells], a primary source of innate immunity in early embryo development (Yoshimoto et al., 2012), persist into adulthood and remain functionally distinct from their HSC-derived counterparts. The ontogeny and contribution of these YS-derived lymphocytes has been reviewed previously (Yamane, 2018; Ghosn et al., 2019). Finally, the existence of EMP-derived NK cells, possessing a potent degranulation response, has been reported recently (Dege et al., 2020). This is particularly striking as NK cells are considered to be of lymphoid origin. However, it is unclear whether these EMP-derived NK cells are part of the myeloid lineage or if these findings have revealed lymphoid potential in EMP. Similarly, it is not clear to what extent EMP-derived NK cells persist into adulthood (Sun et al., 2011; Wu et al., 2014; Corat et al., 2017; Schlums et al., 2017).

HSC-INDEPENDENT MACROPHAGES PARTICIPATE IN EMBRYONIC ORGANOGENESIS

The role of macrophages in tissue remodeling is an exciting field of ongoing research (Hoeffel and Ginhoux, 2018; Wittamer and Bertrand, 2020). The discovery of adult YS-derived TRM populations with an M2-like non-inflammatory phenotype, associated with wound healing and tissue repair, hints at potential roles in embryonic organogenesis (Takahashi et al., 1998; Rae et al., 2007; Pucci et al., 2009; Fantin et al., 2010; DeFalco et al., 2014; Italiani and Boraschi, 2014; Munro et al., 2019; Shigeta et al., 2019). However, identifying unique and specific roles of HSC-independent cells is complicated by the fact that embryonic organ development spans across all hematopoietic waves. Below we highlight the instances where specific roles for HSC-independent macrophages have been identified (**Figure 1B**).

HSC-Independent Embryonic Macrophages Guide Vascular Network Organization in Developing Organs

Vascular networks are established by tip- and stalk- endothelial cells. Endothelial tip-cells, guided by vascular endothelial growth factor (VEGF) gradients, drive the direction of the vessel while the endothelial stalk-cells follow and establish the vessel lumen (Herbert and Stainier, 2011). During embryonic organogenesis, macrophages have been shown to play an essential role in organizing endothelial networks. A role for HSC-independent macrophages in blood vessel anastomosis was first described in detail in the mouse embryonic hindbrain where it is entirely dependent on pMP/early EMP-derived macrophages (Fantin et al., 2010; Rymo et al., 2011). These macrophages invade the brain in a CSF1-dependent manner. Subsequently, upon brain vascularization, the macrophages closely associate with tip-endothelial cells. Macrophage depletion in the brain [*Pu.1* (Scott et al., 1994; McKercher et al., 1996) and *Csf1* KO (Wiktor-Jedrzejczak et al., 1990; Cecchini et al., 1994)], but not specific depletion of FL-derived macrophages (*Lysm*-Cre-mediated diphtheria toxin) (Clausen et al., 1999), significantly reduces the number of vessel intersections and thereby limits the overall complexity of the brain vascular network (Fantin et al., 2010; Rymo et al., 2011).

Hematopoietic stem cells-independent macrophages also play a role in kidney and testis vascular network formation. In mouse embryos, the mesonephros (a temporary kidney structure) and the gonads (which will give rise to the testis in males) are established near the extending nephric ducts around E9. Further extension of the nephric duct results in the generation of uretic buds and the metanephros (precursor to the adult kidneys) around E10-E11.5 (Takasato and Little, 2015). Proliferating primitive pMP/early EMP-derived macrophages (E7.5 pulsed *Csf1r*-Mer-iCre-Mer) are first detected in the gonadal region around E10.5 (DeFalco et al., 2014). By E11.5-E13.5, these macrophages closely associate with and engulf EC of the mesonephros vascular plexus and testis vasculature. Depletion of fetal macrophages (*Cx3cr1*-Cre-mediated diphtheria toxin) results in an enlarged mesonephros vascular plexus, reduced migration of EC into the testis, and impaired development of the coelomic artery. Blood vessels start entering the metanephros between E11.5-E12, and YS-derived macrophages are consistently found perivascular at developing vascular fronts (Rae et al., 2007; Hoeffel et al., 2015; Munro et al., 2019). Analysis of macrophage depleted E12.5 kidney explants (anti-CSF1R depletion) showed increased numbers of unconnected endothelial structures and a reduction in vascular network size, consistent with a role for macrophages in vessel anastomosis.

The developing heart harbors macrophages derived from both the HSC-independent and HSC-dependent hematopoietic waves (*Ccr2*-GFP, *Cx3cr1*-GFP) (Leid et al., 2016). HSC-independent macrophages (E7.5 pulsed *Rosa26-tdCsf1r*-MerCre) appear in the heart around E12.5 and predominantly populate the myocardium where they accumulate near perfused coronary vessels. Genetic depletion of macrophages ($Csf1^{op/op}$) results in retarded primitive coronary plexus development. However, specific depletion of HSC-derived macrophages (*Ccr2* KO) does not affect primitive coronary plexus development, indicating that HSC-independent macrophages are responsible for the modulation of the myocardial vascular network.

Finally, recent RNA-seq of a human Hofbauer cells (Zulu et al., 2019), a fetus-derived macrophage population found in the placenta, suggests that they may play a role in angiogenesis and remodeling (Thomas et al., 2021). Although Hofbauer cells have also been identified in mice (Takahashi et al., 1991), their role has not yet been investigated *in vivo*.

It is tempting to postulate a generalized role for HSC-independent macrophages in the establishment of vascular networks during embryonic organogenesis. In support of this, HSC-independent macrophages have a similar role in organizing vascular networks in zebrafish, independently of specific organs (Fantin et al., 2010).

HSC-Independent Macrophages Directly Support Organogenesis

Hematopoietic stem cells-independent macrophages have also been directly implicated in embryonic organ development. The central nervous system is arguably one of the best-studied systems in this context, with YS-derived microglia playing a role in multiple perinatal brain developmental events (Li and Barres, 2018; Low and Ginhoux, 2018). Around E14.5, microglia accumulate near developing axonal tracts and their genetic (*Pu.1* KO)/antibody-based depletion (anti-CSF1R) or their inappropriate activation (E13.5 lipopolysaccharide maternal immune activation), affects the development of interneuronal connections and dopaminergic axon outgrowth (Squarzoni et al., 2014). Similarly, in the peripheral nervous system, macrophages/microglia are found in close contact with developing sensory neurons in dorsal root ganglia from E11. Genetic (*Pu.1* KO) or antibody-based depletion (anti-CSF1R) of these macrophages alters the developmental trajectory of the sensory neurons (Angelim et al., 2018).

During gonad development, macrophages associate with and engulf mislocated germ (E10.5–E11.5) and Sertoli (E12.5) cells, and their absence (*Cx3cr1*-Cre/diphtheria toxin) results in irregularly branched and shortened testis cords (DeFalco et al., 2014). Furthermore, depletion of macrophages during embryogenesis ($Csf1^{op/op}$, anti-CSF1R depletion) but not postnatally (*Ccr2* KO, anti-CSF1 depletion at birth) results in impaired spermatogenesis after birth (Lokka et al., 2020). Similar observations have been made in kidney development where the clearance of rostral nephrogenic cells and uretic bud formation are delayed in the absence of YS-macrophages (*Cx3cr1*-Cre/diphtheria toxin) (Munro et al., 2019). These results suggest that embryonic macrophages are participating in gonad/testis and kidney development.

Yolk sac-derived osteoclasts are essential for normal skeletal development in the embryo and their absence (*Csf1r* KO model and *Csf1*-Cre-mediated *Tnfrsf11a* KO) disrupts tooth eruption, skull formation, long bone formation, and their timely hematopoietic colonization (Yoshida et al., 1990; Dougall et al., 1999; Dai et al., 2002; Jacome-Galarza et al., 2019). This phenotype is not observed when HSC-derived macrophages are deleted (*Flt3*-Cre-mediated and *Csf1r* KO) (Jacome-Galarza et al., 2019).

Finally, heart development also depends on HSC-independent macrophages which, interestingly, originate locally from HE cells populating the endocardium (Nakano et al., 2013; Yzaguirre and Speck, 2016; Shigeta et al., 2019). An important phase in heart development is the establishment and remodeling of the heart valves which starts around E9.5 and concludes after birth. Specific depletion of endocardial macrophages (*Nfatc*-Cre-mediated *Csf1r* KO) demonstrated that they are essential for heart valve development and that macrophages of other sources cannot compensate for their loss (Shigeta et al., 2019).

HSC-Independent Macrophages Support HSC Formation

Arguably the most striking function of HSC-independent macrophages is that they can affect HSC ontogeny. This has been studied in detail in zebrafish. HSCs generated in the dorsal aorta of zebrafish enter the circulation via the postcardinal vein (PCV) (Bertrand et al., 2010; Kissa and Herbomel, 2010; Lam et al., 2010). This requires newly formed HSC to traverse the mesenchyme separating the two vessels. Primitive macrophages accumulate in this subaortic mesenchyme and, via metalloproteinases mediated

extracellular matrix degradation, create tracks for the HSC to enter the subaortic mesenchyme. These primitive macrophages then join the PCV from where they migrate to the zebrafish FL equivalent, known as the caudal hematopoietic tissue (CHT; Travnickova et al., 2015). Once the HSC reaches the CHT, a specific set of primitive VCAM+ macrophages (usher macrophages) interact with and "capture" passing HSPC and guide them into the CHT (Li D. et al., 2018).

In mice, HSC-independent macrophages also play an important role in HSC ontogeny. At E10.5, HSC-independent macrophages are found in close association with EC and IAHC in the AGM, where they possibly participate in moving KIT+ IAHC cells towards the aortic lumen (Mariani et al., 2019). The CXCL3 chemokine (expressed amongst others by HE and IAHC) is important for the macrophage accumulation in the AGM (Mariani et al., 2019). The deletion of its receptor, *Cxcr3*, results in increased numbers of macrophages in the YS and reduced numbers in the AGM. Reduction of the number of macrophages in the AGM, either by genetic (*Cxcr3* KO) or chemical (clodronate and CSF1R inhibitor BLZ945) depletion, negatively affects HSC generation in the AGM (Mariani et al., 2019). Furthermore, both direct and indirect (transwell) co-culture experiments of AGM-derived aortic endothelial cells (including HE) with aortic macrophages result in an increase of the hematopoietic colony-forming capacity of the endothelial cells (Mariani et al., 2019). These results indicate an essential role for macrophage secreted factors in AGM EHT. RNA-seq of the aortic macrophages revealed that despite having an immune phenotype associated with anti-inflammatory or M2 type phenotype, they have a distinct pro-inflammatory transcriptome (Mariani et al., 2019). Currently, it is unclear if these macrophages promote emergence of all, or only subsets, of HSC.

CONCLUDING REMARKS

In the last decade, it has become clear that HSC-independent hematopoietic cells have previously unanticipated roles in both embryos and adults. They have been found to participate in organogenesis and persist in adults as distinct hematopoietic populations. There are however still many open questions about their exact role, origin, and contributions. In this context, the development of more precise and efficient genetic tracing models would be beneficial. Specifically, models that can efficiently differentiate wave 2 (late-EMP and LMP) from wave 3 (HSC) are needed.

It is also essential to acquire more detailed knowledge of the different hematopoietic waves, both mechanistically and in terms of their exact sites of origin. Indeed, the observation that heart-specific HE can give rise to a specialized population of macrophages, raises the question whether other specialized hematopoietic cells are produced in a site or organ-specific way. Both in the AGM and the YS, multiple sites of hematopoietic emergence have been described (Muller et al., 1994; Medvinsky and Dzierzak, 1996; de Bruijn et al., 2000; Li et al., 2012; Frame et al., 2016; Kasaai et al., 2017). Closer investigation of these known sites as well as the identification of new sites could reveal the existence of new, functionally unique, hematopoietic populations. Furthermore, understanding if and how the distinct hematopoietic cells generated by the different waves interact to optimize blood production is equally fascinating. Altogether such knowledge could provide cues to develop better strategies for *in vitro* generation of HSCs and/or specific blood lineages from embryonic- and induced pluripotent stem cells (ES and IPSC). IPSC generated from different cellular sources may be inherently primed towards specific hematopoietic lineages. Additionally, it may be beneficial to incorporate mature hematopoietic cells into *in vitro* blood production protocols. Along these lines, it has been recently reported that macrophages can support the *in vitro* production of mature enucleated erythroid cells (Lopez-Yrigoyen et al., 2019).

Finally, findings in animal model systems are starting to be confirmed in humans. Macrophages have been found to accumulate in the human AGM at the time of HSC formation (Travnickova et al., 2015), and single-cell sequencing indicates that human microglia are also derived from HSC-independent hematopoietic waves (Bian et al., 2020). In conclusion, it is now well established that HSC-independent hematopoiesis is essential for embryonic organogenesis and its progeny can, and does, persist after birth. This has opened up a new and fascinating field of hematopoietic research.

AUTHOR CONTRIBUTIONS

WHN and ML wrote the manuscript. MZHF produced the figure. GL revised and edited the manuscript. All authors approved the final manuscript.

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Fetal-Derived Immune Cells at the Roots of Lifelong Pathophysiology

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Tissue-resident innate immune cells exert a wide range of functions in both adult homeostasis and pathology. Our understanding of when and how these cellular networks are established has dramatically changed with the recognition that many lineages originate at least in part from fetal sources and self-maintain independently from hematopoietic stem cells. Indeed, fetal-derived immune cells are found in most organs and serous cavities of our body, where they reside throughout the entire lifespan. At the same time, there is a growing appreciation that pathologies manifesting in adulthood may be caused by adverse early life events, a concept known as "developmental origins of health and disease" (DOHaD). Yet, whether fetal-derived immune cells are mechanistically involved in DOHaD remains elusive. In this review, we summarize our knowledge of fetal hematopoiesis and its contribution to adult immune compartments, which results in a "layered immune system." Based on their ontogeny, we argue that fetal-derived immune cells are prime transmitters of long-term consequences of prenatal adversities. In addition to increasing disease susceptibility, these may also directly cause inflammatory, degenerative, and metabolic disorders. We explore this notion for cells generated from erythro-myeloid progenitors (EMP) produced in the extra-embryonic yolk sac. Focusing on macrophages and mast cells, we present emerging evidence implicating them in lifelong disease by either somatic mutations or developmental programming events resulting from maternal and early environmental perturbations.

Keywords: erythro-myeloid progenitors, macrophages, mast cells, developmental origins of health and disease, layered hematopoiesis, cancer, neurological disease, atopic disease

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INTRODUCTION

It is now widely recognized that many non-communicable diseases have developmental origins, brought about by somatic mutations or environmental perturbations during gestation and in early life. Immune dysregulation is a common denominator in the etiology of these diseases, and can even directly cause pathology. Indeed, immune cells have many functions beyond protective immunity, for example in controlling tissue homeostasis. The first immune cells seed developing tissues during organogenesis, and unlike previously thought, appear fully functional already at these early stages. Moreover, we have recently come to realize that fetal-derived cells persist and self-maintain in

Abbreviations: AGM, aorta-gonad-mesonephros; BM, bone-marrow; DETC, dendritic epidermal T cells; EMP, erythro-myeloid progenitor; HSC, hematopoietic stem cell; IBS, irritable bowel syndrome; LTi, lymphoid tissue inducer; MIA, maternal immune activation; NK, natural killer; YS, yolk sac.

adult tissues. This is true for macrophages and mast cells derived from erythro-myeloid progenitors (EMP) generated in the yolk sac (YS) before the emergence of hematopoietic stem cells (HSC). Their ontogeny and proliferative capacity make EMP-derived cells particularly vulnerable to early life perturbations and identify them as potential transmitters of long-term effects on health and disease.

Here, we explore this notion, focusing on EMP and their cellular progeny. Because they allow establishing *in vivo* lineage and cause-consequence relationships between perturbation of immune development and pathology, we will primarily discuss experimental animal studies. However, where possible, we will also discuss relevant human data, especially those that benefitted from recent technological advancements such as single-cell RNA-sequencing. We will briefly summarize our current understanding of fetal hematopoiesis and its contribution to adult tissue-resident immune compartments/landscapes. Having established their normal developmental trajectories, we will then discuss a growing body of literature supporting the notion that mutations affecting EMP or exposure to adverse early life environments render macrophages and mast cells pathogenic in conditions as diverse as neurological or atopic disease and cancer.

Layered Hematopoiesis

Traditionally, HSC found in the bone marrow (BM) have been regarded as the sole, lifelong source of all immune cells. This view has changed with the recognition that many lineages originate at least in part from fetal sources and self-maintain independently from HSC. Indeed, we now appreciate that fetal-derived cells comprise varying proportions of the resident immune compartments in most adult organs and serous cavities.

The production of hematopoietic progenitors is initiated early during mammalian development. Although low-grade hemogenic capacity might also exist in the BM during a brief perinatal window (Yvernogeau et al., 2019), *de novo* hematopoiesis is otherwise restricted to fetal stages. Fetal hematopoiesis occurs in several waves that differ in time and space but partially overlap (**Figure 1**). These distinct waves also differ in their lineage output, as we will discuss in more detail below.

Hematopoietic Waves

The first hematopoietic progenitors are produced in the extra-embryonic YS. In the mouse, this occurs on day 7 of embryonic development (E7.0) (Wong et al., 1986). This so-called **primitive** wave generates erythrocytes, megakaryocytes (Tober et al., 2007), and possibly also the first macrophages that colonize the embryo proper, at least microglia in the brain parenchyma (Alliot et al., 1999; Ginhoux et al., 2010). Primitive hematopoiesis is followed by the production of **erythro-myeloid progenitors (EMP)**, a second wave sometimes called transient or **transient-definitive** (Moore and Metcalf, 1970; Palis et al., 1999; Tober et al., 2007). EMP are generated between E8.5 and E10.5 from hemogenic endothelial cells of the YS (Gomez Perdiguero et al., 2015; McGrath et al., 2015). In addition to their erythrocyte, granulocyte, and megakaryocyte potential, EMP readily differentiate into macrophages, monocytes, granulocytes, and mast cells *in vivo* (Gomez Perdiguero et al., 2015; Hoeffel et al., 2015; Mass et al., 2016; Gentek et al., 2018a; Li et al., 2018). Finally, definitive HSC emerge in a third wave that originates from the main arteries of the embryo proper, particularly the aorta-gonad-mesonephros (AGM) region (Boisset et al., 2010), although hemogenic activity has been reported at additional sites including the umbilical and vitelline arteries, placenta, heart and the hindbrain-branchial arch region of the head (Alvarez-Silva et al., 2003; Gekas et al., 2005; Zeigler et al., 2006; Li et al., 2012, 2016; Gordon-Keylock et al., 2013; Solano et al., 2014; Shigeta et al., 2019). Like EMP, HSC derive from hemogenic endothelial cells (De Bruijn et al., 2002; Boisset et al., 2010). They transiently colonize the fetal liver to expand and ultimately settle in the BM, where they persist lifelong. HSC are operationally defined by their potential for self-renewal and multi-lineage reconstitution upon transplantation into irradiated adult hosts. Based on these criteria, additional populations of HSC have been described (Beaudin et al., 2016). However, their contribution to adult BM HSC remains controversial. Resolving this requires appropriate models that do not rely on transplantation but instead, allow assessment of persistence and lineage output under physiological conditions *in vivo*., e.g., using the Polylox barcoding model (Pei et al., 2017). Therefore, we propose to refer to these progenitors as fetal-restricted or **transient HSC** and distinguish them from **adult-type HSC** found in the BM.

This complex process generates what has been termed a "**layered immune system,**" in which certain lineages have distinct origins throughout life, whereas others remain entirely or in part of fetal origin. While originally postulated for innate-like B1 and conventional B(2) cells (Herzenberg and Herzenberg, 1989; Herzenberg, 2015), this concept now appears to be more globally applicable, in particular to long-lived tissue-resident innate cells, including macrophages, mast cells, and innate lymphocytes (Gentek et al., 2018b; Schneider et al., 2019; Dege et al., 2020; Simic et al., 2020).

Human Hematopoiesis

Similarly to mice, also human hematopoietic waves appear sequentially at distinct sites, with EMP-like cells in the YS that produce erythrocytes, as well as macrophages, megakaryocytes (reviewed in Julien et al., 2016) and mast cells (Popescu et al., 2019; Bian et al., 2020). Once the blood circulation is established, EMP colonize the fetal liver where they are joined by definitive HSC coming from the AGM (Julien et al., 2016). Recent single-cell RNA-sequencing studies of human fetal tissue provide further evidence for the evolutionary conserved hematopoietic development and molecular programs of individual cell types (Popescu et al., 2019; Bian et al., 2020; Cao et al., 2020), supporting the notion that also in humans EMP generate long-lived immune cells that are self-maintained independently of HSC. At least for tissue-resident macrophages, this is now an accepted concept, since transplant patients harbor donor macrophages in transplanted organs such as the lungs (Eguíluz-Gracia et al., 2016; Nayak et al., 2016), skin (Kanitakis et al., 2011), and heart (Bajpai et al., 2018) for many years. Taken together, evidence is mounting that adult human tissues harbor

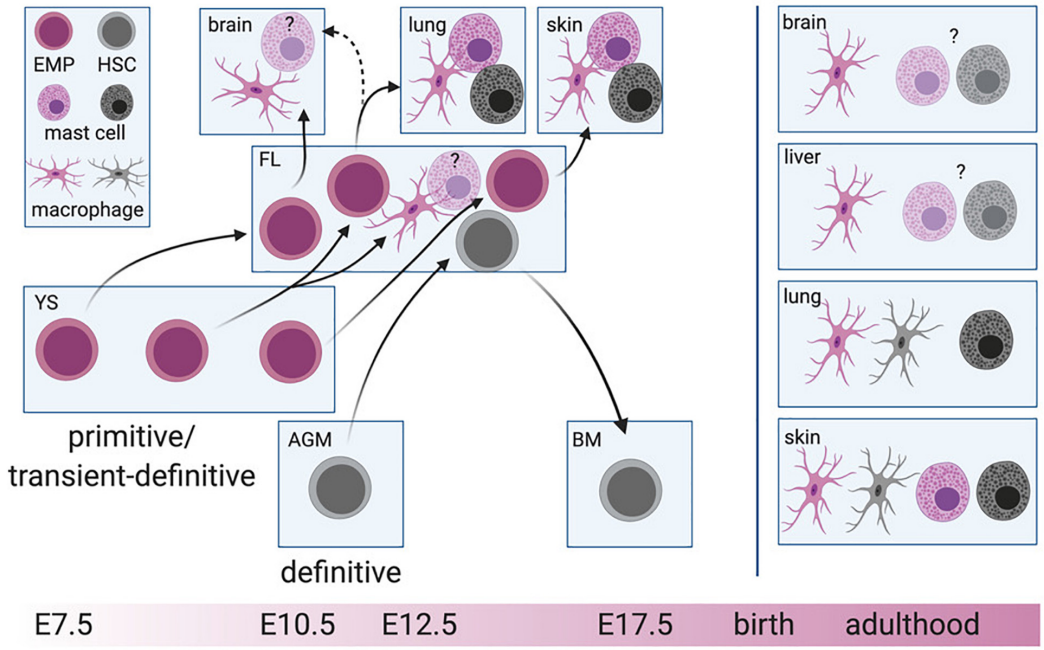

FIGURE 1 | Layered hematopoiesis. The primitive and transient-definitive waves in the yolk sac (YS) give rise to erythro-myeloid progenitors (EMP) that will colonize the fetal liver (FL) consecutively. EMP arising at early stages will differentiate into macrophages and probably mast cells in tissues developing early during embryogenesis (brain, liver), while EMPs at later stages remain in the FL and will give rise to the same cell types driven by the demand of later developing tissues, such as the lung and skin. Hematopoietic stem cells (HSC) develop in the aorta-gonad-mesonephros (AGM) region before colonizing the FL. After the formation of the bone marrow (BM), HSC migrate to the BM cavity where they will constantly give rise to short-lived macrophages and mast cells. The cell of origin for mast cells and macrophages is color-indicated. For simplicity, blood-circulating intermediate precursor stages between EMP and macrophages are not depicted. Created with BioRender.com.

fetal-derived cells, therefore, supporting the use of the mouse as a powerful model system for studying human immune cell development and function.

Distinct Ontogeny = Distinct Function?

Layered hematopoiesis has fundamental implications: Developmentally distinct immune cells might exert discrete, non-overlapping functions, at least within defined stages of development or non-homeostatic conditions, as we have previously discussed (Geissmann and Mass, 2015; Schultze et al., 2019). This is true for pancreatic and lung cancer, in which macrophages derived from fetal progenitors and monocytes have distinct tumor-promoting and anti-tumor roles (Zhu et al., 2017; Loyher et al., 2018). Particularly the continuous development of new tools to efficiently target macrophages of distinct origins will pave the way to understanding complementarity functions of developmentally distinct macrophages. This has been shown in a stroke model, where activation and proliferation of EMP-derived microglia rely on a transient influx of monocyte-derived macrophages, which thereby conjointly control the regeneration of neuronal tissue (Werner et al., 2020).

Their developmental pattern also renders layered lineages particularly vulnerable to early genetic and environmental perturbations, which might translate into long-lasting or even permanent effects on later-life health and disease. While this might also apply to transient and adult-type HSC, as recently discussed (Apostol et al., 2020), we here focus on HSC-independent hematopoiesis, i.e., EMP and their effector cell progeny, because their developmental trajectories are increasingly well understood in health, and evidence is mounting that disruption of their normal blueprint mediates adult disease.

EMP–From Transient Fetal Hematopoiesis to Lifelong Immune Landscapes

Although this three-wave model is widely accepted and experimental data are usually interpreted within the framework of its nomenclature, it likely represents an oversimplification of fetal hematopoiesis: Different progenitors share expression of common surface markers (e.g., CD45, Csf1r, CD41, Kit, CD16/32) and their production partially overlaps in space and time. Therefore, at present, no single fate-mapping model can unequivocally pinpoint distinct pre-HSC waves with precision, and conclusions on the origins of immune cells should be drawn with caution and from several *in vivo* models in a complementary manner. Negligence to do so-i.e., (over)interpretation of *in vivo* data from single fate-mapping models, intermingling with *in vitro* assays that do not necessarily reflect *in vivo* lineage output, and not accounting for developmental events during embryogenesis-has stirred an ongoing debate about

the exact nature and identity of hematopoietic progenitors, their emergence and their contribution to distinct cell types (Perdiguero et al., 2015; Sheng et al., 2015; Ginhoux and Guilliams, 2016; Perdiguero and Geissmann, 2016; Palis, 2017).

One lingering controversy is the question whether EMP also generate lymphocytes. It has long been appreciated from *ex vivo* and progenitor transplantation assays that lymphoid potential precedes the onset of adult-type HSC-dependent hematopoiesis and that certain innate(-like) lymphocytes even remain of fetal origin throughout life (Hayakawa et al., 1985; Liu and Auerbach, 1991; Payer et al., 1991; Huang et al., 1994; Godin et al., 1995; Yokota et al., 2006; Yoshimoto et al., 2011; Lin et al., 2014). A series of recent fate-mapping studies have corroborated this notion *in vivo* with the demonstration that several lymphoid lineages such as dendritic epidermal T cells (DETC), lymphoid tissue inducer (LTi), and natural killer (NK) cells first emerge independently of adult-type HSC (Gentek et al., 2018b; Dege et al., 2020; Simic et al., 2020).

In trying to further pinpoint the exact sources of the first lymphocytes, the concept of YS-derived lympho-myeloid-restricted progenitors (LMP) has been put forward (Böiers et al., 2013), and LMP have been proposed to be the first progenitors seeding the developing thymus (Luis et al., 2016). However, the LMP denotation is in part based on co-expression of genes associates with both, myeloid and lymphoid lineages (Böiers et al., 2013; Zhu et al., 2020), which does not necessarily equal differentiation into these lineages. Indeed, this notion has recently been challenged with the demonstration that YS progenitors, despite transient expression of lymphoid-associated transcripts (*Il7r*, *Rag2*, *Rag1*), do not generate lymphocytes *in vivo* (Elsaid et al., 2020).

The LMP controversy illustrates how difficult it often remains to assign the origin of fetal-derived immune cells to specific progenitors, even with genetic fate mapping. The models used often rely solely on the temporal distinction of waves, the resolution of which is insufficient for progenitors produced between E8.5 and E10.5. Whilst DETC and fetal LTi are likely not EMP-derived based on the absence of labeling in several models (Gentek et al., 2018b; Elsaid et al., 2020; Simic et al., 2020), it has recently been suggested that the first fetal NK cells originate at least in part from EMP (Dege et al., 2020). However, this was assessed in a single model with relatively low labeling induced at time points for which the contribution of other progenitors cannot be excluded (Dege et al., 2020). Thus, the true physiological contribution of EMP to the first lymphocytes remains to be determined *in vivo* using additional, complementary approaches.

When Is an EMP and EMP?

Possibly the most heavily debated controversy concerns the precise origins of fetal-derived immune cells found in adult organs, in particular tissue-resident macrophages. While many scientists might dismiss this as a pure developmental biologists' or even semantics problem, we believe it is essential to precisely dissect the ontogeny of those immune cells that remain lifelong within tissues, where they undergo continuous genetic and epigenetic changes that may eventually influence or perturb organ homeostasis. In an attempt to delineate different progenitor waves, the nomenclature of "early" and "late" EMP (or EMP1 and EMP2) has been introduced, which proposes that early EMP (produced at E7.5) belong to the primitive wave, while late EMP (starting at E8.5) express the transcription factor c-Myb and thus originate from the second, definitive wave of hematopoiesis (Hoeffel et al., 2015; Ginhoux and Guilliams, 2016). Because of these discrepancies in the definition of pre-HSC waves and the tools used to target them, different groups consider e.g., microglia, the brain-resident macrophages, derived either purely from the primitive (Alliot et al., 1999; Ginhoux et al., 2010; Hoeffel et al., 2015) and/or from the second wave (Kierdorf et al., 2013; Gomez Perdiguero et al., 2015; De et al., 2018). The concept of early and late EMP has also been applied to mast cells, and it has been speculated that their mast cell progeny differs in their longevity (Li et al., 2018). However, this has not been experimentally addressed, and could simply reflect responses to environmental differences encountered by progenitors recruited at distinct time points.

Indeed, it stands to reason that the strict segregation of early and late EMP based on timing (i.e., the time point of label induction in inducible fate-mapping models) and c-Myb expression is rather artificial and does not reflect the real-life scenario. Rather, early and late EMP might represent two extremes of the same wave, "captured" by different labeling approaches. We propose that EMP represent a continuous product of the YS hemogenic endothelium generated between E7.5 and E10.5, which intrinsically have the same differentiation potential, irrespective of their time of emergence. This is supported by clonogenic assays for EMP obtained from the YS at these different time points and fetal liver EMP at E12.5 (Gomez Perdiguero et al., 2015; Dege et al., 2020). *In vivo*, EMP heterogeneity and their actual lineage output would be dictated by stage-specific signals co-opting cell-autonomous transcriptional networks to meet the current demands for immune cells. Such a demand-driven model could easily be reconciled with existing experimental data: Macrophages are considered integral to organogenesis and thus, need to colonize tissues as they develop. At E7.5, YS EMP therefore primarily give rise to microglia, while at E10.5, they mainly produce macrophages for tissues that develop later during embryogenesis, e.g., the lung (**Figure 1**). Although their functions during development remain largely enigmatic, mast cells might contribute to fine-tuning of the nerve and vasculature networks, at least in the cornea (Liu et al., 2015). Rather than at the onset of organ development, they might thus be required only at later stages, in line with EMP starting to produce mast cell-committed progenitors at E12.5.

To respond to these demands, EMP need to produce committed progenitors that can invade tissues via the circulation. This can occur via different routes, some of which involve migration to the fetal liver (Stremmel et al., 2018), where they provide a hematopoietic reservoir at least until E16.5. The subsequent dynamics of EMP in the fetal liver and other hematopoietic organs, as well as their various differentiation trajectories that contribute to the layered immune system in each organ remain largely elusive, but their reconstruction will be

feasible with the advent of single-cell technologies in combination with novel fate-mapping models.

Developmental Programming of EMP-Derived Cells by Adverse Early Life Environments

It is now firmly established that the likelihood of developing a non-communicable disease in adulthood is strongly influenced by environmental factors in early life, including the fetal period (Barker, 2004). This concept is known as DOHaD and is deeply rooted in epidemiological studies, which have since been backed up by experimental data. Pioneering work focused on the consequences of maternal malnutrition on offspring health and found correlations with obesity, cardiovascular disease, hypertension, and diabetes (Roseboom et al., 2001; Barker et al., 2009; Ravelli et al., 2010). However, similar phenomena have since been observed for a wide range of adversities and (often chronic) pathologies, ranging from other dietary and lifestyle factors (e.g., maternal obesity, smoking), maternal disease and infection (such as maternal allergy), exposure to environmental pollutants (e.g., diesel exhaust, endocrine-disrupting chemicals) and psycho-social stress, which collectively increase the susceptibility to chronic inflammatory, atopic, auto-immune and neurological disease as well as cancer. A dysregulated immune response is common to all these pathologies.

Whilst similar considerations apply to environmental perturbations in the perinatal period, which is arguably important for shaping the immune system in response to microbial exposures and colonization, fetal development normally occurs in a tightly controlled intra-uterine environment and thus, represents a critical window of vulnerability. As outlined above, the fetal period also overlaps with key events of immune development, strongly suggesting that environmental insults experienced *in utero* impact later-life health and disease by programming offspring immunity.

EMP-Derived Cells as Mediators of DOHaD

At least three requirements must be met for environmental perturbations during fetal development to have long-lasting effects that can persist into adulthood: Signals must (1) be conveyed to the developing fetus, either directly by crossing the placental barrier, or by eliciting an inflammatory response at the fetal-maternal interface. Such signals must (2) be sensed by physiological systems equipped to respond to environmental stimuli, and (3) these systems must persist and undergo long-lasting imprinting or programming. This applies to the immune system (Palmer, 2012; Marques et al., 2013; Balistreri et al., 2019), and in particular to long-lived fetal-derived cells such as macrophages and mast cells, making them prime candidate mediators of long-term adverse effects (**Figure 2**).

Programming of these immune cells might act on different levels and manifest in several ways that are non-mutually exclusive. Adverse environments could directly act on individual cells, modulating their effector functions, causing e.g., a long-lasting shift in the balance between type I and II immunity (Veru et al., 2014). Adverse conditions during development could also affect the cellular longevity and self-maintenance capacity of individual cells, thereby ultimately modifying the composition or cellular dynamics of adult immune cell populations, which might result e.g., in higher-than-normal dependence on recruitment of BM progenitors. Functional perturbations might also result from impaired or altered crosstalk of programmed immune cells with each other and the non-hematopoietic stroma within their niches (Hsu et al., 2014; Guilliams and Scott, 2017; Naik et al., 2018; Zhou et al., 2018; Chakarov et al., 2019). Finally, in addition to local effects within their tissues of residence, developmentally programmed immune cells might also impact pathologies systemically or at other sites, for example, via long-range inter-organ trafficking, a phenomenon appreciated in recent years for infectious and inflammatory conditions (Huang et al., 2018; Leyva-Castillo et al., 2019). Thus, programming of fetal EMP-derived immune cells might result in increased susceptibility to a range of pathologies, examples of which we will discuss in the following.

Neurological Disorders

The DOHaD have been described half a century ago, directly linking prenatal nutrition to mental disorders (Zamenhof et al., 1966; Weinberger, 1987). Before the fetal origins hypothesis, the fetus was rather seen as a "perfect parasite"-absorbing what it needed but protected from nutritional damage inflicted on the mother (Susser and Stein, 2009). A major change in society was achieved in 1968 after results of a conference on early brain development and cognition were featured in newspapers stating that early nutritional deprivation causes irreparable damage to the brain and consequently to cognition (Scrimshaw et al., 1968). Since then, scientists aimed at understanding the mechanisms that can influence the developmental programming of the brain using animal models. By now, the research focus has switched from studying maternal undernutrition (e.g., human subjects born during the Dutch hunger winter) to maternal overnutrition, with nowadays ∼39% of women being overweight or obese, as well as pollution and maternal immune activation (MIA) models, with poly(I:C) or lipopolysaccharide (LPS) representing viral and bacterial infection, respectively. Intriguingly, maternal obesity in humans has been associated with cognitive impairment, learning disability, anxiety, and attention deficit hyperactivity disorder (Hatanaka et al., 2017). Similarly, in rodent models maternal obesity leads to marked changes in anxiety, learning behavior and memory in the offspring (Cordner and Tamashiro, 2015).

In light of the regulation of gene expression by metabolites and MIA, epigenetic regulation of gene expression is currently the proposed mechanism of adverse intergenerational effects (Nardone and Elliott, 2016; Contu and Hawkes, 2017). However, the identity of the cell types undergoing adaptations of epigenetic marks upon maternal obesity and their long-term functional consequences for brain development and function are not fully defined.

In the healthy developing brain, microglia interact with neuronal progenitors and regulate their cell numbers (Cunningham et al., 2013). They are key modulators of angiogenesis (Fantin et al., 2010), contribute to myelinogenesis

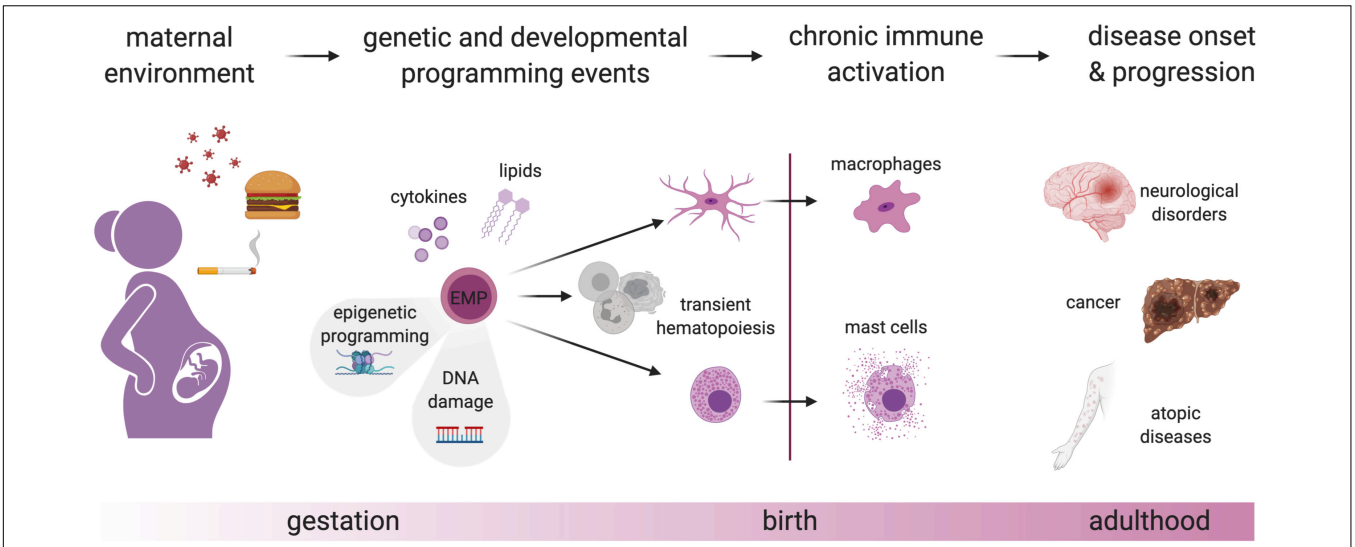

FIGURE 2 | Genetic and developmental programming of fetal-derived immune cells. Perturbation of the maternal environment e.g., by nutrition, smoking, and infection, leads to the production of mediators such as cytokines and lipids that will lead to epigenetic alterations in EMP and thereby, chronic activation of EMP-derived immune cells after birth. This switch from a homeostatic to a pro-inflammatory state due to developmental programming may cause or contribute to different diseases. In addition to naturally occurring somatic mutations in proliferating EMP and their progeny, maternal-derived hazardous materials may lead to DNA damage, which could eventually lead to a change in transcriptional programs and chronic activation of tissue-resident immune cells. Created with BioRender.com.

(Wlodarczyk et al., 2017) and oligodendrogenesis (Shigemoto-Mogami et al., 2014), and control synaptic development and plasticity (Paolicelli et al., 2011; Schafer et al., 2012; Parkhurst et al., 2013; Miyamoto et al., 2016), and brain connectivity (Squarzoni et al., 2014). Microglia regulate brain development and function primarily through phagocytosis and paracrine signaling. Synapse elimination, also called synaptic pruning, is needed to remove the excess of synapses that has been established in the early postnatal stage. Synaptic pruning depends on complement proteins that tag inappropriate synaptic connections for phagocytosis by microglia. Dysregulation of the complement cascade leading to under- or over-pruning has been implicated in neurodevelopmental disorders (Presumey et al., 2017).

In the adult brain, microglia actively contribute to learning and memory by surveying and modulating multiple synaptic structures (Tremblay et al., 2010; Wang et al., 2020), controlling neuronal activity (Badimon et al., 2020), and clearing apoptotic neural progenitor cells in the dentate gyrus (Sierra et al., 2010). In contrast, excessive activation of microglia leads to changes in synaptic transmission (Pascual et al., 2012), enhanced phagocytotic activity, and can contribute to or even cause neurodegenerative diseases (Mass et al., 2017; Song and Colonna, 2018).

This overwhelming collection of the homeostatic functions of microglia, their longevity, and their ability to acquire epigenetic memory (Wendeln et al., 2018) makes them a prime candidate for the intergenerational transmission of persistent changes inflicted by the maternal environment causing neurodevelopmental and neurodegenerative pathologies. Past difficulties in proving this cause-consequence relationship on a transcriptional level may be due to the transient nature of perturbations in microglia development (Matcovitch-Natan et al., 2016), which nevertheless might have far-reaching implications for brain function across the lifespan, during which the body keeps accumulating adverse environmental challenges.

Allergy and Atopic Diseases

Atopic diseases are caused by IgE-mediated allergic reactions that represent exaggerated immune responses to otherwise harmless substances like pollen, dander, and certain types of food. Mast cells are the central mediators of these responses (Reber et al., 2012). Upon the first encounter of such allergens, IgE binds to its high-affinity receptor Fcer1 on mast cells, which get activated following subsequent allergen exposures, resulting in rapid release of a variety of very potent effector molecules such as histamine, serotonin, proteases, and lipid mediators. These cause symptoms of varying severity that range from sneezing, itch, wheeze, rashes, gastro-intestinal manifestations like vomiting and diarrhea to life-threatening anaphylaxis. Depending on the nature of the allergen and exposure route, atopic disease primarily affects the skin and airways, presenting as atopic dermatitis, allergic rhinitis, or asthma. Their overall prevalence has dramatically increased and continues to rise (Asher et al., 2006; Anandan et al., 2010; Pedersen et al., 2011; Backman et al., 2017).

It has previously been recognized that allergic sensitization might occur prenatally (Piccinni et al., 1993; Kihlström et al., 2003; Boyle and Tang, 2006), and a family history of atopy is a well-established risk factor (Tariq et al., 1998; Sugiyama et al., 2007; Indinnimeo et al., 2016). This correlation is consistently higher for maternal compared to paternal atopy (Aberg, 1993; Litonjua et al., 1998; Bracken et al., 2002; Johnson et al., 2002; Liu et al., 2003; Wu et al., 2012), further implicating the maternal microenvironment. A recent study in mice provided

experimental evidence in support of this notion with the demonstration that fetal mast cells sensitized *in utero* by maternal IgE crossing the placental barrier mediate airway and skin inflammation upon postnatal re-exposure (Msallam et al., 2020). Given its potential to quickly amplify disease prevalence from one to the next generation, programming by maternal disease might have contributed to the dramatic surge in atopic disease observed within just a few decades.

Other early life risk factors implicated in atopic disease are air pollution (Hsu et al., 2015; Deng et al., 2016) and parental smoking (Martinez et al., 1992; Raherison et al., 2007), as well as maternal stress (Andersson et al., 2016; Chang et al., 2016; Magnus et al., 2018) and obesity (Reichman and Nepomnyaschy, 2008). For maternal obesity and smoking, the correlation appears to be stronger for offspring allergic asthma (Krämer et al., 2004; Raherison et al., 2007; Ekström et al., 2015), underscoring that differences may exist in the etiology and programming of distinct atopic disorders, which might be explained by locally restricted production of IgE (Coëffier et al., 2005; Takhar et al., 2007). The mechanisms by which these factors impact susceptibility to atopic disease and the cellular targets they are acting on are less well defined. However, mast cells are also involved in the more chronic stages of allergic inflammation (Galli and Tsai, 2012), a process that macrophages might also impact on (Zasłona et al., 2014). Of note, populations of fetal-derived macrophages exist in the skin (Kolter et al., 2019) and airways (Loyher et al., 2018; Liu et al., 2019). Exposure to a perturbed intra-uterine environment could thus render mast cells and macrophages pro-inflammatory by signals other than antigen-specific IgE, which might lower their threshold for activation or prevent them from returning to baseline. This could be mediated by an inflammatory response in the mothers, which represents a shared feature of the above-mentioned early life risk factors. As introduced, MIA and even more complex environmental perturbations like pollution and maternal obesity can be modeled *in vivo*. Combined with genetic tools, future work can thus determine if and how mast cells and macrophages are programmed into a chronic inflammatory state promoting atopic diseases. Although causality with atopic disease remains to be established, evidence is mounting that mast cells are indeed hyperactivated by stress experienced during development. This will be discussed in the following.

Irritable Bowel Syndrome

Early life stress is also a major risk factor for gastrointestinal diseases, including irritable bowel syndrome (IBS) (Talley et al., 1994; Bradford et al., 2012). IBS is a chronic disorder characterized by abdominal pain, diarrhea, bloating, and vomiting. Its etiology is incompletely understood, but deregulated mast cell activation is widely recognized as a key pathological event (Zhang et al., 2016; Boeckxstaens, 2018). In patients, the number of mast cells located in the proximity of enteric nerves is significantly increased, as is the frequency of their degranulation, features that correlate with the degree of abdominal pain (Park et al., 2003; Barbara et al., 2004). Furthermore, mast cell activation syndrome patients are often affected by similar gastrointestinal symptoms (Hsieh, 2018), and pharmacological targeting of mast cells using antagonists of receptors for histamine and serotonin or mast cell stabilizers has proven beneficial for some patients (Klooker et al., 2010; Zhang et al., 2016). Substantial evidence links early life stress, mast cell hyperactivation and susceptibility to IBS. In a porcine model, early weaning stress induces chronic diarrhea and intestinal permeability, indicative of IBS-like symptoms, which are accompanied by elevated numbers and baseline degranulation of intestinal mast cells are observed (Pohl et al., 2017).

Similarly, in mice, early life stress potentiates mast cell-glia and -neuron interactions in the myenteric plexus in a histamine-dependent manner (McClain et al., 2020). While mast cells normally interact with the enteric nervous system to regulate intestinal homeostasis, i.e., bowel movements, barrier defense and maintenance, inappropriate mast cell activation as observed following early life stress appears to sensitize enteric neurons and glia, resulting in chronic pain, altered gut permeability and motility (Barbara et al., 2007; McClain et al., 2020). Mechanistically, mast cell hyperactivation could be the consequence of epigenetic inactivation of CRF2 (corticotropin-releasing factor receptor subtype 2), which normally limits mast cell degranulation through inhibiting store-operated calcium signaling (Ayyadurai et al., 2017). Future studies should account for the particular vulnerability of (immune) development during the prenatal period and use genetic models to establish if hyperactivation of intestinal mast cells and potentiation of their neuronal interactions are causal.

Cancer

Macrophages and mast cells densely populate the stroma of most solid tumors, where they can have either tumor-promoting or -suppressive functions. At present, this complexity represents a major hurdle for the clinical exploitation of these cells to diagnostic, prognostic or therapeutic benefit. Their impact on tumor growth can be different within distinct cellular neighborhoods (Schürch et al., 2020) and thus, is at least in part determined by local microenvironmental signals. However, developmental characteristics might equally contribute. Sizeable populations of fetal-derived immune cells persist in most healthy adult tissues, and could thus be recruited to malignant lesions. Intriguingly, a recent study suggests that specific interactions between fetal-like macrophages and fetal-associated endothelial cells provide an immuno-suppressive environment promoting hepatocellular carcinoma (Sharma et al., 2020), a phenomenon that might be more globally true. Furthermore, fetal-derived macrophages originating from YS EMP appear to promote tumor progression and fibrosis in a murine model of pancreatic ductal adenocarcinoma, whereas monocyte-derived macrophages might be involved in anti-tumor immunity (Zhu et al., 2017). Of note, macrophages phenotypically resembling these developmentally distinct populations can also be identified in human tumors. Similarly, in murine lung carcinoma, fetal-derived interstitial macrophages co-exist with monocyte-derived ones, and promote tumor growth and spread, respectively (Loyher et al., 2018).

In addition to their ontogeny, environmental challenges experienced during development might impact the functions of tumor-associated macrophages and mast cells. Epidemiologically, the risk of developing cancer has been associated with "stressful"

adverse childhood experiences, such as trauma, maltreatment, or abuse, albeit inconsistently (Felitti et al., 1998; Fuller-Thomson and Brennenstuhl, 2009; Brown et al., 2010). This is in part attributable to the retrospective nature of these studies and self-reporting of stressful experiences. However, a prospective birth cohort study found a two-fold increased risk for cancer in individuals who experienced more than 2 stressful events in childhood, even when confounding factors were accounted for Kelly-Irving et al. (2013). In line with these findings, a recent study reported a higher melanoma burden in mice prenatally exposed to maternal stress (Hong et al., 2020). Whilst attributed to impaired T cell immunity, T cell recruitment, and activation are regulated by myeloid cells. EMP-derived mast cells persist in the adult (Gentek et al., 2018a; Li et al., 2018), and mast cells show signs of hyperactivation in *in vivo* models of maternal stress, as discussed above. Moreover, patients suffering from mast cell activation syndrome are also at a higher risk of developing solid tumors, including melanoma (Molderings et al., 2017). Programming of fetal-derived mast cells e.g., by prenatal stress might thus contribute to an overall melanoma-promoting environment.

Genetic Perturbations in EMP and Their Progeny

Histiocytosis

Histiocytoses are rare diseases characterized by aberrant expansion of histiocytes, which is a historical term describing macrophages or dendritic cells. Langerhans cell histiocytosis (LCH) is one example, which presents a remarkable diversity of phenotypes ranging from subtle skin lesions and mild neurological symptoms to life-threatening disseminated disease. Since the discovery of recurrent somatic mutations in the MAPK signaling pathway, particularly $BRAF^{V600E}$ (Badalian-Very et al., 2010; Satoh et al., 2012; Berres et al., 2014; Haroche et al., 2015; Diamond et al., 2016), known to be oncogenic in several human cancers (Davies et al., 2002), histiocytoses are now considered inflammatory myeloid neoplasms. Much like normal tissue-resident macrophages until recently, the mutant histiocytes were also thought to derive predominantly from BM progenitors. Yet, diverse phenotypes, particularly neurodegenerative and behavioral deficits that were retrospectively diagnosed in many patients (Cohen-Aubart et al., 2018; Héritier et al., 2018) as well as spontaneous regression of pediatric histiocytoses, are difficult to reconcile with mutated cells infiltrating from the BM, and hence, their causes remained a matter a debate.

Once the YS origin of adult microglia in mice was established (Ginhoux et al., 2010) and genetic models were available to target microglial progenitors, we introduced the $BRAF^{V600E}$ mutation into the EMP lineage (Mass et al., 2017) to address the possibility that the clinical outcome of histiocytosis is not dictated by mutation of distinct progenitors in the adult BM (Berres et al., 2014, 2015), but rather by affecting one of the fetal hematopoietic waves. Indeed, all mice born with mutated microglia (∼15% $BRAF^{V600E+}$) developed behavioral deficits and displayed chronically activated microglia, which eventually resulted in paralysis and neurodegeneration. In contrast, $BRAF^{V600E}$ expression in all $CD11c^{+}$ cells, i.e., dendritic cells and some tissue-resident macrophages, resulted in myeloid-cell tumors in the lung and spleen while targeting the whole hematopoietic system caused a leukemic phenotype and prenatal lethality (Mass et al., 2017). Due to the intentionally low targeting efficiency of EMP using the $Csf1r^{MeriCreMer}$ model, we did not observe a large population of BRAF-mutated macrophages in other tissues. However, higher tamoxifen doses or different fate-mapping models may allow characterization of other organs, e.g., the bone or liver, where EMP-derived macrophages may play a causative role in the development of histiocytic lesions.

Mastocytosis

Mastocytosis is an umbrella term for a heterogeneous group of rare disorders characterized by aberrant clonal expansion of mast cells, for which curative treatments are not available. These disorders are classified as myeloproliferative neoplasms and are often benign, but can also become malignant or associated with additional hematological malignancies (Valent et al., 2017). Symptoms caused by the release of bioactive mast cell mediators include skin reactions such as itching, gastrointestinal complications like nausea and diarrhea, as well as bone, joint and muscle pain, fatigue, and an increased risk of anaphylaxis.

Different forms of mastocytosis are distinguished according to the affected sites, disease onset, and clinical course. Reflecting the high abundance of dermal mast cells in health, mastocytosis usually involves the skin. In cutaneous mastocytosis, mast cell expansion is restricted to the skin, whereas systemic forms also manifest at additional sites such as the BM and internal organs like the liver, spleen, lymph nodes, and the gastrointestinal tract. Pediatric disease is predominantly of the cutaneous type, generally follows a milder course and usually regresses by adolescence. Adult onset mastocytosis, on the other hand, is frequently systemic and does not normally regress. The majority of adult patients present with stable, indolent disease and symptoms ranging from mild to moderate, however, adult mastocytosis can also progress into aggressive systemic disease or mast cell leukemia (Georgin-Lavialle et al., 2013; Berezowska et al., 2014), both of which have more severe symptoms and dismal prognosis.

Despite substantial progress in understanding the etiology of mastocytosis on a cellular and molecular level, the biological differences underlying the remarkably distinct clinical features of pediatric and adult mastocytosis remain enigmatic. Mastocytosis is caused by mutations in *KIT*, the tyrosine kinase receptor for stem cell factor, on which mast cell development and maintenance depends. With very few exceptions (Beghini et al., 2001; Akin et al., 2004; Tang et al., 2004; Hartmann et al., 2005; Zhang et al., 2006; Wasag et al., 2011; Speight et al., 2013), these mutations are somatic and activating in nature, resulting in constitutive or ligand-independent KIT signaling, enabling deregulated mast cell expansion. Such *KIT* mutations have been identified in aberrant mast cells of virtually all adult- and the majority of pediatric-onset mastocytosis patients (Frieri and Quershi, 2013). The most common mutation is a substituting point mutation in codon 186 (D186V), which has been reported in about 80% of adult patients. Although

this particular mutation is less abundant in pediatric patients, it is still found in almost 40% of cases, and of the remainder, another approximately 40% carry activating mutations in other regions of the *Kit* gene (Bodemer et al., 2010; Frieri and Quershi, 2013). Auto-activating Kit mutations are thus a shared feature of pediatric and adult mastocytosis. While different types of mutations may be of prognostic relevance, they unlikely account for the striking clinical differences between the transient pediatric and chronic adult forms. Rather, different cells of origin (i.e., EMP- vs. HSC-derived) might be underlying the distinct clinical entities, paralleling the considerations for histiocytosis.

Intriguingly, spontaneous regression of pediatric and persistence of adult-onset mastocytosis bear close resemblance to the kinetics of normal mast cell development. In mice, YS EMP-derived mast cells are gradually diluted and, in most tissues, ultimately largely replaced by adult-type HSC-derived ones (Gentek et al., 2018a; Li et al., 2018). This change occurs in the first weeks of life, corresponding to puberty, which also marks the age at which pediatric (usually < 15 years) and adult-onset (>15 years) are clinically distinguished, as well as the time regression is observed in pediatric-onset patients. Thus, it is tempting to speculate that pediatric and adult mastocytosis have different cells of origin, namely YS-derived EMP and HSC. In support of this notion, activating *KIT* mutations have been identified in HSC and more committed downstream progenitors in the BM and peripheral blood of adult patients with systemic disease (Afonja et al., 1998; Jara-Acevedo et al., 2015; Jawhar et al., 2015; Grootens et al., 2019), but not pediatric patients. While this hypothesis awaits experimental confirmation, such a scenario would be reminiscent of activating *BRAF* mutations that have different pathological consequences depending on the progenitor they are affecting.

Immune Cell Mosaicism in Cancer

Malignant transformation is a multi-hit process that requires cooperation between mutations in oncogenes and tumor suppressor genes within one cell. However, in addition to these cell-autonomous events, tumor growth further depends on interactions between malignant cells and their microenvironment (Hanahan and Weinberg, 2011). The so-called tumor stroma is densely populated by immune cells, which can have tumor-promoting or -suppressive functions that appear to be determined locally within their cellular neighborhoods (Schürch et al., 2020). Intriguingly, a recent study suggests that specific interactions between fetal-like macrophages and fetal-associated endothelial cells provide an immuno-suppressive environment promoting hepatocellular carcinoma (Sharma et al., 2020). However, although sizeable populations of fetal-derived immune cells persist in most healthy adult tissues, whether their mutation is causally involved in the emergence of solid tumors has not been addressed.

Here, we discuss a possible cooperative mechanism between tumor and EMP-derived cells that we term **intercellular complementation** and that would allow pre-malignant cells to evade immune surveillance. In genetics, complementation typically describes the combination of two genomes containing distinct recessive mutations that results in a mutant phenotype. In our case, neoplastic mutation of one cell type would be complemented by mutation of a neighboring tissue-resident immune cell, and only collectively would these mutations promote tumor growth. Such phenomena have been described in the fruit fly, where tumorigenesis is initiated by cooperating oncogenic mutations in Ras and Notch affecting neighboring epithelial cells (Brumby and Richardson, 2003), whilst mutations in Ras and genes affecting cell polarity cooperate to confer metastatic behavior (Pagliarini and Xu, 2003). The unrestricted growth of "winner" over "loser" epithelial cells is also termed cell competition, and applies not only to malignant settings, but also represents a well-known mechanism in developing tissues. Conversely, more recent work implicated cell competition in restraining clonal outgrowth of super-fit (pre-malignant) clones in tissues with high mutational burden (Bowling et al., 2019). This concept stems from deep sequencing work revealing a vast degree of mutational diversity in tissues from aged, healthy/non-diseased humans such as the skin (Martincorena et al., 2015), esophagus (Martincorena et al., 2018), and endometrium (Anglesio et al., 2017). Since every proliferating cell, including long-lived tissue-resident immune cells, accumulates a high number of somatic mutations throughout its lifetime, we hypothesize that immune cell mosaicism may play not only a contributing but causative role in cancer development and progression.

Intercellular Complementation of Mast Cells in Neurofibroma

Neurofibromatosis type 1 (NF-1) is a common genetic disorder caused by loss-of-function mutations in the *NF1* tumor suppressor gene, which encodes neurofibromin, a GTPase activating protein negatively regulating the activity of the proto-oncogene Ras. These mutations can arise spontaneously, though often are congenital. Patients frequently develop plexiform neurofibroma derived from Schwann cells, which are benign, but difficult to resect. Despite being a genetic disease, symptoms, clinical course, and severity are highly variable. This is at least in part explained by the fact that loss of heterozygosity (LOH) for *NF1* in Schwann cells alone is not sufficient to induce neurofibromas (Zhu et al., 2002). Strikingly, heterozygosity for Nf1 in mast cells elicits tumor formation in mice with biallelic loss of Nf1 in Schwann cells (Yang et al., 2008). Mechanistically, Nf1 heterozygosity appears to render mast cells more sensitive to Kit ligand, which attracts them to peripheral nerves and likely regulates their expansion and/or survival within the growing tumor. Consequently, pharmacological inhibition of Kit signaling inhibits tumor formation and attenuates tumor growth.

Although the tumor-promoting role for MC in neurofibroma has been demonstrated using BM transplantation into adult recipient mice, it is important to note that the onset for tumor development often is in childhood, and this is particularly true for plexiform neurofibroma (Ferner et al., 2007). It is therefore tempting to speculate that LOH in Schwann cells and mutations in EMP-derived mast cells complement one another to facilitate tumor growth. Since they are likely to experience less selection pressure than neoplastic Schwann cells, mast cells might be less prone to developing drug resistance and thus, represent the better

therapeutic targets. It will be important to address if mutations in mast cells complement neoplastic cells also in other tumors with mast cell infiltrates, and whether this in part explains the conflicting results implicating them as either beneficial (Biswas et al., 2014; Siiskonen et al., 2015) or detrimental (Tóth-Jakatics et al., 2000; Ribatti et al., 2003a,b).

Intercellular Complementation of Tissue-Resident Macrophages

Similar to mast cells, also macrophages seem to participate in intercellular complementation. This is the case for example in a sporadic colorectal tumor model where reciprocal BM chimera studies indicate that tumor-associated macrophages (TAMs) with a constitutively active cytoplasmic hematopoietic cell kinase promote tumorigenesis (Poh et al., 2017). However, these macrophages derive from adult BM monocytes and are typically recruited to pre-existing malignant lesions, indicating that genetic mosaicism of TAMs is not the main driver of the disease. To our knowledge, there is so far no single study demonstrating the initial cellular interaction of fetal-derived tissue-resident macrophages and a pre-malignant cell resulting in tumor development. While following the early stages of malignant transformation may be experimentally challenging in mice, *Drosophila* is a powerful tool to manipulate different cell types genetically and in a mosaic fashion, and there are some parallels to mammals concerning macrophage development (Gold and Brückner, 2015). Here, a screen could be set up, e.g., by introducing Ras^{V12} or other oncogenes into hemocytes, the tissue-resident macrophages of the fruit fly, while using RNAi or overexpression in other cell types such as endothelial cells or neurons via mosaic analysis with a repressible cell marker (MARCM) to characterize the combination of two distinct genetic alterations that result in tumorigenesis.

In more general terms, the contribution of EMP-derived macrophages to the pre-metastatic niche can be studied in mice by genetically manipulating these cells using common fate-mapping drivers such as $Csf1r^{MeriCreMer}$ or $Cx3cr1^{CreERT}$ models (Mass, 2018) in combination with metastatic cell lines. Since the liver is a common site for metastatic disease, Kupffer cells, as the resident macrophage population lining the hepatic sinusoids, are prime candidates to prevent or promote tumor metastasis. Kupffer cells are scavengers that phagocytose and eliminate circulating dead and dying cells, commensal bacteria, and other waste products that pass through the liver sinusoids. Thus, a homeostatic Kupffer cell is the first line of defense against incoming metastatic tumor cells (Bayon et al., 1996; Keirsse et al., 2018). In contrast, perturbance of its homeostatic function e.g., diminished phagocytic activity via depletion of Dectin-2 (Kimura et al., 2016) or persistent immune activation enhances cancer cell metastasis (Keirsse et al., 2018). In summary, it is becoming increasingly evident that EMP-derived macrophages are not just bystanders reacting to inflammatory events in their tissue of residence, but that they are active modulators of adult pathophysiology.

Is Neurodegeneration a Kind of Cancer?

As described above, a $BRAF^{V600E}$ mutation in the EMP lineage results in mutant microglia, thereby causing neurodegeneration (Mass et al., 2017). Similarly, another oncogene expressed in microglia-Ras^{V12} - is sufficient to activate microglia and lead to photoreceptor degeneration (Moriuchi et al., 2020). In contrast, microglial deletion of tumor suppressor genes such as Transforming growth factor-β activated kinase 1 (TAK1) (Goldmann et al., 2013) or p53 (Su et al., 2014; Aloi et al., 2015) is neuroprotective, suggesting that the underlying functional dichotomy of cancer genes in microglia may represent a mechanism that drives neurodegeneration in a non-cell-autonomous manner. It is therefore conceivable that other mutations accumulating during aging that are usually found in malignant cells will lead to chronic activation of microglia resulting in increased cell proliferation, cytokine expression, and phagocytosis. Since microglia are now considered to be a major genetic risk factor in many age-related neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Bartels et al., 2020) it may well be that certain forms of these diseases are indeed due to "cancerous microglia"-a hypothesis that can likely be tested via deep sequencing of cell nuclei to detect mosaicism (Lee et al., 2018) in the near future. Encouragingly, albeit that the cellular target remains unknown in humans, there are ongoing phase 1–3 clinical trials using cancer kinase inhibitors to treat Alzheimer's Disease (Fagiani et al., 2020).

Open Questions and Future Directions

One overarching question remaining is why the animal kingdom relies on consecutive hematopoietic waves, with only the adult-type HSC remaining active later throughout life. We believe that evolution has selected for this layering to supply immune cells that meet the stage-specific demands of developing tissues.

Why Are Certain Immune Cells Needed Early?

At earlier stages, rather than providing protective immunity, these demands are likely homeostatic in nature. Indisputably, oxygen supply by YS-derived red blood cells is needed as soon as the heart starts beating, a time point at which the embryo proper is developmentally likely not equipped to give rise to HSC. This may be explained by the need for a state of physiological hypoxia in embryos, which is important for the proliferation and survival of hematopoietic precursors (Simon and Keith, 2008). Similarly, macrophages seem indispensable throughout early embryogenesis owing to their contribution to angiogenesis (Fantin et al., 2010), neurogenesis (Cunningham et al., 2013), BM formation (Jacome-Galarza et al., 2019), and many other developmental processes that we are just now beginning to understand. They are phylogenetically the oldest immune cell type, originally discovered by Ilja Metchnikoff in starfish larvae (Metchnikoff, 1905). Macrophage-like hemocytes are the only immune cells also in other invertebrates such as *Drosophila*, where they have prominent roles in development and immunity (Parsons and Foley, 2016), similar to tissue-resident macrophages in mice. For mast cells, the reasons for their production before the onset of HSC hematopoiesis are currently less clear. In both mice and humans, they colonize embryonic tissues with some delay compared to macrophages, indicating they might not contribute to the initial steps of organogenesis, but rather, organ maturation. Indeed, mast cells appear to regulate corneal

nerve and vasculature as well as mammary gland branching (Lilla and Werb, 2010; Liu et al., 2015). Beyond our present focus on EMP-derived lineages, similar considerations also apply to other lineages with layered ontogeny, such as innate lymphocytes.

Protective Immunity and Immune Priming *in utero*?

In addition to developmental functions, fetal immune cells might protect from infections occurring during pregnancy or, following *in utero* priming, postnatal life. Infectious threats would arguably have to occur at frequencies high enough to impose a strong selection pressure for establishing immune cells this early. This might well be the case in nature and could explain why the developing fetus also invests energy in generating other, short-lived cell types such as granulocytes and NK cells, which will be replaced just a few days later.

At least longer-lived fetal immune cells might also undergo immune priming. Although it is the longstanding belief that the fetus is sterile, data is emerging that the human microbiome is seeded before birth and DNA of bacteria, fungi, and viruses has been detected in amniotic and meconium fluid (Stinson et al., 2019). These studies remain controversial due to contamination issues during sample acquirement and processing, but evidence is mounting that the fetal immune system can detect and respond to microbial compounds and other immune-stimulatory agents present at the fetal-maternal interface, regardless of their source. Sensitization of fetal mast cells by maternal IgE is in keeping with this (Msallam et al., 2020). While this promotes allergy postnatally, such intra-uterine immune priming does not necessarily have to be pathological, but could also be protective during later-life exposures.

Are Fetal-Derived Immune Cells Mediators of Lifelong Pathology?

Whatever their physiological functions, the presence of fetal-derived, proliferating immune cells in virtually all adult tissues makes the organism vulnerable to developmental programming events during gestation, as well as the accumulation of somatic mutations. These perturbations may shift their functions from homeostasis- to inflammation-promoting. Using mice as model organisms and applying environmental challenges such as MIA, maternal obesity, or smoking, we may be able to establish cause-consequence relationships between developmental programming of EMP-derived cells and pathophysiology in the offspring, which cannot be deduced from epidemiology. Combining such models with the ever-growing toolbox to target developmentally distinct immune cell populations or their hematopoietic progenitors will allow us to dissect their precise roles in adult disease onset and progression. Delineating the basic mechanisms shaping the functions of EMP-derived cells might ultimately inform if and how we could reverse their programming toward restoring homeostasis. Finally, such mechanistic studies in animals can now be complemented with deep sequencing efforts of single human cells, where somatic mutations in nuclear or mitochondrial DNA might serve as a readout for cellular origin, thus allowing us to study immune cell origin not only in patients, e.g., after transplantation, but also in healthy subjects.

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RG and EM wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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Physiological and Aberrant γ-Globin Transcription During Development

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The expression of the fetal Gγ- and Aγ-globin genes in normal development is confined to the fetal period, where two γ-globin chains assemble with two α-globin chains to form $\alpha_2\gamma_2$ tetramers (HbF). HbF sustains oxygen delivery to tissues until birth, when β-globin replaces γ-globin, leading to the formation of $\alpha_2\beta_2$ tetramers (HbA). However, in different benign and pathological conditions, HbF is expressed in adult cells, as it happens in the hereditary persistence of fetal hemoglobin, in anemias and in some leukemias. The molecular basis of γ-globin differential expression in the fetus and of its inappropriate activation in adult cells is largely unknown, although in recent years, a few transcription factors involved in this process have been identified. The recent discovery that fetal cells can persist to adulthood and contribute to disease raises the possibility that postnatal γ-globin expression could, in some cases, represent the signature of the fetal cellular origin.

Keywords: globin genes, transcription factors, hereditary persistence of fetal hemoglobin, juvenile myelomonocytic leukemia, erythropoiesis

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ERYTHROPOIESIS DURING DEVELOPMENT

During mammalian development, hematopoiesis is regulated both spatially and temporally: it begins in the yolk sac, it goes through a transitory phase in the fetal liver and then is definitively established in the thymus and bone marrow (Dzierzak and Bigas, 2018). The first erythroid precursors emerge from the yolk sac as soon as the embryo grows too big to be supplied with oxygen by diffusion and give rise to primitive erythroid cells (EryPs). These cells are released in the bloodstream when they are still nucleated and are characterized by the expression of embryonic globins. A second wave of cells migrating into the fetal liver from the yolk sac support fetal hematopoiesis until birth, in the interval between primitive and definitive hematopoietic stem cell (HSC)-dependent hematopoiesis. Of interest, recent studies suggest that in mouse, this second fetal transient hematopoietic wave of yolk sac-derived erythro-myeloid progenitors (EMPs) may persist postnatally (Epelman et al., 2014; Gomez Perdiguero et al., 2015). HSCs arise from the hemogenic endothelium of the embryonic aorta-gonad-mesonephros (AGM), the vitelline and umbilical arteries, and from the placenta (Dzierzak and Philipsen, 2013; Lacaud and Kouskoff, 2017; Dzierzak and Bigas, 2018). These cells migrate first into the FL and then to the bone marrow (BM)—their long-term adult resident location—where they will last throughout life and will generate all types of blood cells, including erythrocytes (Orkin and Zon, 2008; Palis, 2014; Dzierzak and Bigas, 2018).

The different types of erythroid cells produced at the different hematopoietic stages have many common characteristics, including the main steps of progressive differentiation and maturation

from early progenitors to erythroblasts and finally to red blood cells (RBCs) (Kina et al., 2000; Aisen, 2004; Chen et al., 2009; Baron, 2013; Palis, 2014). However, importantly, they can be in part distinguished by differences in cell morphology and in the expression of embryo/fetal vs. adult globins.

GLOBIN GENES

In humans, the α-globin cluster contains three functional genes: the embryonic, HBZ (ζ-globin) and the two fetal/adult HBA2 and HBA1 duplicated genes (α2- and α1-globin) (Stamatoyannopoulos, 2005). The β-globin cluster contains five active genes: the embryonic HBE (ε-globin) gene, the two highly homologous fetal HBG2 and HBG1 genes (Gγ- and Aγ-globin, respectively) and the two adult HBD and HBB genes (δ- and β-globin, the latter accounting for about 98% of adult β-like globin) (**Figure 1**). Each locus is under the control of a set of distal enhancers (Grosveld et al., 1987; Higgs et al., 1990). The genes contained in the α-globin and β-globin loci are sequentially expressed in a stage-specific manner that maintains the 1:1 ratio between the α-like and β-like globin chains, in a process known as "hemoglobin switching" (Forget, 1990; Stamatoyannopoulos, 2005; Sankaran et al., 2010a).

Interestingly, the presence of fetal-specific (γ) genes and thus of a fetal (γ) to adult (β) globin switch is unique to humans and old-world monkeys: most species, including mice, have only one switch, from embryo/fetal to definitive globin genes expression, occurring early in development (Stamatoyannopoulos, 1991; Sankaran et al., 2010a; Philipsen and Hardison, 2018; **Figure 1**). In mice transgenic for the human β-globin locus, the switching of human globin genes parallels the switching of mouse genes, with γ genes being switched off between E11.5 and E13.5, together with εγ and βh1 mouse embryo/fetal genes (Strouboulis et al., 1992; Peterson et al., 1995). Each developmental switch is accompanied by a profound chromatin remodeling within the loci: the interaction ("loop") between the promoter of the gene active at a given time with the common distal enhancers [locus control region (LCR)] is progressively favored, with inactive globin genes being looped out (Tolhuis et al., 2002; Palstra et al., 2003). Whereas only EryPs of yolk sac origin expresses embryonic ζ-, human ε- and mouse εγ-globin genes, the other globin genes are more promiscuously expressed by cells of different origin. McGrath et al. (2011) showed that the first cells expressing adult globins, prior to the generation of HSC-derived erythroblasts, are indeed the transient population of EMP-derived erythroid cells.

PHYSIOLOGICAL AND NON PHYSIOLOGICAL γ-GLOBIN EXPRESSION

The switching from γ- to β-globin expression is the most intensively studied because the persistence of γ-globin expression in adult stages is a hallmark of a very heterogeneous spectrum of conditions. These can be benign, as in the case of the few F cells (cells expressing HbF) found in normal adults (Boyer et al., 1975) and in HPFH (hereditary persistence of fetal hemoglobin) (Forget, 1998), or associated with disease, such as in response to transient or chronic anemias (Weatherall, 2001) or leukemias (Weatherall et al., 1968; Sheridan et al., 1976). As an additional reason of interest, the ability of reactivating γ-globin in the adult is considered as a possible strategy to cure β-hemoglobinopathies (Wienert et al., 2018). The cause of γ-globin expression in adult cells remains largely unknown and is thought to rely on different mechanisms, both maturational and/or directly related to defects intrinsic to the HBB locus (Zago et al., 1979; Stamatoyannopoulos, 2005). Although these aspects are strictly intertwined, and it is almost impossible to sharply separate them, this review will focus on the latter, in particular on the major transcription factors (TFs) that, by directly binding to the HBB locus, act as selective on/off switches of γ-globin expression in normal and aberrant conditions.

THE TRANSCRIPTIONAL SWITCHES OF γ-GLOBIN EXPRESSION

The observation that the main differentiation and maturation steps leading to RBC formation are common to YS-, EMP-, and HSC-derived erythroblasts (Kina et al., 2000; Aisen, 2004; Chen et al., 2009; Palis, 2014) suggests that these cells may also rely on a common set of transcription factors directing erythroid differentiation and globin gene regulation. Indeed, embryonic/fetal and adult cells share a common set of ubiquitous (such as NF-Y, that binds all globin promoters, although with different affinity (Liberati et al., 1998; Zhu et al., 2012; Martyn et al., 2017)) and erythroid-specific activators/coactivators [first of all GATA1 (Ferreira et al., 2005; Love et al., 2014; Katsumura et al., 2017; Barbarani et al., 2019), NFE2(Gasiorek and Blank, 2015; Kim et al., 2016), KLF1(Perkins et al., 2016), and TAL1 (Kang et al., 2015)]. Moreover, the expression of γ-globin genes in adult cells, as in HPFH, suggests that adult cells represent an environment permissive for the expression of both embryo/fetal and adult globin genes. It is thus likely that the specific timing of γ-globin expression might require specific activators/repressors acting at different times. The number of transcription factors directly involved in the activation/repression of γ-globin transcription is surprisingly small, and their main characteristics are briefly reviewed here below (**Figure 2**).

TRANSCRIPTION FACTORS AFFECTING THE EMBRYO/FETAL TO ADULT SWITCHING

γ-Globin Repressors

BCL11A, also known as CTIP1 (Coup-TFII interacting protein), is a C2H2-type zinc-finger protein (Avram et al., 2000). Alternative splicing generates four major protein isoforms, sharing a common N-terminus: eXtra-Long (XL), Long (L), Short (S), and eXtra-Short (XS) (Liu et al., 2006). BCL11A plays important roles in non-erythroid hematopoietic cells,

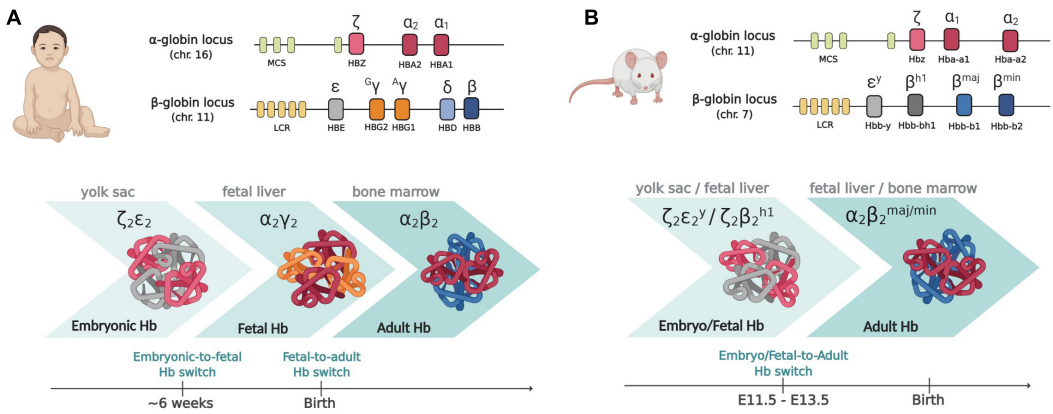

FIGURE 1 | The globin gene loci in humans **(A)** and mice **(B)**. Upper panels: the globin gene loci. MCS (multispecies conserved sequences) and LCR (locus control region) are the distal enhancers coordinating the developmental expression of the genes within the cluster. Lower panels: the different types of globin chains expressed at the different developmental stages and the corresponding sites of hematopoiesis are shown. Please note that whereas in humans two subsequent switching events take place, in mouse, as the majority of mammals, there is only one switch from embryo/fetal to adult genes, occurring early in development. Created with BioRender.com.

including B cells (Liu et al., 2003), dendritic cells (Ippolito et al., 2014), and hematopoietic stem cells (HSCs) (Tsang et al., 2015; Luc et al., 2016). Outside hematopoiesis, BCL11A is essential for central nervous system development (Basak et al., 2015; Funnell et al., 2015; Greig et al., 2016) and possibly for the differentiation of other lineages, such as breast (Khaled et al., 2015) and pancreas (Peiris et al., 2018) cells. Its specific role in γ-globin silencing was identified by genome-wide association studies (GWAS) aiming to identify eQTLs (expression quantitative trait loci) associated with high levels of postnatal HbF (Menzel et al., 2007; Lettre et al., 2008; Uda et al., 2008). Its conditional knockout within the erythroid compartment [obtained by disrupting the erythroid-specific BCL11A enhancer (Bauer et al., 2013; Canver et al., 2015)] impairs HbF silencing in adult erythroid cells, without altering erythropoiesis in the mouse (Sankaran et al., 2008, 2010b; Xu et al., 2013). This latter observation made BCL11A-targeted inactivation in erythroid cells a promising approach to reactivate γ-globin in β-hemoglobinopathies (Sankaran et al., 2010a; Wienert et al., 2018; Zeng et al., 2020). Interestingly, in human cells, S and XS isoforms are specific of YS primitive and FL erythropoiesis, whereas XL and L are specific of BM definitive erythropoiesis (Sankaran et al., 2009). Notably, the mouse *Bcl11A* pattern of expression is different, with the XL isoform being already present in FL definitive erythroid cells. This delay in BCL11A-XL in humans could explain the different timing of γ-globin switching in human vs. mouse (Sankaran et al., 2009). The specific γ-globin repression in adult cells is mediated by its binding to the consensus sequence GGTCA, present in several discrete sites within the β-locus (Liu et al., 2018). Among them, the site in the distal CCAAT box region of the γ-promoter, which contains the −117 residue, whose G > A mutation causes HPFH, indeed abolishes BCL11A-XL binding (Martyn et al., 2018).

BCL11A is activated by *KLF1/EKLF* (Borg et al., 2010; Zhou et al., 2010) (Kruppel-Like Factor-1), an erythroid-specific zinc finger TF originally identified because of its ability to bind to CACCC motifs (Miller and Bieker, 1993) and now recognized as a critical regulator of many aspects of erythropoiesis (Perkins et al., 2016). The KLF1 gene knockout in the mouse results in embryonic lethality at around stages E14–E15 due to lethal anemia because of the inability to activate β-globin (Nuez et al., 1995; Perkins et al., 1995) and mutations in the CACCC box of the β-globin promoter that abolish its binding causing thalassemia (Feng et al., 1994). Thus, KLF1 promotes the switching from γ- to β-globin gene expression both directly, by activating the β-globin promoter and indirectly, by activating BCL11A. Finally (Basak et al., 2020), recently demonstrated that the oncofetal protein LIN28B (Piskounova et al., 2011; Shyh-Chang et al., 2013), already known to increase γ-globin expression (Lee et al., 2013, 2015; de Vasconcellos et al., 2014), blocks BCL11A translation. The failure of the above regulatory circuits converging on BCL11A results in elevated HbF.

SOX6 is a HMG box transcription factor, characterized by the presence of a high-mobility group domain (HMG) (Wegner, 1999). SOX6 is expressed in several tissues, including cartilage, testis, neural cells, and erythroblasts (Hagiwara, 2011). Mice with a chromosomal inversion (p^{100H}) disrupting the *Sox6* gene, or carrying a targeted inactivation of Sox6 die perinatally, secondary to cardiac or skeletal myopathy (Hagiwara et al., 2000). *Sox6*-null mouse fetuses and pups are anemic and have defective RBCs (Dumitriu et al., 2006). In erythroid cells, SOX6 has indeed a dual role: it stimulates erythroid cell survival, proliferation, and terminal maturation during definitive murine erythropoiesis (Cantu et al., 2011), and it directly silences embryo/fetal globin genes (Yi et al., 2006; Xu et al., 2010). The first aspect is mediated by the activation of SOCS3, whose overexpression recapitulates the proliferation arrest imposed by SOX6 (Cantu et al., 2011); the second requires the direct binding and repression of the embryonic εγ-globin promoter and the cooperation with BCL11A, via direct physical interaction, to silence γ-globin in adult erythroid cells (Yi et al., 2006; Xu et al., 2010).

The DRED complex (direct repeat erythroid-definitive) is a 540-kDa complex containing the nuclear orphan receptors

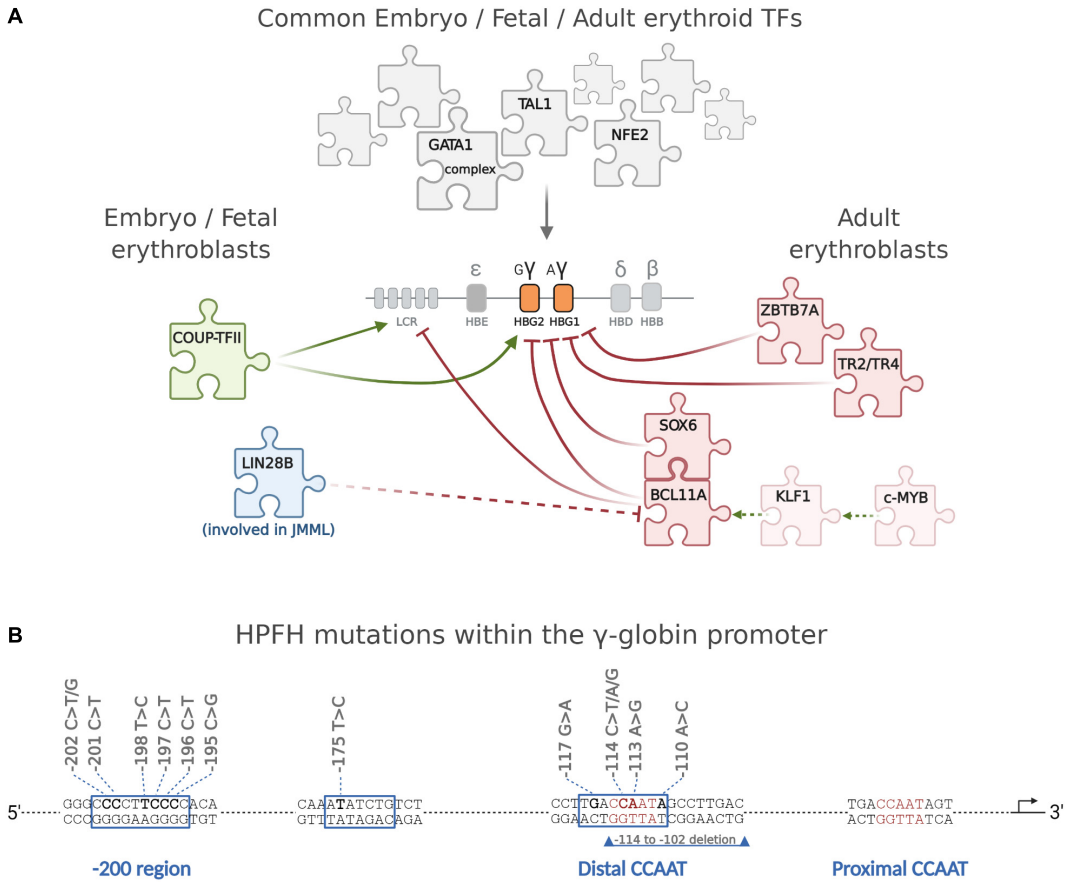

FIGURE 2 | The major transcription factors directly regulating the differential expression of γ-globin during development. **(A)** The expression of γ-globin in embryonic, fetal, and adult cells is regulated by a large common set of ubiquitous (such as, for example, NF-Y) and erythroid-specific transcription factors, the most important of which are GATA1 and its complexes (Love et al., 2014), NFE2, and TAL1. A very small number of TFs, discussed in the text, are instead directly involved in the time-specific expression of γ-globin and in its deregulation when it persists in the adult. c-MYB activates KLF1 that in turn activates BCL11A, which cooperates with SOX6 in repressing γ-globin. ZBTB7A and TR2/TR4 repress γ-globin independently from BCL11A. Of interest, whereas different γ-globin-specific repressors have been identified so far, little is known about early specific activation of embryo/fetal globin genes, and COUP-TFII is the only γ-globin-specific activator identified so far. The oncofetal LIN28B protein, expressed at high levels in JMML cells concomitantly expressing high γ-globin, blocks BCL11A translation. **(B)** Schematic representation of the γ-promoter showing the position of HPFH mutations. Created with BioRender.com.

TR2 and TR4, expressed in many tissues, including erythroid cells (Tanabe et al., 2002; Lin et al., 2017). TR2 and TR4 form homodimers or heterodimers binding to GGTCA repeat sequences with variable spacing, a consensus common to non-steroid nuclear receptors (Lee et al., 1998). The double conditional knockout of TR2 and TR4 in mouse erythroid cells results in increased embryonic εy and βh1 globins (Cui et al., 2015). In line with this result, the −117HPFH point mutation, associated with high HbF, reduces TR2/TR4 binding (Tanabe et al., 2002). Finally, TR2 and TR4 have been proposed directly repress GATA1 transcription, suggesting a wider role in erythroid maturation (Tanabe et al., 2007b).

ZBTB7A/LRF is a C2H2 zinc finger TF belonging to the POK (BTB/POZ and Krüppel) group of transcriptional regulators (Davies et al., 1999). ZBTB7A is expressed in various hematopoietic lineages (Maeda, 2016). However, its knockout shows a specific erythroid defect, with mouse embryos dying around E16.5 because of severe anemia, demonstrating that ZBTB7A is required for definitive erythropoiesis (Maeda et al., 2009). Adult-stage knockout of Zbtb7a results in erythropoietin-unresponsive macrocytic anemia, reversed by BIM knockout (Maeda et al., 2009). ZBTB7A specifically represses embryonic and fetal globin gene expression, independently from BCL11A, probably through the interaction with components of the nucleosome remodeling deacetylase (NuRD) complex (Masuda et al., 2016).

COUP-TFII, THE SELECTIVE ACTIVATOR OF γ-GLOBIN IN YOLK SAC-DERIVED CELLS

The COUP-TFII gene (Chicken Ovalbumin Upstream Promoter Transcriptional Factor II, also known as NR2F2/ARP1) encodes for an orphan nuclear receptor. Its expression is high in the mesenchymal component of developing organs

and overall decreases after the completion of organogenesis (Pereira et al., 1995; Lin et al., 2011). Its knockout results in early embryonic lethality (E9.5–E10) (Pereira et al., 1999) caused by defects in angiogenesis and heart development. In the erythroid lineage, Coup-TFII is expressed in the early embryo in the YS and in FL, and it declines around day E12.5 (Filipe et al., 1999; Cui et al., 2015; Fugazza et al., 2020). Although originally identified as ε- and γ-globin gene repressor on the basis of *in vitro* binding data, its functional role remained elusive (Ronchi et al., 1995; Liberati et al., 2001). Our group recently demonstrated that COUP-TFII is co-expressed with embryonic globin genes in cells of YS origin, where it acts as specific γ-globin activator by binding to the GGTCA motifs present within the β-locus (Fugazza et al., 2020). This last observation opens many questions since the same consensus is bound by BCL11A (Liu et al., 2018) and possibly by TR2/TR4 (Tanabe et al., 2007a) in adult cells. Importantly, COUP-TFII is able to activate γ-globin when overexpressed in adult cells, suggesting once again a very similar cellular environment of fetal vs. adult cells (Fugazza et al., 2020).

INAPPROPRIATE TIMING OF γ-GLOBIN EXPRESSION IN "ADULT-TYPE" CELLS: HEREDITARY PERSISTENCE OF FETAL HEMOGLOBIN

HPFH is a benign condition in which γ-globin remains expressed at high levels in adult life (Forget, 1998). HbF can be high in all red blood cells (pancellular HPFH) or restricted to a small subset of erythroid cells (heterocellular HPFH) (Thein et al., 2009). HPFHs, based on the different types of causative mutations, can be broadly divided in three main categories: deletional HPFH, non-deletional HPFH, and HPFH non-linked with the β-locus. Deletional HPFH is associated with the deletion of large regions of DNA between the γ- and β-globin genes within the β-globin locus, as it happens, for example, in the Sicilian ≈13 kb and in the Italian ≈40-kb deletions[1] (Kountouris et al., 2014). Many deletions include the loss of δ- and β-globin genes resulting in $(\delta\beta)^0$-thalessemia and HPFH (Ottolenghi et al., 1982). The molecular mechanism underlying the elevated HbF is complex and involves the concomitant deletion of the β promoter, which removes its competition with the γ promoters for the upstream LCR and for limiting TFs (Forget, 1998). Non-deletional HPFHs are caused by point mutations described in both γ-globin promoters. These mutations fall into three distinct clusters: the −200 region, the −175 site, and the distal CCAAT box region, around −115 (Forget, 1998; Martyn et al., 2018), where they either disrupt binding sites for γ-globin repressors or create *de novo* binding sites for γ-globin activators. For example, the −196 mutation abolishes the binding of ZBTB7A (Martyn et al., 2018), whereas mutations in the distal CCAAT box region impair BCL11A binding (Liu et al., 2018; Martyn et al., 2018). Instead, the −175T > C mutation creates a *de novo* binding site for the activator TAL1 (Wienert et al., 2015). Despite these evidences, the function of these sequences is more complex. The −198T > C mutation, for example, although being located within the cluster of HPFH mutations that impair the binding of ZBTB7A, is a gain-of-function mutation creating a binding site for the erythroid activator KLF1 (Wienert et al., 2017). The same is true for the mutation at position −113 A > G, which, although lying within the −115 region bound by the repressor BCL11A, creates a new binding site for the activator GATA1, without altering the BCL11A binding (Martyn et al., 2019).

[1] https://www.ithanet.eu/

THE ROLE OF MODIFIERS LOCI

Genome-wide association studies (GWAS) have revealed other two loci, not linked to the β-locus, consistently associated with HbF levels, and with β-globin disorder severity, across various ethnic backgrounds: a region on 2p (Menzel et al., 2007; Lettre et al., 2008; Uda et al., 2008) and the HBS1L-MYB intergenic region on 6q (Craig et al., 1996; Thein et al., 2007). The 2p region turned out to correspond to BCL11A, and the fine mapping of the single nucleotide polymorphisms associated to HbF within this region led to the identification of the intronic enhancer driving the expression of BCL11A in erythroid cells (Bauer et al., 2013; Canver et al., 2015).

The variants within the HBS1L-MYB intergenic region on 6q impair the binding of LDB1, GATA1, TAL1, and KLF1 to the enhancer controlling *c-MYB* expression (Stadhouders et al., 2014). c-Myb is the cellular homolog of *v-Myb*, the avian retroviral oncogene causing myelomas and lymphomas in birds (Wolff, 1996). Of the two major isoforms, isoform 2 (72 kDa) is the dominant one in human erythroid cells (Baker et al., 2010; Wang et al., 2018). In hematopoiesis, *c-MYB* is expressed in immature cells of all hematopoietic lineages (Wang et al., 2018); in erythropoiesis, it is required for the expansion of erythroid progenitors and must be downregulated to allow differentiation (Emambokus et al., 2003). c-Myb-null murine embryos are normal until E13.5, but by E15, they become severely anemic and die, suggesting that c-Myb is required for definitive erythropoiesis (Mucenski et al., 1991). The reduced c-MYB level has a twofold impact on globin genes. Low c-MYB levels, by accelerating the kinetics of erythroid differentiation, would favor the release of early erythroid progenitor cells still synthetizing HbF (Stamatoyannopoulos, 2005; Jiang et al., 2006). In addition, the reduced activation of KLF1 (Bianchi et al., 2010; Suzuki et al., 2013) by MYB would promote γ-globin expression by reducing BCL11A levels.

INAPPROPRIATE TIMING OF γ-GLOBIN EXPRESSION IN "FETAL-TYPE" CELLS PERSISTING AFTER BIRTH: THE CASE OF JUVENILE MYELOMONOCYTIC LEUKEMIA

High HbF levels are a hallmark of different leukemias (Sheridan et al., 1976). However, the expression of γ-globin in juvenile myelomonocytic leukemia (JMML) is peculiar. JMML is a rare and aggressive blood cancer of early childhood

(Loh, 2011; Niemeyer and Flotho, 2019). About 90% of the patients present hyperactivation of the RAS pathway, as a result of mutations in KRAS, NRAS, PTPN11, NF1, or CBL genes, and about 25% of the patients carry chromosome 7 monosomy (Flotho et al., 1999; de Vries et al., 2010). JMML is considered a stem cell disease (Inoue et al., 1987; Busque et al., 1995; Flotho et al., 1999; Cooper et al., 2000). Of interest, increased HbF levels and the presence of fetal red cell traits (Weinberg et al., 1990; de Vries et al., 2010; Helsmoortel et al., 2016a) are present in more than half of JMML patients. These evidences suggested a fetal origin for JMML, confirmed by the retrospective analysis of JMML patient samples collected at birth (Kratz et al., 2005; Matsuda et al., 2010; Stieglitz et al., 2015). However, yolk sac EMPs expressing gain of function PTPN11 mutations recapitulate part of the characteristics of JMML, but they are not able to cause disease in mice (Tarnawsky et al., 2017). Recently, gene expression profiling of JMML samples identified a subgroup characterized by high LIN28B expression and higher HbF levels (Helsmoortel et al., 2016b). LIN28B is an oncofetal protein (Shyh-Chang and Daley, 2013) that induces γ-globin expression (Lee et al., 2013, 2015; de Vasconcellos et al., 2014) and is highly expressed in fetal HSCs (Copley et al., 2013). Interestingly, LIN28B was shown to repress BCL11A-XL by blocking its translation (Basak et al., 2020). Thus, high levels of LIN28B decrease the amount of BCL11A protein, and this results in high HbF. This observation suggests a mechanistic link between LIN28B and high HbF observed in JMML. Whether the 50% of JMML with high HbF arose from a fetal EMP subpopulation expressing high LIN28B deserves further investigation.

CONCLUSION

The existence of γ-globin genes is specific to humans and old-world monkeys. Physiologically, γ-globin expression is confined to fetal life, tightly regulated by a complex network of transcription factors (activators and repressors) and co-regulators. Nevertheless, normal subjects present rare F cells, whose nature is still unclear (Rochette et al., 1994). During development, γ-globin genes are expressed in two different types of erythroblasts, with independent origin, the first originating from the transient, yolk sac-derived EMP population and the second from definitive HSCs (McGrath et al., 2011). These two cell types are very similar in their maturational pathway, and adult cells indeed represent a permissive environment for γ-globin expression, as shown by HPFH. Here, alterations in the few specific transcription factors that regulate γ-globin transcription (or in the sequences bound by them within the HBB locus) allow substantial γ-globin expression. Moreover, recent RNA-seq studies on A and F cells from the same healthy donors show that these cells do not significantly differ in the expression of known γ-globin regulators, suggesting that differences in the transcription of globin genes within the HBB locus itself account for the HbF trait (Khandros et al., 2020).

However, new evidence from the study of JMML unveil an alternative possible scenario, where γ-globin genes could be the marker of the fetal origin of these leukemic cells. The discovery that fetal progenitor-derived cells can persist to adulthood and contribute to disease raises the possibility that, especially in childhood malignancies, such as JMML, HbF can indeed be the signature of the fetal origin of cancer cells.

AUTHOR'S NOTE

While this minireview was in press, an article was published by Liu and colleagues (doi: 10.1038/s41588-021-00798-y) showing that BCL11A competes with NF-Y binding to initiate γ-globin repression at the CCAAT box region.

AUTHOR CONTRIBUTIONS

AR conceived and wrote the manuscript. GB and AL contributed with ideas and discussion. SS and AA contributed in the organization of the manuscript. All authors contributed to the article and approved the submitted version.

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BRIEF RESEARCH REPORT
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The Earliest T-Precursors in the Mouse Embryo Are Susceptible to Leukemic Transformation

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Acute lymphoblastic leukemia (ALL) is the most common malignancy in pediatric patients. About 10–15% of pediatric ALL belong to T-cell ALL (T-ALL), which is characterized by aggressive expansion of immature T-lymphoblasts and is categorized as high-risk leukemia. Leukemia initiating cells represent a reservoir that is responsible for the initiation and propagation of leukemia. Its perinatal origin has been suggested in some childhood acute B-lymphoblastic and myeloblastic leukemias. Therefore, we hypothesized that child T-ALL initiating cells also exist during the perinatal period. In this study, T-ALL potential of the hematopoietic precursors was found in the para-aortic splanchnopleura (P-Sp) region, but not in the extraembryonic yolk sac (YS) of the mouse embryo at embryonic day 9.5. We overexpressed the Notch intracellular domain (NICD) in the P-Sp and YS cells and transplanted them into lethally irradiated mice. NICD-overexpressing P-Sp cells rapidly developed T-ALL while YS cells failed to display leukemia propagation despite successful NICD induction. These results suggest a possible role of fetal-derived T-cell precursors as leukemia-initiating cells.

Keywords: notch signaling, notch intracellular domain, yolk sac, para-aortic splanchnopleura, aorta-gonad-mesonephros region, acute T cell leukemia, hematopoietic stem cell-independent hematopoiesis

INTRODUCTION

In the hematopoietic system, Notch signaling is essential for the commitment of multipotent hematopoietic progenitors (MPP) to the T-cell lineage and it also supports cell growth, proliferation and survival at multiple stages of thymocyte development (Tanigaki and Honjo, 2007; Hozumi et al., 2008; Luis et al., 2016). *Notch 1* is essential for the emergence of hematopoietic stem/progenitor cells (HSPCs) in the mouse embryo (Kumano et al., 2003; Robert-Moreno et al., 2005) and T-cell development in the thymus (Radtke et al., 1999; Han et al., 2002). Notch provides a key regulatory signal in determining T- vs. B-lymphoid cell fate, and is involved in the progression through the early $CD4^{-}CD8^{-}$Double-negative (DN)1, DN2, and DN3 stages of thymocyte development (Schmitt et al., 2004) and in the regulation of TCR-ß rearrangement (Wolfer et al., 2002).

The constitutive activation of Notch signaling has been linked to excessive cell proliferation and arrested differentiation, contributing to the development of cancer (Aster et al., 2000). T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematologic malignancy, comprising 15% of all

newly diagnosed pediatric ALL and generally considered a high risk leukemia (Raetz and Teachey, 2016). Activating mutations of *Notch1* are observed over 70% of pediatric and 65% of adult T-ALL cases (Weng et al., 2004; Sanchez-Martin and Ferrando, 2017; Kimura et al., 2019). The Loss-of-function mutations in FBXW7 are also commonly found in T-ALL and result in inhibition of ubiquitin-mediated degradation of the activated form of Notch (Iacobucci and Mullighan, 2017; Karrman and Johansson, 2017). These mutations cause ligand independent activation and stability of the Notch intracellular domain (NICD), subsequently leading to the increased proliferation and survival of leukemic cells (Staal and Langerak, 2008). Thus, activated *Notch1* mutation plays a major pathogenetic role in human T-ALL.

ALL is the most common leukemia in children. Chromosomal translocations of leukemic cells, including *ETV6-RUNX1*, *TCF3-PBX1*, *BCR-ABL1*, and *KMT2A*, are often observed in these cases. These chromosomal aberrations are reported to have occurred *in utero* and acquire second mutations to drive leukemic transformation (Ford et al., 1993; Gill Super et al., 1994; van der Weyden et al., 2011; Hein et al., 2020). Most infant ALL belongs to B-cell leukemia, 80% of them display MLL chromosomal rearrangement and also poor prognosis (Sanjuan-Pla et al., 2015), known to arise *in utero* (Ford et al., 1993; Hein et al., 2020). The development of T-ALL in infants is extremely rare, but still exists with poor prognosis and *Notch1* mutation in infant T-ALL has also been reported (Mansur et al., 2015). Importantly, *Notch1* mutation was detected in neonatal blood spots (Guthrie test) of the child and infant T-ALL patients, suggesting the in-utero origin of infant and child T-ALL with *Notch 1* mutation, similar to infant B-ALL (Eguchi-Ishimae et al., 2008; Mansur et al., 2015).

There are many leukemia mouse models developed by overexpressing leukemic fusion proteins such as BCR-ABL. However, BCR-ABL overexpression does not always induce leukemia in any progenitor cell types; it has been reported that only pro-B cells or higher progenitors were permissive to B-ALL development (Signer et al., 2010) and that B-progenitors of fetal origin developed more aggressive leukemia with shorter latency than adult BM B-progenitors upon BCR-ABL overexpression (Montecino-Rodriguez et al., 2014). In this sense, it is well known that continuous Notch activation through over-expression of NICD leads to transformation of BM HSPCs into T-ALL, as a mouse model mimicking human T-ALL (Aster et al., 2000; Wendorff and Ferrando, 2020). Therefore, NICD-overexpression may select the leukemia initiating cells that are permissive to progress T-ALL. This notion raises the question whether fetal lymphoid precursors at pre-HSC stage can become a T-ALL initiating cell.

In the fetal hematopoiesis, it is becoming recognized that there are several waves of hematopoiesis prior to the first HSC emergence in the aorta-gonad-mesonephros (AGM) region at E10.5 (Medvinsky and Dzierzak, 1996; Montecino-Rodriguez et al., 2016; Hadland et al., 2017). Traditionally, in searching the first site of HSC emergence in the mouse embryo, lymphoid potential has been intensively investigated using organ culture and stromal cell co-culture because lymphoid potential is considered to suggest the presence of HSC potential. T- and B-lymphoid potentials have been detected in the extraembryonic yolk sac (YS) and/or para-aortic splanchnopleural (P-Sp) region at E8.25–9.5 (Godin et al., 1993, 1995; Nishikawa et al., 1998; Yokota et al., 2006; Yoshimoto et al., 2011, 2012). In addition, we have recently reported the presence of HSC-independent lymphoid progenitors in E10.5 YS and AGM region that directly repopulate only B and T cells without co-culture (Kobayashi et al., 2019). While these B-progenitors are biased to innate-immune B-1 lymphocytes, YS/P-Sp-derived T-precursors develop into $CD4^+$ or $CD8^+$ αβT cells in the recipient mice (Yoshimoto et al., 2012).

Here we examined leukemia propagation of the earliest T-progenitors in the YS and P-Sp by introducing active Notch signaling. We transplanted NICD-induced T cells derived from YS and P-Sp culture into lethally irradiated congenic mice and found massive T-ALL development by P-Sp-derived T cells. Those T-ALL were $CD4^+CD8^+$ double positive (DP) and highly expressed Notch-target genes. Interestingly, YS-derived cells did not develop T-ALL by NICD overexpression. These data indicate the leukemogenic potential of T-precursors at pre-HSC stage in the mouse embryo, suggesting a presence of T-ALL derived from the earliest T-precursors in the fetus.

METHODS

Mice

C57BL/6 (B6), their congenic BoyJ, and NOD/SCID/IL2Rγc$^{-/-}$ (NSG) mice were purchased from Jackson Laboratory and were maintained under the specific pathogen free condition. B6 mice were used for timed mating to produce E9.5 and E10.5 embryos. The embryos were harvested and their somite pair numbers were counted to confirm the proper developmental stage as previously described (Lux et al., 2008; Yoshimoto et al., 2011). The YS and P-Sp tissues were digested with 0.125% collagenase (StemCell Technologies) for 5 min. After E10, P-Sp region is called AGM region. AGM region were digested with 0.25% collagenase for 30 min at 37°C. Sublethally irradiated (150 rad) NSG neonates (day 2–3) were used for E10.5 pre-HSC transplantation. Lethally irradiated (900 rad) congenic BoyJ mice were used as recipients for transplantation of YS- and P-Sp derived NICD-induced T progenitors. Sublethally irradiated adult NSG mice were also used as recipients. The experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Indiana University and the Animal Welfare Committee (AWC) at UTHealth.

In vitro Cultures

YS and P-Sp cells were plated on confluent Delta-like 1-expressing OP9 stromal cells (OP9-DL1, a gift of Dr. Juan Carlos Zuniga-Pflucker, University of Toronto) (Schmitt et al., 2004) in six well plates in induction medium (αMEM, 10% FBS, and 5×10^{-5} M 2-mercaptoethanol) supplemented with 10 ng/ml IL-7 and 10 ng/ml Flt3 ligand. Suspended cells were collected throughout the co-culture period and the phenotype of the non-adherent cells was analyzed by flow cytometry.

Flow Cytometry

Cells from *in vitro* culture or single cell suspension from peripheral blood (PB), spleen, BM, and thymus were stained with various surface antibodies and analyzed using LSRII (Becton Dickinson). The following antibodies were used: anti-mouse AA4.1 (AA4.1), CD19 (1D3), B220 (RA3-6B2), CD3e (145-2C11), Ter119 (TER-119), CD4 (GK1.5), CD8 (53-6.7), CD25 (PC61.5), CD44 (IM7), CCR7 (4B12), CD45.1 (A20), and CD45.2 (104). These Abs were conjugated with FITC, PE, PerCPCy5.5, PE-Cy7, APC or APC-Cy7 in various combinations.

NICD Transduction Into YS/P-Sp Derived Cells and Transplantation

Standard retrovirus infections were performed as previously report with slight modifications (Kobayashi et al., 2017; Rodriguez et al., 2020). YS or P-Sp derived hematopoietic cells, 6–7 days after co-culture with OP9-DL1, were plated at 2.5×10^5 cells per well on a 24 well plate the day before viral transduction. NICD retrovirus vector (Carlesso et al., 1999) was transduced in IMEM medium with 10% FBS, 10 ng/ml SCF, 10 ng/ml IL-7, and 10 ng/ml Flit3-ligand with the virus-containing supernatant plus Polybrene (final concentration 4 μg/mL; Sigma). A multiplicity of infection (MOI) of $\leq$5 was used. The cells suspended in viral supernatant were spinoculated at 1,700 rpm for 50 min, incubated at 37°C and 5% CO_2 for an additional 8 h, then washed and plated in fresh medium overnight. A second transduction was performed on the following day using the same procedure. After a second transduction, cells were cultured on OP9-DL1 with IL7 to enhance cell expansion. One week after beginning the transduction, GFP^+ cells were confirmed in the DN fraction as analyzed by flow cytometory and all the cells were injected into $CD45.1^+$ congenic BoyJ recipient mice with 10^5 BoyJ supportive BM cells. The transplanted mice were monitored daily and WBC count and donor-derived $CD45.2^+GFP^+$ cells were checked with recipient PB every 1–3 week beginning 4 weeks after transplantation.

Histology

Tissues collected from non-transplanted and P-Sp-NICD T cell transplanted mice were fixed in IHC Zinc Fixative (BD Pharmingen) and embedded in paraffin. Each organ paraffin block was serially sectioned at 5 μm and stained with Hematoxylin-Eosin. The slides were examined and scored blind by our pathologist.

Quantitative RT-PCR Analysis

Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA, United States), and reverse transcribed into cDNA (iScript cDNA synthesis kit, Bio-Rad, Hercules, CA, United States). qRT-PCR reactions were performed using gene-specific probes either in a MX3000 system using SYBR Green chemistry (Stratagene, La Jolla, CA, United States), or in a 7900HT Fast system using Taqman probes (Applied Biosystems, Foster City, CA, United States). The sequences of gene-specific probes are following; GAPDH-F, AAGCCCATCACCATCTTCCA, GAPDH-R, TAGACTCCACGACATACTCA, Deltex-F, GCCATGTACTCC AATGGCAACAAG, Deltex-R, CGGGATGAGGTGAAAC TCCATCTT, mIL-7Rα, Mm00434295_m1 (ThermoFisher Scientific), mHes1, and Mm01342805_m1 (ThermoFisher Scientific).

Statistical Analysis

Unpaired student-*t* test was used for statistical analysis.

RESULTS

The Earliest T-Cell Precursors Were Present in the Mouse Embryo at E9.5–10.5

We previously reported that E9.5 YS and P-Sp VE-cadherin $(VC)^+$ endothelial cells (ECs) have T-cell potential detected *in vitro* culture with OP9-DL1 (Yoshimoto et al., 2012). In this study, we isolated E9.5 YS and P-Sp cells and co-cultured them with OP9-DL1 stromal cells and induced CD4CD8 DP and DN T-lymphocytes. These YS- and P-Sp-derived T-cells were engrafted in the thymus and spleen of sub-lethally irradiated NOD/SCID/IL2Rγc$^{-/-}$ (NSG) neonates as naive and memory T cells expressing various TCR repertoires. We also found donor-derived $CD3^+$ T-cells in the PB when E10.5 YS was directly injected into NSG neonates (Yoshimoto et al., 2012). Thus far, there was no report to detect transplantable T cells in the YS or AGM region without co-culture, therefore, we further explored the lymphoid-repopulating ability of E10.5 YS and AGM cells (Kobayashi et al., 2019). When total YS/AGM cells or VE-cad^+CD45^-c-kit^+ cells (from 2.3 to 10 embryo equivalent cells) were injected into sub-lethally irradiated NSG neonates without *in vitro* culture step, only donor-derived T cells were detected in the PB of some recipient mice (4 out of total 32 AGM-transplanted mice and three out of total 23 YS-transplanted mice (**Supplementary Figures 1A,B**; Kobayashi et al., 2019). Those recipients' spleen showed predominant T-cell repopulation ($CD4^+$ and/or $CD8^+$ cells) and a few B cells (**Supplementary Figures 1B,C**). Importantly, these B-cells were B-1 and marginal zone B-cells, but not B-2 cells (**Supplementary Figure 1C**; Kobayashi et al., 2019). In the recipient BM, whereas AGM- and YS-derived $CD4^+$ or $CD8^+$ T-cells were detected, donor-derived $Mac1^+$ cells were barely detected (**Supplementary Figures 1B,D**), indicating E10.5 YS and AGM pre-HSC population contains T-cell biased repopulating cells. These results also suggest that E9.5 VC^+ hemogenic ECs produce T-lymphoid precursors at E10.5.

DN3 Cells Derived From E9.5 P-Sp and YS Express CCR9 and Showed Efficient Thymus Engraftment

Because T-lymphoid potential using OP9-DL1 culture is found in the YS and P-Sp at E9.5, 2 days before HSC detection in the AGM region, we asked their leukemic potential by overexpressing NICD (Carlesso et al., 1999). First, we observed time course of

T-cell development from E9.5 YS and P-Sp cells in the OP9-DL1 culture by flow cytometric analysis to determine the timing of NICD induction. We found $CD45^+$c-kit^+ hematopoietic progenitors around day 4–7 of co-culture (**Figure 1A**). On day 12, $CD45^+Thy1^+$DN population was still dominant and $CD25^+CD44^+$ DN2 and $CD25^+CD44^-$DN3 cells were detected (**Figure 1B**). We confirmed the expression of CCR9, a homing receptor for seeding the thymus, in each DN population, in addition to TCRβ and TCRγ expression (**Figure 1C**). We found that CCR9 expression was the most evident in DN3 and DP populations (**Figures 1C,D**). Therefore, we first tested if $CCR9^+$ cells engraft in the recipient thymus. We sorted DN3 and DP cells from YS and AGM culture and injected 1×10^6 DN3 or DP cells into sublethally irradiated NSG neonates (**Figures 1E,F**). Two weeks after transplantation, we confirmed that only DN3 cells were engrafted in the recipient thymus, and found the thymus repopulated by P-Sp-DN3 cells was bigger than that repopulated by YS- DN3 cells (**Figure 1F**). Thus, we checked the DN3 CCR9 expression as an indicator of transplantable donor cell type during the co-culture.

E9.5 P-Sp Derived T Lymphocytes Developed Leukemia Upon Continuous Notch Activation

In order to determine if YS/P-Sp-derived T-precursors possess transforming capability similar to adult BM HSPC, NICD was retrovirally introduced into YS and P-Sp cells 6–7 days after co-culture with OP9-DL1, at the timing when $CD45^+$c-kit^+ HPCs were produced in the culture (**Figures 1A**, **2A**). After NICD induction, NICD-GFP expressing YS- and P-Sp-derived hematopoietic progenitor cells were expanded on OP9-DL1 again up to 6 days and were injected into lethally irradiated BoyJ recipient mice together with BoyJ BM supporting cells (**Figure 2A**). We confirmed that the injected cells were mostly DN3 cells, containing phenotypic leukemia initiating cells (**Figure 2B**) (Tremblay et al., 2010; Gerby et al., 2014). We also confirmed the CCR9 expression in GFP^+ cells (**Figure 2B**). As soon as 5 weeks after transplantation of NICD-expressing YS- and P-Sp-derived cells into recipient BoyJ mice, recipients transplanted with P-Sp-derived cells started to show leukemic symptoms such as high WBC, comprised largely of $CD4^+CD8^+$ GFP^+ cells in the PB (**Figures 3A,B**). The mice subsequently progressed to a moribund appearance within a few days. In contrast, NICD-YS-derived T-cells failed to give rise to leukemic cells (**Figures 3B,C,K**). Thus, transplantation of NICD-P-Sp cells led to a marked decrease in survival of transplanted recipients due to T-ALL progression compared to NICD-YS cell transplanted hosts (**Figure 3C**). Mice receiving NICD-P-Sp cells became moribund with leukemia with a mean latency of 40 days, while mice repopulated with NICD-YS cells did not develop any signs of disease during the 18 weeks observation. All the mice transplanted with NICD P-Sp-derived cells showed a large thymus with donor-derived GFP^+DP cells whereas NICD YS-derived cells failed to repopulate recipient thymus (**Figures 3D,K**). More than 95% of the leukemic BM cells comprised of donor-derived DP GFP^+ cells (**Figure 3E,K**). The recipients' liver and kidney were enlarged and also extensively infiltrated with leukemic cells (**Figures 3F,G**). Quantitative PCR analysis of Notch targets from total BM cells of leukemia mice revealed upregulations of Hes1, Deltex1 (Targets of Notch signaling) and IL7Rα compared to normal BM cells (**Figure 3H**), in line with the reports that upregulation of IL7Ra is often observed in human T-ALL cells from patients (Zenatti et al., 2011). We also compared thymus DP cells, a normal counterpart of the leukemic T-cells, to P-Sp derived leukemic cells in the recipient BM cells (**Figure 3I**). Notch target gene expression was much higher in the leukemic cells than in the normal DP thymic cells, showing that continuous exogenous NICD signals induced leukemia in P-Sp-derived T-cells. When we examined the recipients transplanted with NICD-YS derived T-cells, we found GFP^+ $CD4^+$, $CD8^+$, and DP cells in the recipient BM but they did not proliferate (**Figure 3K**). Those NICD-YS derived $CD4^+$ and/or $CD8^+$ cells showed an upregulation of IL7R at a higher level than NICD-P-Sp derived T-cells (**Figure 3J**). Thus, although the YS contain autonomously developing T-cell precursors that can engraft in recipient mice, they were not permissive for NICD-induced leukemia. This is in contrast to P-Sp derived cells that contributed in every animal to leukemic development upon continuous NICD expression.

In addition, we continued NICD-P-Sp cell culture on OP9-DL1 up to 35 days (**Figure 2C**) because P-Sp-derived cells showed extensive proliferation *in vitro*. To test if this extensive proliferation ability is cell intrinsic due to NICD-overexpression, we removed external Notch signaling by transferring NICD-overexpressing cells onto regular OP9 cells that do not express DL1 (**Figure 2C**). After 7 days, NICD-GFP^+ population kept DP cells while GFP^- population lost DP cells and shifted to CD8SP. These *in vitro* results indicate the cell-autonomous proliferation of P-Sp-derived DP cells by NICD-overexpression.

E10.5 AGM Cells Possess Leukemic Potential Upon NICD Overexpression

Because E10.5 AGM cells contain T-cell repopulating cells (**Supplementary Figure 1**) (Kobayashi et al., 2019), we next asked if freshly isolated AGM cells can transform T-ALL by overexpressing NICD (**Figure 4A**). We dissected and digested E10.5 YS and AGM cells and retrovirally transduced NCID. The following day, we transplanted NICD-expressing YS and AGM cells into sublethally irradiated adult NSG mice (1 e.e/recipient mouse, $N = 5$ for each NICD-expressing YS and AGM cells). Before transplantation, we confirmed that NICD-GFP was successfully transduced into YS and AGM cells (**Figure 4B**). We examined GFP^+ donor cells in the PB of the recipient mice after transplantation over time. Most recipient mice showed less than 1% of donor cells. Only one mouse transplanted with NICD-expressing AGM cells showed increased $GFP^+CD45.2^+$ cells (**Figure 1C**). Interestingly, only AGM cells expressing NICD developed T-ALL in two out of five transplanted mice with longer latency compared to cultured E9.5 NICD-expressing P-Sp cells, while YS cells did not develop leukemia without showing any morbidity within the observation period (**Figure 4D**). The NICD-AGM-derived leukemic mice showed enlarged liver

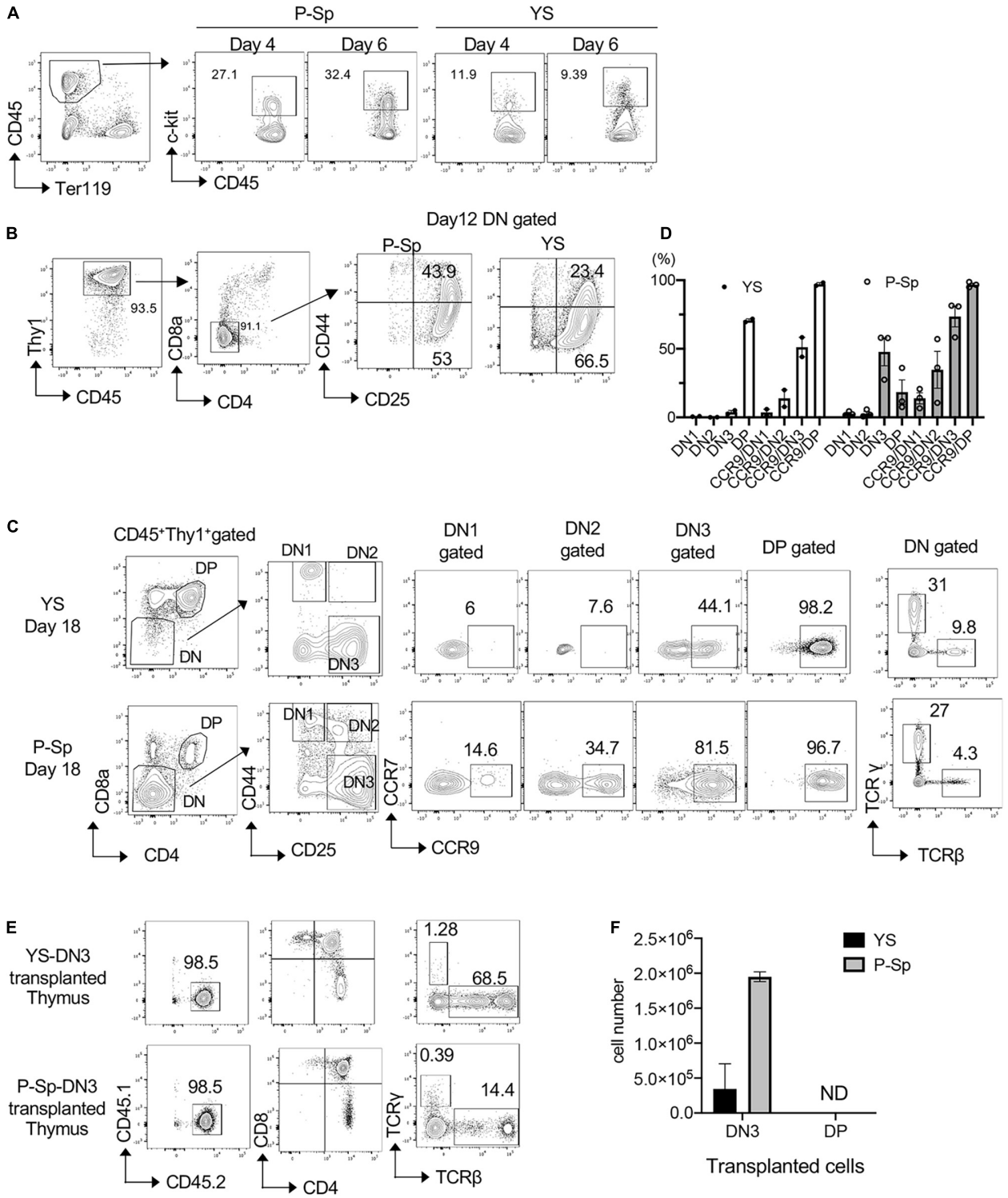


FIGURE 1 | Induction of DN2 phenotype by NICD transduction. **(A)** E9.5 YS and P-Sp were cultured on DL1-OP9, and $CD45^{+}$c-kit^{+} hematopoietic progenitors were measured on different time points, one of the three independent experiments shown. **(B)** Representative FACS plots showing DN phenotype on day 12 of YS and P-Sp co-culture on OP9-DL1. **(C)** CCR9 expression in each DN populations of YS and P-Sp cells co-cultured with DL1-OP9 for 18 days. One of the three independent experiments is depicted. TCRβ and TCRγ expressions in DN cells were also confirmed. **(D)** The percentage of DN stages and $CCR9^{+}$ cells in each DN population of YS and P-Sp co-culture with OP9DL1, depicted in panel **(C)**. Thymus engraftment of DN3 cells from YS- and P-Sp co-culture with OP9-DL1 cells **(E)** and their repopulated cell numbers in the recipient thymus **(F)**.

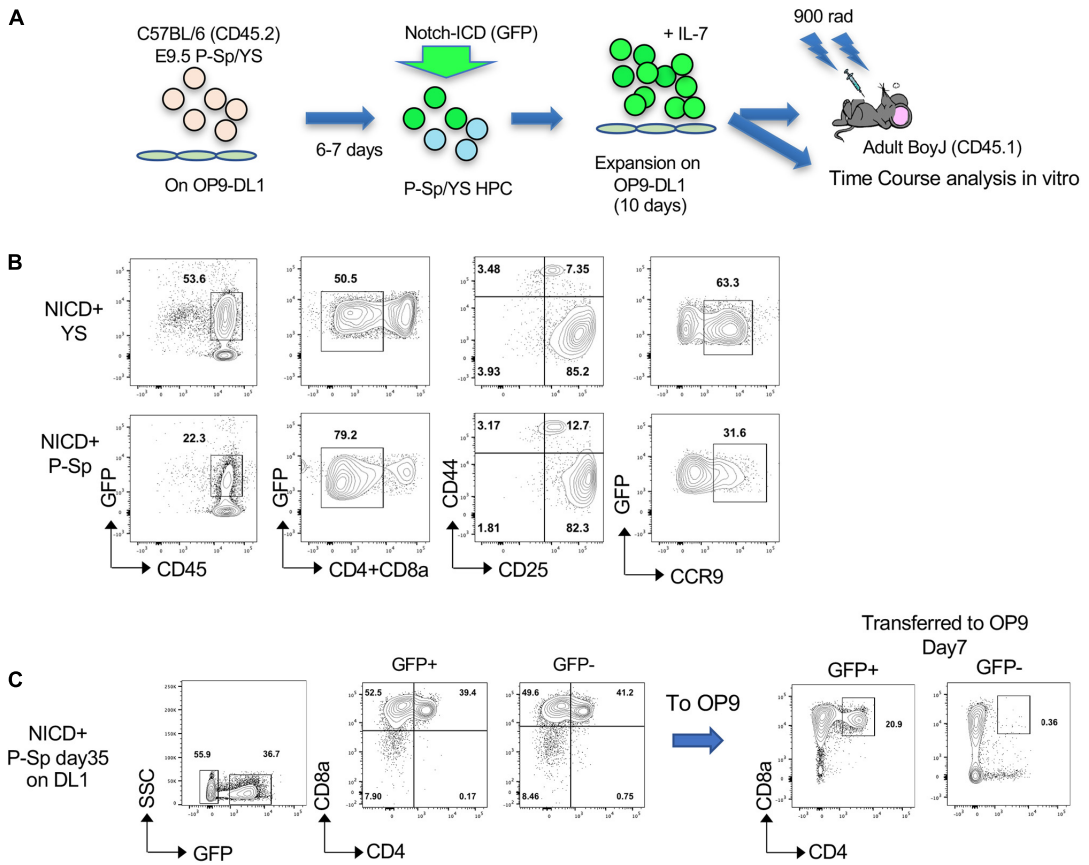
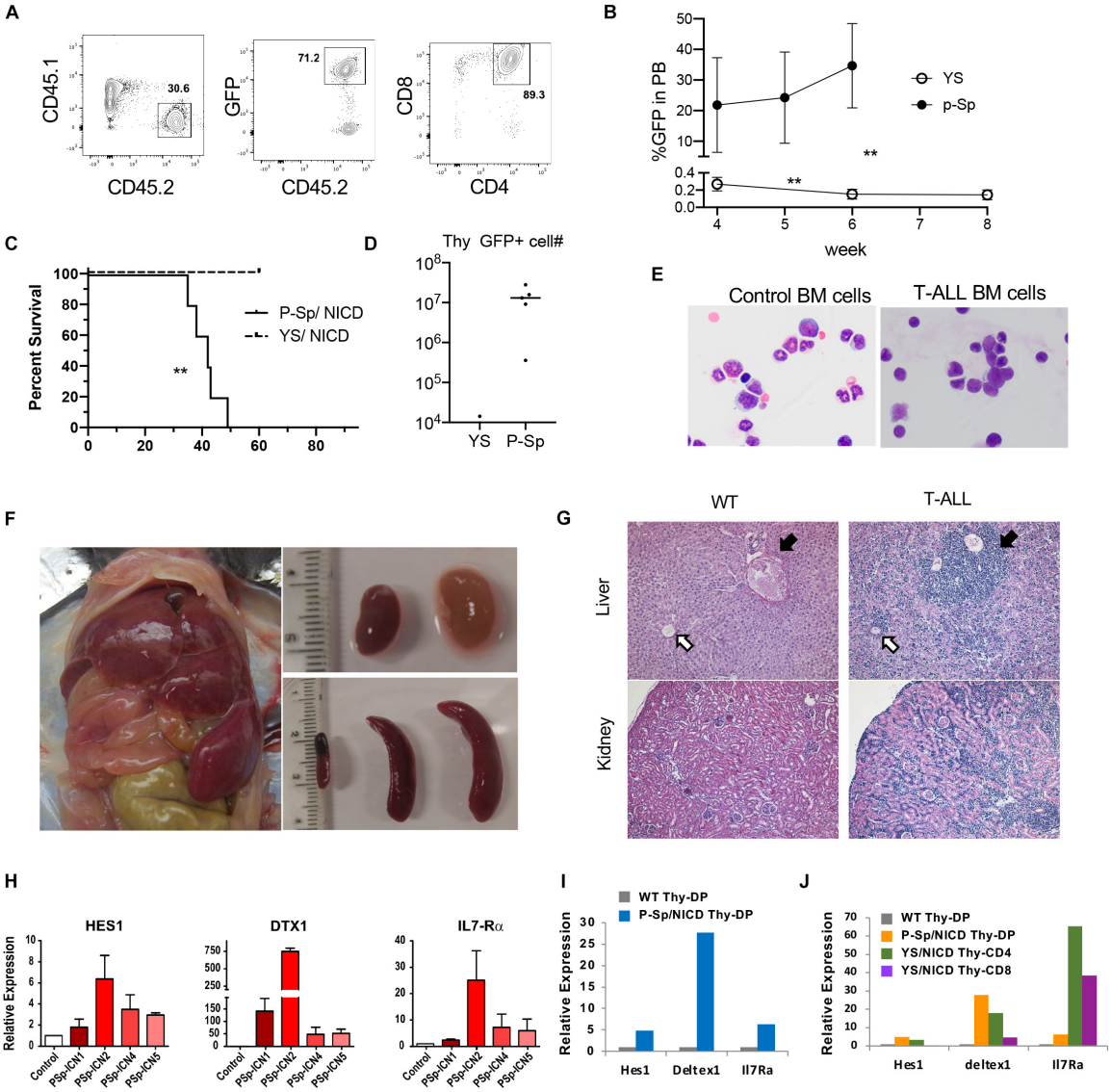


FIGURE 2 | NICD introduction into hematopoietic progenitors derived from YS and P-Sp in the OP9-DL1 culture. **(A)** Schematic experimental stream for NICD induction and Transplantation. **(B)** NICD-induced GFP$^+$ DN cells express CD25 and CCR9 on day 12 of co-culture with OP9-DL1 before transplantation. **(C)** P-Sp cells with NICD were harvested at day 35 and GFP$^+$ or GFP$^-$ cells were sorted and transferred onto OP9 (without DL1), subsequently analyzed after day 7 (right panel).

and spleen (**Figures 4E,F**). The leukemic spleen was occupied with CD4$^+$CD8$^+$GFP$^+$ cells (**Figure 4G**), but thymus was not detected in any recipient mice. Recipient mice that did not develop T-ALL showed no donor-derived cells in the PB. Of note, one recipient mouse transplanted with NICD-YS cells was found to have an enlarged spleen when it was terminated (**Supplementary Figure 2A**). These cells were GFP$^+$CD4$^+$ cells, but not CD4$^+$CD8$^+$, seemed to have developed unusual transformation of CD4$^+$ cells. While AGM-derived T-ALL cells occupied the recipient BM, YS-derived GFP$^+$CD4$^+$ or GFP$^+$CD8$^+$ cells were detected at a small percentage of the recipient BM (**Supplementary Figure 2B**). Taken together, E10.5 AGM cells possess T-ALL transforming ability.

DISCUSSION

In this study, we demonstrated that E9.5 P-Sp-derived cells co-cultured with OP9-DL1 possess T-ALL-initiating potential by overexpressing NICD. Traditionally, T-lymphoid potential has been detected in the YS and/or P-Sp region as early as at a pre-circulation stage (E8.25) using *ex vivo* thymic organ culture or OP9-DL1 co-culture (Nishikawa et al., 1998; Cumano et al., 2001; Yoshimoto et al., 2012). These reports have shown the T cell potential of the tested cells *in vitro* culture but never displayed the presence of committed T-progenitors. However, we have recently shown the presence of T-precursors among VC$^+$c-kit$^+$ HSC-precursors (pre-HSCs) in E10.5 YS and AGM region, which engrafted in the immunodeficient mice without co-culture (Kobayashi et al., 2019). Therefore, it is assumed that hemogenic ECs that have T-cell "potential" at E9.5 or before give rise to transplantable T-precursors by E10.5. Thus, we propose the possibility that fetal T-precursors at a pre-HSC stage can develop leukemia in postnatal life. However, we have not determined which cell types were responsible for the T-ALL propagation *in vivo*. Because E9.5 P-Sp cells reportedly contain "pro-HSCs" that can gain a transplantable ability by aggregation culture with OP9 (Rybtsov et al., 2014), it is possible that T-ALL initiating cells at E9.5 P-Sp developed *via* HSCs in the OP9-DL1 co-culture. However, considering the fact that mono T-cell repopulating ability was detected in AGM region in mice and humans (Ivanovs et al., 2011; Kobayashi et al., 2019) and that freshly isolated E10.5 AGM cells developed leukemia upon NICD overexpression, it is also plausible that HSC-independent T-cell precursors may have



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Cell # Transferred	P-Sp (n=5)					YS (n=7)				
	8.0 x 10e6 (derived from 1e.e)					2.8 x 10e6 (derived from 1e.e)				
% Survival	0%					100%				
Flow	% Donor	% GFP	% DP	% CD4	% CD8	% Donor	% GFP	% DP	% CD4	% CD8
		(% in Donor)					(% in Donor)			
PB	51.4 ±28.3	82.1 ±35.1	55.9 ±38.4	30.4 ±38.9	11.8 ±20.8	0.5 ±0.25	33.2 ±10.4	1.4 ±1.44	9.1 ±10.3	67.8 ±17.8
BM	96.1 ±10.4	81.4 ±28.4	57.8 ±41.6	1.3 ±1.8	18.4 ±20.8	0.5 (n=1)	24.5	4.4	10.9	70.7
Sp	62.8 ±16.2	85.3 ±31.0	53.6 ±23.3	20.2 ±21.6	24.4 ±32.5	0.8 (n=1)	38.3	0.7	15.2	75.2
Thy	48.6 ±30.9	88.5 ±18.0	79.9 ±12.2	9.6 ±9.12	10.1 ±13.7	0.1 (n=1)	38.1	0.0	52.5	27.5

FIGURE 3 | Leukemia propagation by the cultured P-Sp-NICD, not YS-NICD. **(A)** Representative dot plots of recipient PB repopulated with P-Sp-NICD at 6 weeks after transplantation. **(B)** Percentage of GFP$^+$CD45.2 cells in recipient PB are plotted (n = 5–7, $^{**}P < 0.01$). **(C)** Survival curve are depicted ($^{**}P < 0.01$). **(D)** Thymus engraftment by P-Sp-derived NICD-GFP+ T-ALL cells. **(E)** May–Giemsa staining of ctr BM cells and recipient BM cells with P-Sp-NICD are shown. **(F)** Liver, spleen, and kidney are markedly enlarged. **(G)** Upper panel: liver, leukemic cells infiltrate in the portal area and around central vein as well as in the liver sinusoids. Black arrow: portal area, white arrow: central vein. Lower panel: kidney, leukemic cell infiltration was observed. **(H–J)** Upregulations of Notch targets in leukemic cells were measured by qPCR. Each RNA from recipient spleen were applied and compared to normal BM cells **(H)**. Those targets in leukemic thymic DP cells were compared to normal thymus DP cells **(I)**. Small populations of GFP$^+$NICD-YS-derived CD4$^+$ or CD8$^+$ T-cells were sorted and evaluated for Notch targets **(J)**. **(K)** P-Sp- and YS-derived cells engrafted in the recipient organs including PB, BM, spleen, and thymus.

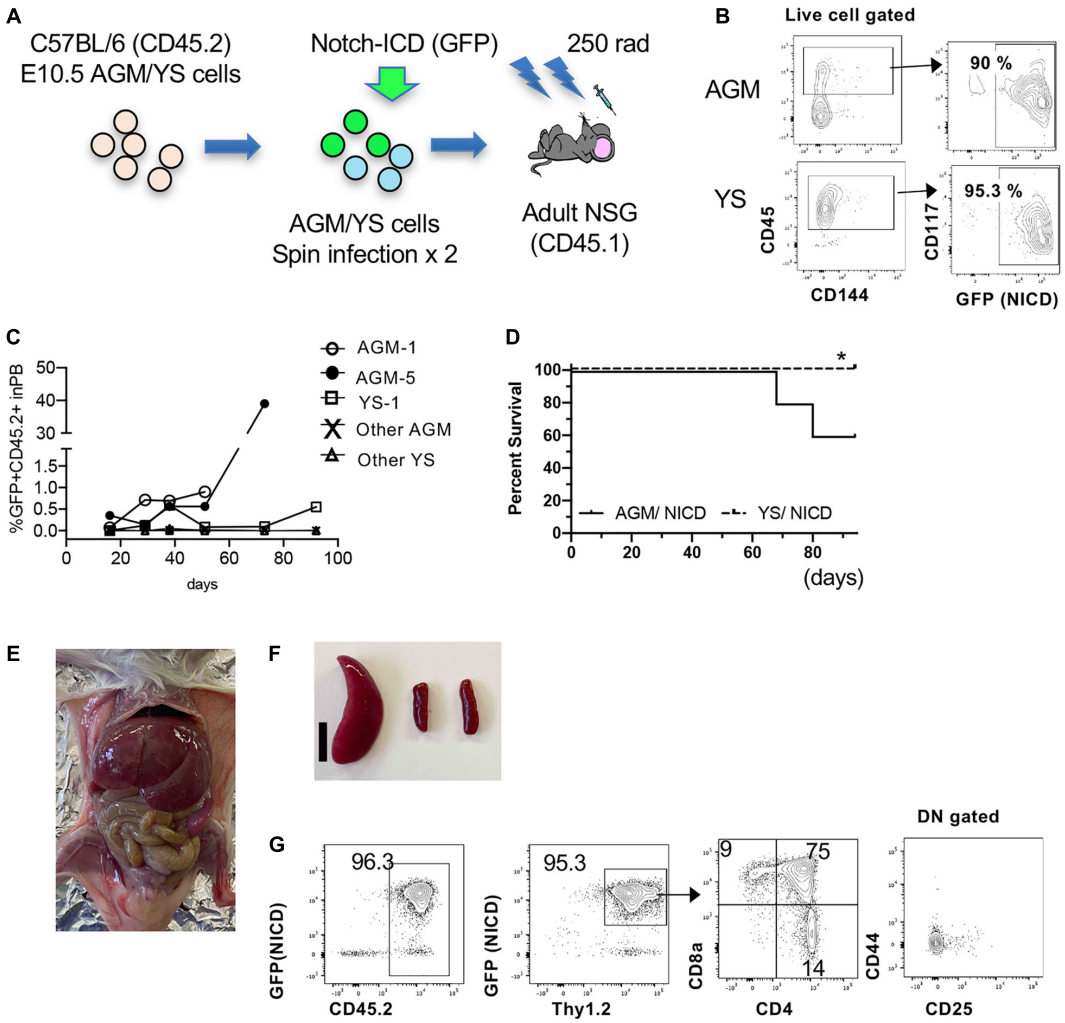

FIGURE 4 | Leukemia propagation by the freshly isolated AGM-NICD. **(A)** Schematic experimental stream for NICD induction into freshly isolated AGM/YS cells and Transplantation (without co-culture). **(B)** CD45$^+$AGM and YS cells showed more than 90% NICD-GFP induction. **(C)** GFP+CD45+ donor cell percentage in the recipient PB. **(D)** Survival curve of mice transplanted with AGM-NICD or YS-NICD cells is depicted (*N* = 5 each). * YS-derived cells showed GFP$^+$CD4 proliferation in one recipient spleen. **(E,F)** Leukemic mice transplanted with AGM-NICD cells showed enlarged liver and spleen. Bar: 1 cm. **(G)** FACS plots of the leukemic spleen derived from AGM-NICD cells.

developed leukemia. A recent report examining gene signatures of human embryonic thymus, AGM, fetal liver, and fetal blood using single-cell RNA-sequencing has identified the pre-thymic lymphoid progenitors in the AGM region (Zeng et al., 2019). These data suggest that AGM region contains T-precursors that seed the fetal thymus in humans, and we propose that the T-ALL initiating cells found in the P-Sp region may belong to the initial wave of thymus seeding cells. Further investigation is required to determine the leukemic initiating cells in the embryo.

In the OP9-DL1 culture, P-Sp cells showed greater proliferation than YS cells. In general, the erythro-myeloid capacity is more abundant in the YS while more lymphoid and HSC potential is detected in the P-Sp. (Nishikawa et al., 1998; Cumano et al., 2001; Yokota et al., 2006; Yoshimoto et al., 2012). The difference of hematopoietic capacity might have contributed to the different outcome of leukemia development in this study. Another fact to be considered may be the target cell population of NICD transfection. It is noted that c-kit$^+$CD45$^+$ percentage was less in the YS than P-Sp in the co-culture (**Figure 1A**), which might reflect the progenitor numbers permissive to leukemic transformation.

We confirmed YS- and P-Sp-derived DN3 cells engrafted in the recipient thymus, and transplanted NICD-expressing cells included CCR9$^+$DN3 cells, in line with the previous report that DN3 contains self-renewing leukemic cells in a T-ALL mouse model (Tremblay et al., 2010; Gerby et al., 2014). In addition, NICD-expressing P-Sp T-ALL cells in the leukemic mice showed high IL7R expression, which is in line of human T-ALL. Human T-ALL cells collected from patients at diagnosis often express IL7Ra (Zenatti et al., 2011). In addition, gain of function mutations in *IL7R* have been found in 10% of T-ALL cells, which induce Jak1/Jak3 and STAT5 activation (Ribeiro et al., 2018).

It has also been reported that IL7R is essential for T-ALL development (González-García et al., 2019). Thus, high IL7R expression, known to be activated by *Notch 1* (Weng et al., 2004), is critically important for human T-ALL development. Accordingly, high IL7R-expressing T-ALL cells developed from P-Sp region by *Notch1* overexpression seem recapitulate human T-ALL. However, NICD YS-derived T-cells failed to progress leukemia despite successful NICD induction and higher IL7R expression. It is generally considered that leukemogenic events are not sufficient to induce leukemia in all blood cells; rather, they need to occur in a selective hematopoietic lineage and at a specific progenitor stage in order to develop leukemia (Signer et al., 2010). Therefore, the difference between YS- and P-Sp-derived T-cells may be a key to understanding the leukemogenic capacity of embryonic T-cells that induce pediatric leukemias.

Recent advance in the analysis of hematopoietic development in the mouse embryo has established a new paradigm of several waves of fetal hematopoiesis that initiate before HSC emergence, which may last longer than previously considered (Montecino-Rodriguez et al., 2016; Palis, 2016; Dzierzak and Bigas, 2018). For example, tissue-resident macrophages (e.g., brain microglia) and a part of mast cells are derived from the early YS progenitors and function even in the adult (Gomez Perdiguero et al., 2015; Gentek et al., 2018). It has also been indicated that several waves of thymopoietic cells are present during fetal to neonatal periods (Ikuta et al., 1990; Ramond et al., 2014; Montecino-Rodriguez et al., 2018), some of which are originated from early embryonic stages, presumably of HSC-independent. Montecino-Rodriguez et al. (2018) have shown that, using PU.1 hypomorphic embryos, the initial wave of thymopoietic cells is less dependent on PU.1 compared to the adult T-cell development and showed different gene expression patterns between fetal and adult T-cell progenitors. Ramond et al. (2014) also reported at least two waves of thymus seeding progenitors and their different properties in terms of Vγ3 generation and the cell cycle. Furthermore, Zeng et al. (2019) has reported the single-cell RNA-sequencing of various hematopoietic organs at different developmental stages in human embryo and has identified a distinct type of pre-thymic lymphoid progenitors in the AGM region. Although it remains unknown whether these embryonic early T-progenitors are derived from HSCs, their analysis segregating several ETP subsets suggests the presence of several waves of T-progenitor emergence even in the human embryo. In this sense, it has been reported that infant T-ALL has distinct genetic and epigenetic features compared to childhood T-ALL (Doerrenberg et al., 2017). Since *Notch1* mutation has been detected in neonatal blood spots in both infant and child T-ALL cases, these two pediatric T-ALL seems to be derived from prenatal period but could be from different waves of fetal hematopoiesis.

In conclusion, we found a leukemia-initiating capacity in the earliest T-precursors in P-Sp/AGM region prior to HSC emergence. It is possible that YS- and P-Sp/AGM produce different T-cell waves with different biological signatures. Further investigation is required to determine whether these fetal T-cell waves contribute to T-ALL propagation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at Indiana University and UTHealth at Houston.

AUTHOR CONTRIBUTIONS

JD performed the experiments and wrote the manuscript. AC provided the reagents, interpreted, and discussed the results obtained. MY and MK conceived and performed the experiments and wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.634151/full#supplementary-material

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Defining the Emerging Blood System During Development at Single-Cell Resolution

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Developmental hematopoiesis differs from adult and is far less described. In the developing embryo, waves of lineage-restricted blood precede the ultimate emergence of definitive hematopoietic stem cells (dHSCs) capable of maintaining hematopoiesis throughout life. During the last two decades, the advent of single-cell genomics has provided tools to circumvent previously impeding characteristics of embryonic hematopoiesis, such as cell heterogeneity and rare cell states, allowing for definition of lineage trajectories, cellular hierarchies, and cell-type specification. The field has rapidly advanced from microfluidic platforms and targeted gene expression analysis, to high throughput unbiased single-cell transcriptomic profiling, single-cell chromatin analysis, and cell tracing—offering a plethora of tools to resolve important questions within hematopoietic development. Here, we describe how these technologies have been implemented to address a wide range of aspects of embryonic hematopoiesis ranging from the gene regulatory network of dHSC formation via endothelial to hematopoietic transition (EHT) and how EHT can be recapitulated *in vitro*, to hematopoietic trajectories and cell fate decisions. Together, these studies have important relevance for regenerative medicine and for our understanding of genetic blood disorders and childhood leukemias.

Keywords: single-cell RNA sequencing, embryonic haematopoiesis, single-cell ATAC sequencing, lineage hierarchy, single-cell genomics, endothelial to hematopoietic transition (EHT), hematopoietic stem cells (HSC)

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INTRODUCTION

The well-characterized, gradual maturation of hematopoietic stem cells (HSCs) into functional blood and immune cells serves as a conceptual model for stem-cell-related processes like hierarchical organization, lineage commitment, cell fate decision, and malignant transformation. Historically, breakthroughs in our understanding of hematopoiesis have been intimately correlated with technological advances (Doulatov et al., 2012; Jacobsen and Nerlov, 2019). Accordingly, the current era of single-cell genomics has already had fast and valuable impact even on the most central views of hematopoietic differentiation, questioning the dogma of the classical, stepwise lineage commitment of hematopoiesis (Laurenti and Gottgens, 2018; Jacobsen and Nerlov, 2019).

The blood system is developed at early stages of ontogeny to support the growing embryo at the initiation of heartbeat. Intriguingly, these first primitive blood cells develop independently of HSCs in the yolk sac at murine embryonic day (E)7 and consist of erythrocytes, megakaryocytes,

and macrophages, critical for the embryo's basic needs (Palis, 2016). Prior to the emergence of HSCs, a second wave of erythromyeloid progenitors (EMPs) initiates in the yolk sac around E8, and progenitors with lymphoid potential emerge both in the yolk sac and embryo proper at around E9.5 (Yoshimoto et al., 2011, 2012; Boiers et al., 2013; Palis, 2016; Ghosn et al., 2019). Definitive HSCs (dHSCs) responsible for life-long maintenance of the blood system are first observed at E10.5 in the mouse system, arising in the aorta–gonad–mesonephros (AGM) region (Dzierzak and Bigas, 2018). dHSCs arise alongside HSC-independent progenitors from endothelial cells lining the dorsal aorta in a process known as endothelial to hematopoietic transition (EHT; Ottersbach, 2019; Zhu et al., 2020). EHT is additionally involved in the formation of EMPs and likely also lympho-myeloid progenitors in the yolk sac (Frame et al., 2016; Palis, 2016). Circulating HSCs colonize the fetal liver (FL) approximately at E12 and undergo a massive expansion phase before finally migrating to the bone marrow at E17.5, the main site of hematopoiesis during the life time of an individual (Ema and Nakauchi, 2000; Gao et al., 2018). Of note, the FL niche harbors both HSC-dependent as well as HSC-independent progenitors during development, adding to heterogeneity and complexity (Schematic overview of hematopoietic development is shown in **Figure 1**).

This review summarizes the impact of single-cell genomics on hematopoietic ontogeny research, outlining the suitability of different methods for the investigation of various processes. It focuses on representative and key studies (outlined in **Figure 2**) in mouse and human that illustrate how technological advances offer a possibility to resolve previously elusive conceptual questions regarding embryonic and fetal hematopoiesis, as well as discusses future challenges and possibilities.

SINGLE-CELL qPCR ANALYSIS SHED LIGHT ON EMERGING HSCs

The development of microfluidic chips allowing for simultaneous quantitative PCR (qPCR) reactions of hundreds of single cells at nanoliter volumes paved the way for the single-cell (sc)-genomic era of the last decade (Warren et al., 2006; Spurgeon et al., 2008). Compared to more recent scRNA-sequencing (scRNA-seq) methods, sc-qPCR analysis offers higher sensitivity and specificity but substantially lower throughput both in cell numbers and features. Additionally, the targeted approach of sc-qPCR demands a high level of prior knowledge of the test cells for efficient primer-panel design. Thus, early single-cell experiments in embryonic hematopoiesis focused on EHT, where hematopoietic stem and progenitor cells (HSPCs) emerge from the hemogenic endothelium (HE) of the dorsal aorta. dHSCs are rare in the early embryo (Kumaravelu et al., 2002), and consequently, the target populations are small, while the gene targets are distinct (endothelial/hematopoietic) and well described. Using a green fluorescent protein (GFP)-reporter mouse model for hematopoietic-associated *Runx1* activity, Swiers et al. (2013) isolated and performed sc-qPCR analysis on a total of 803 cells, from E8.5 to E11.5. Despite using a primer panel consisting of only 18 established endothelial or hematopoietic gene markers, it could be shown that single GFP positive cells lost endothelial potential around E9.5 and gradually activated a hematopoietic molecular signature. Thus, single-cell analysis made it possible to reveal that the endothelial wall consists of HE that is molecularly specified toward hematopoietic fate already 2 days before definitive hematopoiesis emerges.

In a similar approach, the formation of primitive blood was investigated at four different timepoints from E7.0 to E8.5 (Moignard et al., 2015). Here, a Runx1-GFP reporter mouse was used in combination with the vascular endothelial growth factor receptor FLK1, to catch early hematopoiesis and mesoderm with hematopoietic potential, respectively. In total, five populations and almost 4,000 single cells were analyzed against primers targeting relevant transcription factors. The high number of cells from a putative hierarchy of cell states allowed for the visualization of trajectories for blood development and the bifurcation of blood and endothelium. The focus on transcription factors, furthermore, made possible the identification of regulatory networks that specify early blood formation, specifically *Sox7*, *Hoxb4*, and *Erg* factors. Thus, the sensitivity of sc-qPCR is powerful for mechanistic insights into developmental processes when combined with high cell numbers and carefully selected primer panels.

Similar to mouse, HSCs in human development also emerge through EHT in the AGM region, and from around Carnegie stage (CS), 13 (day 27) hematopoietic clusters can be seen in the dorsal aorta (Ivanovs et al., 2017). Understanding of gene regulatory networks behind EHT and how dHSCs emerge in human is of clinical relevance to generate functional HSCs from human pluripotent stem cells (hPSCs). However, conventional analysis of dHSCs generation within the human embryo is challenging due to a combination of limited access to material as well as rarity and heterogeneity of relevant cell populations.

Using the power of single-cell technologies, Guibentif et al. (2017) explored the EHT process in hPSCs differentiated toward blood using sc-qPCR analysis. CD34-positive cells were index sorted on day 10 of differentiation, a timepoint when HE cells, HSC-like populations, as well as a mixture of EHT-related cells are present in the culture. A total of 437 cells and a panel of 91 genes, related to the EHT process, were analyzed. Based on molecular signatures, a rare but distinct population of immediate precursors to hematopoietic progenitors, coexpressing endothelial and key hematopoietic genes, was observed. Combining molecular and cell surface (index) data allowed for prospective isolation of these EHT cells and subsequent functional analysis, which revealed that hematopoietic potential preceeds complete downregulation of the endothelial program.

Taken together, although limited in throughput and targeted in nature, the high sensitivity and accuracy of sc-qPCR analysis has been useful for answering questions related to transcription factor activity and gene regulatory networks. As such, sc-qPCR

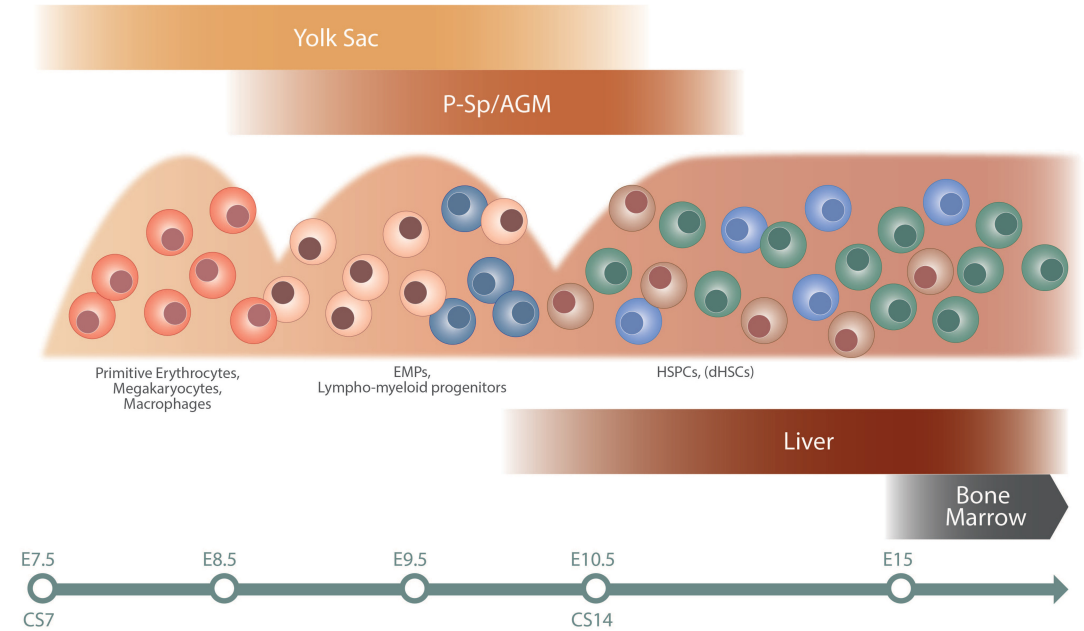

FIGURE 1 | Schematic overview of hematopoietic development. The different waves of hematopoietic cells are illustrated, as well as site of emergence (top) and colonization (bottom). The timeline at the bottom indicates approximate time in mouse (E) and human (CS). The first primitive wave of hematopoietic cells emerges in the yolk sac at approximately E7 in mouse and CS7 in human. This is followed by a second wave of EMPs and lymphoid/lympho-myeloid progenitors in mouse, a wave not fully characterized in human (Ivanovs et al., 2017). The third wave initiates in the AGM region and gives rise to HSPC and dHSCs. Progenitors from the second and third waves colonize the fetal liver. The bone marrow is colonized later in development, around E15 in mouse, and becomes the dominating hematopoietic niche around birth. AGM, aorta–gonad–mesonephros; CS, Carnegie stage; E, embryonic day; EMPs, erythro-myeloid progenitors; dHSCs, definitive hematopoietic stem cells; HSPCs, hematopoietic stem and progenitor cells; P-Sp, paraaortic splanchnopleura.

methods could still complement current less-sensitive protocols for global sc-genomics.

USING SINGLE-CELL RNA-SEQ TO STUDY ORGANOGENESIS AND EMERGING HSCs

The advent of scRNA-seq represents an advancement of single-cell genomics from targeted to global, unsupervised gene expression analysis (Tang et al., 2009; Picelli et al., 2013; Klein et al., 2015; Macosko et al., 2015; See et al., 2018). Similar to sc-qPCR, early scRNA-seq studies focused on preHSCs in the mouse embryo. Using flow cytometry, a total of approximately 100 endothelial cells and different types of preHSCs from the AGM region were purified at E11 as well as HSCs from FL at E12 and E14 (Zhou et al., 2016). Despite the low throughput, this approach, together with the possibility to measure expression of >5,000 genes/cell, allowed for investigation of the dynamic change in gene expression, as cells transform from endothelial cells to functional HSCs. Importantly, the preHSC population was found to be molecularly heterogeneous, highlighting the need for high-throughput single-cell methods. A more comprehensive transcriptional map of EHT in E9-11 AGM mouse embryos as well as gene regulatory networks and trajectories involved in the EHT process have recently been published (Baron et al., 2018; Bergiers et al., 2018; Zhu et al., 2020).

Recently, analysis of HSPCs formation by scRNA-seq methods has been extended to human embryos. As in the mouse system, there are only a few known surface markers that enrich for early preHSCs. However, exploiting the high-throughput capacity of droplet-based sequencing (10xGenomics) together with the low-throughput but more sensitive well-based RNA-seq protocol, the heterogeneity of the dorsal aorta in the human AGM region in early CS10–15 embryos could be dissected, resulting in the visualization of a landscape for HSC generation in the AGM region (Zeng et al., 2019). Importantly, hemogenic endothelial cells was observed and identified as positive for the cell surface marker CD44. These were primed toward the hematopoietic lineage coexpressing endothelial genes (e.g. *CDH5*, *SOX7*, and *ERG*) and hematopoietic transcription factors (e.g. *RUNX1*, *MYB*, and *ANGPT1*).

A marked feature of HSC emergence is that it occurs in discrete anatomical sites, specifically in clusters at the ventral wall of the dorsal aorta (Ivanovs et al., 2017). To characterize the HSC-promoting mechanisms of this niche, spatial transcriptomics was recently performed using laser capture dissection coupled with RNA sequencing (LMO-seq) at CS15–17 (Crosse et al., 2020). Additionally, the transcriptome of 2300 single cells from the dorsal aorta were examined by scRNA-seq. Together, this approach allowed for defining the molecular mechanisms behind EHT within the aortic niche as well as the identification of cell–cell interactions. Within the identified ventrally enriched signaling pathways, a novel factor, endothelin, secreted in the

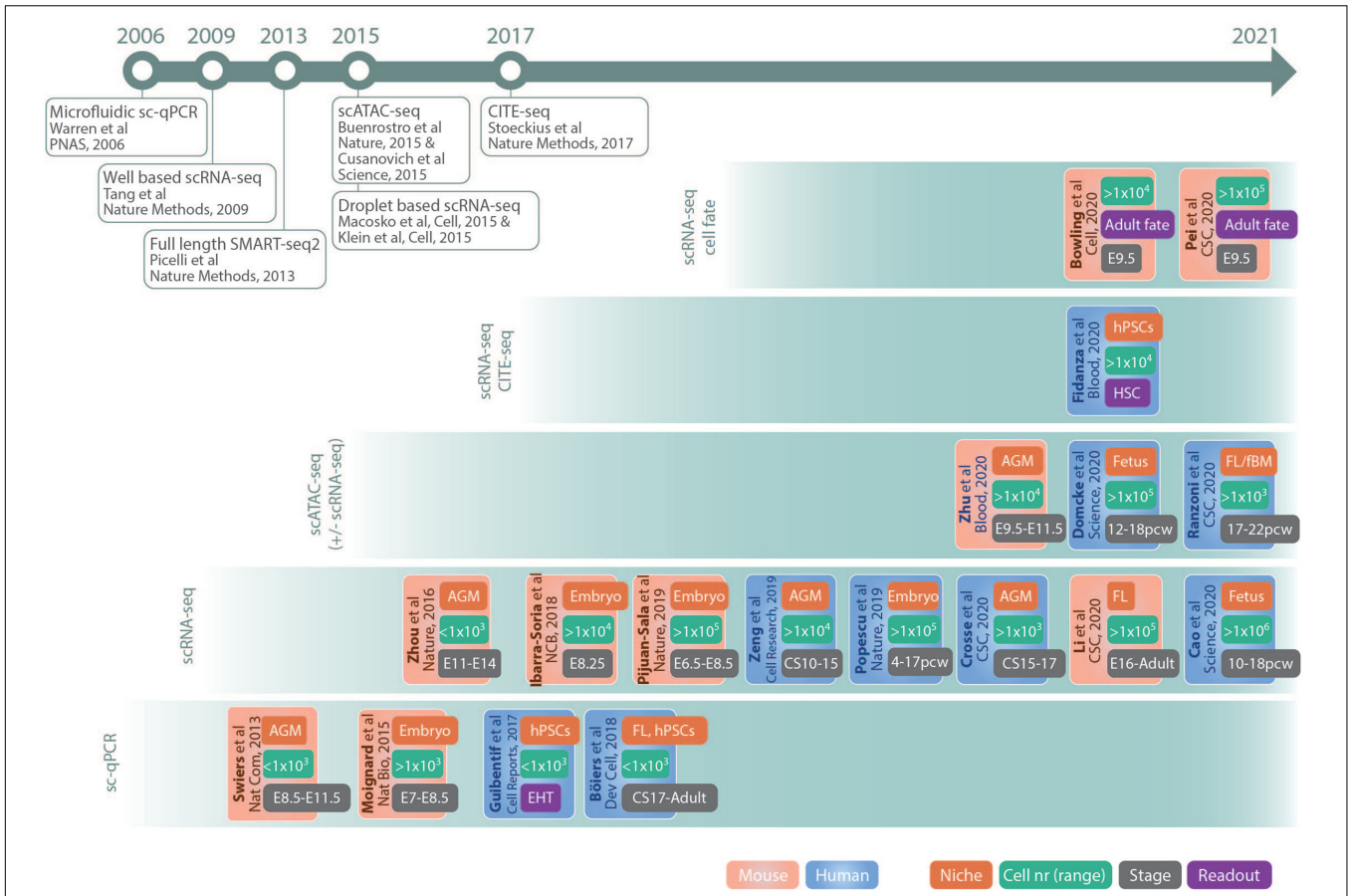

FIGURE 2 | Schematic drawing of key studies highlighted in the text. The references are grouped in panels according to the main method used and listed according to publication date. The color code of the boxes indicates if the study was performed in mouse (*red*) or human (*blue*). The different tags in the boxes indicates main fetal origin of cells investigated (*niche*, *red*), nr of cells sequenced (*cell nr*, *green*), approximate developmental age (*stage*, *gray*), and readout (*purple*). Publications of important single-cell methods are listed in the timeline at the top. AGM, aorta–gonad–mesonephros; fBM, fetal bone marrow; CS, Carnegie stage; E, embryonic day; EHT, endothelial to hematopoietic transition; FL, fetal liver; hPSCs, human pluripotent stem cells; pcw, postconceptual weeks; HSCs, hematopoietic stem cells. Abbreviations Journals: CSC, Cell Stem Cell; Dev Cell, Developmental Cell; NCB, Nature Cell Biology; Nat Bio, Nature Biotechnology; Nat Com, Nature Communications.

ventral domain of dorsal aorta, was found to promote the development of aortic clusters. This finding was subsequently validated by the authors in both mouse and human models as a potential important factor to promote formation of HSCs *ex vivo* (Crosse et al., 2020).

The increasing throughput capacity of single-cell transcriptomics has enabled studies of whole embryos to address conceptual questions regarding organogenesis, overcoming previous confounding factors such as heterogeneity, lack of cell-type-specific surface markers, and rare populations. Investigating whole mouse E8.25 embryos using droplet-based sequencing, >20,000 single cells could be characterized and 20 major cell types identified, including the brain, gut, and blood precursors. Focusing on the endothelial cell transitioning to blood, *Alox5*, involved in leukotriene production, was found to be expressed in the transition to EMP, and further functional validation found leukotriene to promote development of hematopoietic progenitors (Ibarra-Soria et al., 2018). These data represented a snapshot of the transcriptome during organogenesis but was later followed by other, time-course studies providing a cell atlas of both mouse and human blood development (Pijuan-Sala et al., 2019; Cao et al., 2020). In the study by Pijuan-Sala et al. (2019), early developmental stages from E6.5 to E8.5 were explored. Furthermore, a transcriptional map from embryo chimeras of $Tal^{-/-}$, an important transcription factor in hematopoietic development, was created to characterize the defect in early mesoderm specification (Pijuan-Sala et al., 2019). Cao et al. compared hematopoiesis at different sites in human fetal development by investigating different organs. The heterogeneity map generated was similar to what has been observed in scRNA-seq studies using other methods (Popescu et al., 2019; Bian et al., 2020), and interestingly, the human data set could be integrated and compared with a mouse embryonic cell atlas (Cao et al., 2020).

An important goal in regenerative medicine is to generate functional HSCs from hPSCs. EHT includes the formation of both HSC-dependent as well as HSC-independent progenitors. Thus, which progenitors produced by the hPSC culture and

the corresponding embryonic wave it recapitulates is difficult to state. It is also unknown if *de novo* HSCs are formed. The holistic, unsupervised, and high-throughput data generated by scRNA-seq allow for defining the PSC-differentiation process, including cell hierarchies, molecular regulation, and genetic networks, as well as for resolving heterogeneity of HSPCs formed in the culture (Angelos et al., 2018; Han et al., 2018). Recently, scRNA-seq followed by trajectory analysis revealed the cellular heterogeneity and differences between hPSCs differentiated toward blood and fetal HSCs (Fidanza et al., 2020). Furthermore, candidate surface markers with the potential to prospectively isolate distinct populations within the differentiation hierarchy were identified. Lately Cellular Indexing of Transcriptomes and Epitopes by Seq (CITE-seq; Stoeckius et al., 2017), a modified version of scRNA-seq, has been introduced where cells are stained with antibodies coupled to unique oligonucleotides that are subsequently included in the sequencing library, thus making possible direct correlation of immunophenotype and transcriptome. CITE-seq was applied to validate the cell surface markers identified by scRNA-seq in Fidanza et al. (2020), comprehensively defining the cellular and immunophenotypic hierarchy of hPSC differentiation *in vitro*. Importantly, by performing machine learning and comparing the data with published single-cell transcriptome data from the human embryo (Popescu et al., 2019), distinct cell types could be identified in the *in vitro* data set. Studies like this will help in identifying factors and differences that can improve culture conditions to generate functional HSCs for future clinical applications.

CELL HIERARCHIES AND TRAJECTORIES RESOLVED BY SINGLE-CELL GENOMICS

Adult murine hematopoiesis is viewed as a textbook example of a hierarchical structure, with the self-renewing, multipotent HSC on top of a range of increasingly lineage-committed hematopoietic progenitors (Kondo et al., 1997; Akashi et al., 2000). The hematopoietic hierarchy is constantly revised for new progenitors and complexity, while comparable cell states are recognized in human hematopoiesis (Doulatov et al., 2012; Jacobsen and Nerlov, 2019). Even when progenitor populations are carefully isolated based on expression of multiple cell-surface markers, high-throughput scRNA-seq experiments have revealed a heterogeneity resembling a differentiation continuum rather than a stepwise commitment through defined intermediate cell states (Nestorowa et al., 2016; Velten et al., 2017; Buenrostro et al., 2018; Laurenti and Gottgens, 2018).

Lineage commitment and cell fate decisions during ontogeny are less defined. However, clear differences compared to adult have been observed. Distinct fetal progenitors have been identified both in mouse and human, whereas certain immune cells are formed mainly during fetal development (Boiers et al., 2013; Notta et al., 2015; Beaudin et al., 2016; Ghosn et al., 2019). Importantly the different hematopoietic waves overlap in time as well as niche, adding to the heterogeneity. Popescu et al. created a transcriptional map of the human blood system during development, covering a time window from 4 to 17 postconceptual weeks (pcw) and impressively investigated 140,000 FL cells, as well as immune cells from other tissues like skin and kidney (Popescu et al., 2019). In total 27 different cell states were identified, including nonhematopoietic cells like hepatocytes. Hematopoietic lineage trajectory showed an HSC/multipotent progenitor (MPP) cluster differentiating toward lymphoid, myeloid, and megakaryocyte–erythroid–mast cell (MEM) progenitor populations, where HSCs/MPPs were situated at the branching point of lineage commitment, with early transcriptome priming toward all the different lineages (Popescu et al., 2019). This extensive source of data has already been used as a reference material for later studies (Cao et al., 2020; Fidanza et al., 2020).

Understanding lineage potential and commitment during ontogeny is of particular interest, as some mutations that give rise to childhood leukemia occurs *in utero*, resulting in children being more prone than adults to develop acute lymphoblastic leukemia (ALL), particularly B-ALL (Greaves, 2018). In an attempt to identify differences in fetal and adult lymphoid lineage commitment, a fetal progenitor expressing interleukin 7 receptor (IL7R) was characterized in early human development (Boiers et al., 2018). Taking advantage of the sensitivity of sc-qPCR analysis, lineage-affiliated genes were investigated and $CD19^{-}IL7R^{+}$ progenitors observed to comprise a unique lineage program, coexpressing myeloid and B-lymphoid-associated genes at the single-cell level. This developmentally restricted progenitor is interesting, as it represents a possible target childhood-leukemia-initiating cell emerging *in utero* (Greaves, 2018).

Transcriptional control of gene expression involves interaction of transcription factors with cis-regulatory elements such as promoters and enhancers. A limitation of scRNA-seq is the high-dropout levels resulting in impaired detection of low-expressed cell-type-specific transcription factors likely restricting identification of key regulators of cell function and priming. Additionally, a recent study utilizing transcribed barcodes together with scRNA-seq revealed that scRNA-seq alone could not fully resolve subtle changes involved in early hematopoietic differentiation (Weinreb et al., 2020), suggesting that other complementary methods could be used to further dissect the intricate dynamics of the process. Lately, assay for transposase-accessible chromatin using sequencing (ATAC-seq) was developed and quickly adapted for single-cell analysis (Buenrostro et al., 2015; Cusanovich et al., 2015). ATAC-seq measures chromatin accessibility, and the resulting data provide epigenetic information including active transcription factor binding sites, promotor as well as distal element usage, and accessible enhancers (Buenrostro et al., 2015; Cusanovich et al., 2015; Corces et al., 2016). ATAC-seq interrogation of distal elements have also been suggested to be superior in cell-type classification compared to RNA-seq (Corces et al., 2016). Thus, scATAC-seq provides a complementary viewpoint of heterogeneity and differentiation to scRNA-seq.

By means of a barcoded indexing approach, Domcke et al. (2020) were able to perform scATAC-seq on $\sim 8 \times 10^5$ single cells generating a cell atlas of embryonic gene regulation

covering 15 organs and 59 human fetal samples ranging between 12 and 18 pcw. These data include a comprehensive analysis of blood differentiation over time and also shows that hematopoietic cell types are similar across fetal organs (Domcke et al., 2020).

Ranzoni et al. (2020) explored lineage priming and commitment in human ontogeny at single-cell transcriptome as well as single-cell epigenetic level. Focusing on later stages of FL hematopoiesis (17–22 pcw) and including fetal BM from the same donor, HSCs and progenitors were sorted and RNA-seq performed according to SMART-seq2 (Picelli et al., 2013). The immunophenotype of the sorted populations could thus be linked to the transcriptome data. New surface markers were identified from the most highly expressed genes within the HSC/MPP cluster and used to improve purity of prospectively isolated populations. Moreover, chromatin accessibility was linked with lineage commitment, and integration of scRNA-seq and scATAC-seq data revealed high correlation between cell types. However, within the HSC/MPP population, a change in chromatin accessibility was observed before the onset of lineage commitment, indicative of priming at the chromatin level. The study highlights that lineage commitment and cell fate choice may occur at multiple levels and thus the benefit of combining chromatin accessibility with transcriptome analysis (Buenrostro et al., 2018; Weinreb et al., 2020).

DESCRIBING FETAL TO ADULT TRANSITION WITH SINGLE-CELL METHODS

Fetal and adult HSCs differ molecularly, in lineage potentials, as well as in cell cycle activity (Ema and Nakauchi, 2000; Bowie et al., 2006; Kim et al., 2007; Yuan et al., 2012; Beaudin et al., 2016; Popescu et al., 2019). Fetal HSCs are more self-renewing and proliferating than the adult counterpart, not adapting an adult-like state until around 3 weeks after birth (Bowie et al., 2006). How this transition from fetal to adult is regulated was recently investigated in mouse development, where scRNA-seq was combined with bulk ATAC-seq and ChIP-seq to investigate the transcriptional and epigenomic landscapes at different timepoints from E16 to adult (Li et al., 2020). The postnatal samples clustered separately from fetal and adult samples, indicating a gradual switch toward adult state. Furthermore, looking at fetal and adult identity scores, the changes in fetal and adult gene expressions were found to be uncoordinated causing heterogeneity. Of note, an increase in interferon just before birth proceeded the transition and was shown to induce adult transcriptional programs and made the cells vulnerable to transformation. This might have importance in our understanding of childhood leukemia, where some mutations have been shown to occur *in utero* (Greaves, 2018).

A conceptual question in fetal hematopoiesis is whether HSCs acquire a lineage preference during fetal life that is retained in adulthood. Typically, lineage trajectories are computationally inferred by the similarity of cells and their relative distance to each other and thus does not necessarily reflect the clonal relationship between cells (Weinreb et al., 2020). To link transcriptome with lineage fate, transcribable inherited marks can be introduced in the genome of the target cell allowing for the progeny of that cell to be traced. This allows for investigation of the clonal relationships of cells in conjuncture with their transcriptomic identity. Such approaches could answer lineage relationship during development and link it to gene expression signature. New techniques are rapidly evolving, where transcriptome and lineage tracing can be combined, using for instance transcribed barcodes introduced into the genome by CRISPR-Cas9. Using these kinds of methods, developmental lineage relationships during gestation in zebrafish, and later also mouse, have been investigated (Alemany et al., 2018; Kester and van Oudenaarden, 2018; Chan et al., 2019). Further development of the methods by introducing an inducible Cas9 enabled labeling at different developmental time points (Bowling et al., 2020). The study is a clear proof of concept, and lineage bias of fetal HSCs was also investigated. By labeling embryos at E9.5, when dHSCs are formed, labeled clones and their progeny could be traced in adult mice together with the transcriptome of HSCs and progenitors. Investigated clones did not show any significant lineage preference; however, they were unevenly distributed across bones, showing a bias in localization within the niche. A similar strategy, utilizing a *PolyloxExpress* barcoding system, has also been used to study cell fate of fetal HSCs in adult mice (Pei et al., 2020). Although only in their infancy, these state-of-the-art methods have already shown great promise of deciphering the earliest fate decisions. Additionally, with versatile induction of tracing, they could be applied to different developmental timepoints and genetic backgrounds to study numerus biological questions. However, these models are depending on barcode diversity as well as high labeling efficiency. Furthermore, detection levels of the unique barcodes need to be high (Wagner and Klein, 2020). By using a DNA barcode library, cells from diverse models, including human, can be labeled; however, the cells cannot be investigated *in situ* (Weinreb et al., 2020). Other methods evolving for human settings are to use somatic mutations, which can be detected with whole genome sequencing, and used to trace history of cells. By amplifying single cells in culture, enough material can be obtained for sequencing and subsequent tracing of different mutations to draw pyogenic trees. It has been shown that such approaches can trace cells back as early as to gastrulation (Lee-Six et al., 2018), and indeed, a recent study is using somatic mutation tracing to understand lineage relationships during ontogeny (Chapman et al., 2020).

CONCLUDING REMARKS

The evolution of sc-genomic techniques has opened new possibilities for understanding developmental hierarchies and differentiation landscapes. Future studies are likely to make further use of the current efforts to increase cell throughput, such as Scifi-seq (Datlinger et al., 2019). Together with multiomics, such as ASAP-seq (Mimitou et al., 2020), ECCITE-seq

(Mimitou et al., 2019), and combined scATAC/RNA-seq (10xGenomics), these protocols are important for developmental hematopoiesis, as they will improve detection of rare and embryo-specific cell types as well as make it possible to integrate cell fate, transcriptomics, proteomics, and epigenetic changes at single-cell resolution also when tissue is sparsely available, such as during embryonic development. These techniques will also be important from a disease perspective, where rare mutations in congenital immune and blood disorders can be investigated and compared to existing human developmental maps. In addition, many mutations that lead to childhood ALL occurs *in utero*, creating a preleukemic state. Integrating mutational status of a cell with transcriptional status may allow for identification of leukemic stem cells in a heterogeneous leukemic bulk sample and thus makes it possible to identify and characterize these cells and may allow for the development of new treatments. Undeniably, sc-genomics will continue to have critical impact on developmental hematopoiesis research and subsequently for our understanding of congenital immune and blood disorders, childhood leukemia, cell-replacement therapy, and for regenerative medicine as a whole.

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MINI REVIEW
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JAK-STAT in Early Hematopoiesis and Leukemia

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Hematopoietic stem cells (HSCs) produce all the terminally differentiated blood cells and are controlled by extracellular signals from the microenvironment, the bone marrow (BM) niche, as well as intrinsic cell signals. Intrinsic signals include the tightly controlled action of signaling pathways, as the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway. Activation of JAK-STAT leads to phosphorylation of members of the STAT family to regulate proliferation, survival, and self-renewal of HSCs. Mutations in components of the JAK-STAT pathway are linked with defects in HSCs and hematologic malignancies. Accumulating mutations in HSCs and aging contribute to leukemia transformation. Here an overview of hematopoiesis, and the role of the JAK-STAT pathway in HSCs and in the promotion of leukemic transformation is presented. Therapeutic targeting of JAK-STAT and clinical implications of the existing research findings are also discussed.

Keywords: JAK-STAT, STATs, hematopoiesis, hematopoietic stem cells, leukemia, STAT5

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INTRODUCTION

Hematopoietic stem cells (HSCs) produce all the terminally differentiated blood cells (**Figure 1**) and are controlled by extracellular signals from the microenvironment or niche, and intrinsic cell signals that include signaling pathways. HSCs are ideal for advanced therapies, because of their multipotent and self-renewing properties. The niche supports HSC maintenance, regulation,

Abbreviations: HSCs, hematopoietic stem cells; JAK-STAT, Janus kinase-signal transducer and activator of transcription; BM, bone marrow; HSPCs, hematopoietic stem and progenitor cells; LT-HSCs, long-term self-renewing HSCs; ST-HSCs, short-term self-renewing HSCs; MPPs, multipotent progenitors; OPPs, oligopotent progenitors; AML, acute myeloid leukemia; LSC, leukemic stem cell; AGM, aorta-gonad-mesonephros; AECs, arterial endothelial cell; HECs, hemogenic endothelial cells; E, mouse embryonic day; EC, endothelial cell; MSCs, mesenchymal stem cells; SECs, sinusoidal ECs; OLCs, osteolineage cells; NES, nestin; SCF, stem cell factor; CXCL12, C-X-C motif chemokine 12; NG2, nerve/glial antigen 2; LEPR, leptin receptor; CAR, CXCL12-abundant reticular cells; Foxc1, forkhead box C1 protein; CXCL4, C-X-C motif chemokine 4; Dll4, Delta-like Notch ligand 4; ROS, reactive oxygen species; TPO, thrombopoietin; CXCR4, C-X-C motif chemokine receptor 4; TYK2, tyrosine kinase 2; IFN-β, interferon beta; Ser, serine; CTCL, cutaneous T-Cell lymphoma; MPNs, myeloproliferative neoplasms; PV, polycythemia vera; Pf4-Cre, Platelet factor 4-Cre recombinase; ETP, early T-cell Precursor; ALL, acute lymphoblastic leukemia; IL-7, interleukin 7; MDS, myelodysplastic syndromes; FLT3, fms-related receptor tyrosine kinase 3; FLT3-ITD, FLT3-internal tandem duplication; miR, microRNA; lncRNA, long non-coding RNA; PTCL, peripheral T-cell lymphoma; LGL, large granular lymphocytic; T-ALL, T-cell acute lymphoblastic leukemia; EBF1, early B cell factor 1; CLL, chronic lymphocytic leukemia; miRNA, microRNA; HSCT, HSCs transplantation; CARs, chimeric antigen receptors; TALEN, transcription activator-like effector nucleases; CRISPR, clustered regularly interspaced short palindromic repeats; JAKinib, JAK inhibitor; FDA, Food and Drug Administration; CMML, chronic myelomonocytic leukemia; CML, chronic myelogenous leukemia; AKIs, aurora kinase inhibitors; mRNA, messenger RNA; ASO, antisense oligonucleotides; siRNA, small interfering RNA; HDAC, histone deacetylase; NMSC, non-myelinating Schwann cells; LMPPs, lymphoid-primed multipotential progenitors; CLPs, common lymphoid progenitors; CMPs, common myeloid progenitors; MEPs, megakaryocyte/erythrocyte progenitors; GMPs, granulocyte/macrophage progenitors; NK-cells, natural-killer cells.

self-renewal and proliferation (Crane et al., 2017). Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway activation leads to phosphorylation of STATs that regulate hematopoiesis, and HSCs proliferation, survival and self-renewal. Dysregulation of the JAK-STAT pathway has been associated with various malignancies. STAT5, a member of the STAT family, controls normal lympho-myeloid development (Wang and Bunting, 2013) and plays a critical role in leukemia. Leukemia, characterized by overproduction of abnormal blood cells and defects in HSCs, is considered an age-related disease and its incidents rose continuously in the last decades (Hao et al., 2019). Albeit extensive research in this field, a lot of questions on the underlying molecular mechanisms of JAK-STAT in HSCs in normal lympho-myeloid development and leukemia remain unanswered. A better understanding of the mechanisms and signaling pathways in HSCs will contribute to improving already existing therapeutic approaches and design novel ones for hematopoietic malignancies. Here, a short overview of the advances on HSCs biology and the role of the JAK-STAT pathway in early hematopoiesis and leukemia, together with therapeutic implications of the existing research findings are discussed.

HEMATOPOIESIS

Hematopoietic Stem and Progenitor Cells, and Aging

Hematopoiesis generates all the terminally differentiated cellular blood components (**Figure 1**). HSCs can be found reposed or they proliferate and differentiate, depending on their internal programming and the external signals from the microenvironment (Nakamura-Ishizu et al., 2014). HSCs have the unique potential for multi-potency and self-renewal (Seita and Weissman, 2010) and in adults are mainly situated in the bone marrow (BM). HSCs continuously replenish the blood throughout the lifetime (Orkin and Zon, 2008; Dzierzak and Philipsen, 2013) and can functionally reconstitute the entire blood system in an irradiated recipient by stem cell transplantation (Appelbaum, 2007). Hematopoietic stem and progenitor cells (HSPCs) pool contains long-term self-renewing HSCs (LT-HSCs), short-term self-renewing HSCs (ST-HSCs), and non-self-renewing multipotent progenitors (MPPs) (Seita and Weissman, 2010; Zhang et al., 2019). Distinct myeloid-biased MPP subsets work together with lymphoid-primed MPP cells to guide the generation of blood components. MPPs are produced in parallel by HSCs, at different levels and kinetics depending on the hematopoietic needs in normal or regenerating conditions. In the latter case, the myeloid-biased MPPs are overproduced by HSCs for a short time, to support myeloid amplification and rebuilding of the hematopoietic system (Pietras et al., 2015). The multi-lineage priming of MPPs is linked to low-level activation of expression programs for various lineages. Lineage fate choice is then connected with activation of a specific expression program while the rest are switched-off. Recent single-cell technologies have questioned the rigid past model of hematopoiesis of MPPs advancing to oligopotent progenitors (OPPs), and then to lineage-committed and mature effector cells. The fluidity of HSC differentiation is today represented more by a continuum than a rigid step-by-step procedure. Heterogeneous populations are organized hierarchically, with gradual highly flexible progression during differentiation (**Figure 1**; Laurenti and Gottgens, 2018; Jacobsen and Nerlov, 2019).

Mutations during HSCs development lead to leukemia, myelodysplasia, or BM failure. HSCs are susceptible to age-related changes triggered by intrinsic and extrinsic factors. Aged HSCs feature defective repopulating and homing capacity, increased mobilization and myeloid lineage-biased skewing, decreased fitness, and epigenetic/genetic changes (Lee J. et al., 2019). Many hematological malignancies, including acute myeloid leukemia (AML), are age-dependent. Aging is also connected to expanded clonal hematopoiesis (Konieczny and Arranz, 2018). High fitness of the young HSC pool serves to maintain the existing condition, while in an aged HSC pool the low fitness allows accumulation of mutations and epigenetic changes to improve fitness. For example, *Bcr-Abl* provides an advantage to aged B-lymphoid progenitors compared to young ones, leading to increased *Bcr-Abl* leukemogenesis (Henry et al., 2010, 2011). HSCs or other progenitors when undergoing a mutation can give rise to a leukemic stem cell (LSC), which features a dysregulated self-renewal program (**Figure 1**). LSCs differentiate into the hematopoietic lineage carrying the mutation/s or remain as immature progenitor cells, called leukemic blast cells (Buss and Ho, 2011; Hanekamp et al., 2017; Vetrie et al., 2020).

Bone Marrow Niche

The BM niche constitutes a specialized microenvironment, composed of diverse cell types to support maintenance, induction, differentiation and proliferation of HSCs in embryos and adults. Definitive HSCs develop from the hemogenic endothelium within the aorta-gonad-mesonephros (AGM) region, then migrate to the fetal liver and finally to the adult BM (Gao et al., 2018). Single-cell transcriptomics analysis has defined two molecularly distinct arterial endothelial cell (AEC) populations and putative HSC-primed hemogenic endothelial cells (HECs) in the dorsal aorta of the AGM region, whose number peaked at mouse embryonic day (E) 10.0 and decreased thereafter (Hou et al., 2020). Primitive vascular endothelial cells (ECs) from E8.0 experienced an initial arterial fate choice to become HSC-primed HECs, followed by a hemogenic fate conversion (Hou et al., 2020). Similarities in the development of HSC-primed HECs between mouse and human embryos exist (Zeng et al., 2019; Hou et al., 2020).

The BM niche includes mesenchymal stem cells (MSCs), ECs [AECs and sinusoidal ECs (SECs)], osteolineage cells (OLCs), non-myelinating Schwann cells and progeny of HSCs (such as megakaryocytes and macrophages) located together with the HSCs in the extracellular matrix (**Figure 1**; Yu and Scadden, 2016; Mendez-Ferrer et al., 2020; Mitroulis et al., 2020). Different niche cell populations regulate the balance between HSC proliferation and quiescence during homeostasis or regenerative hematopoiesis. Identification of MSCs, which are important for HSCs function, has relied on reporter mouse models for markers including Nestin (NES), Stem Cell Factor (SCF), CXC chemokine ligand 12 (CXCL12), nerve/glial antigen 2 (NG2), and Leptin receptor

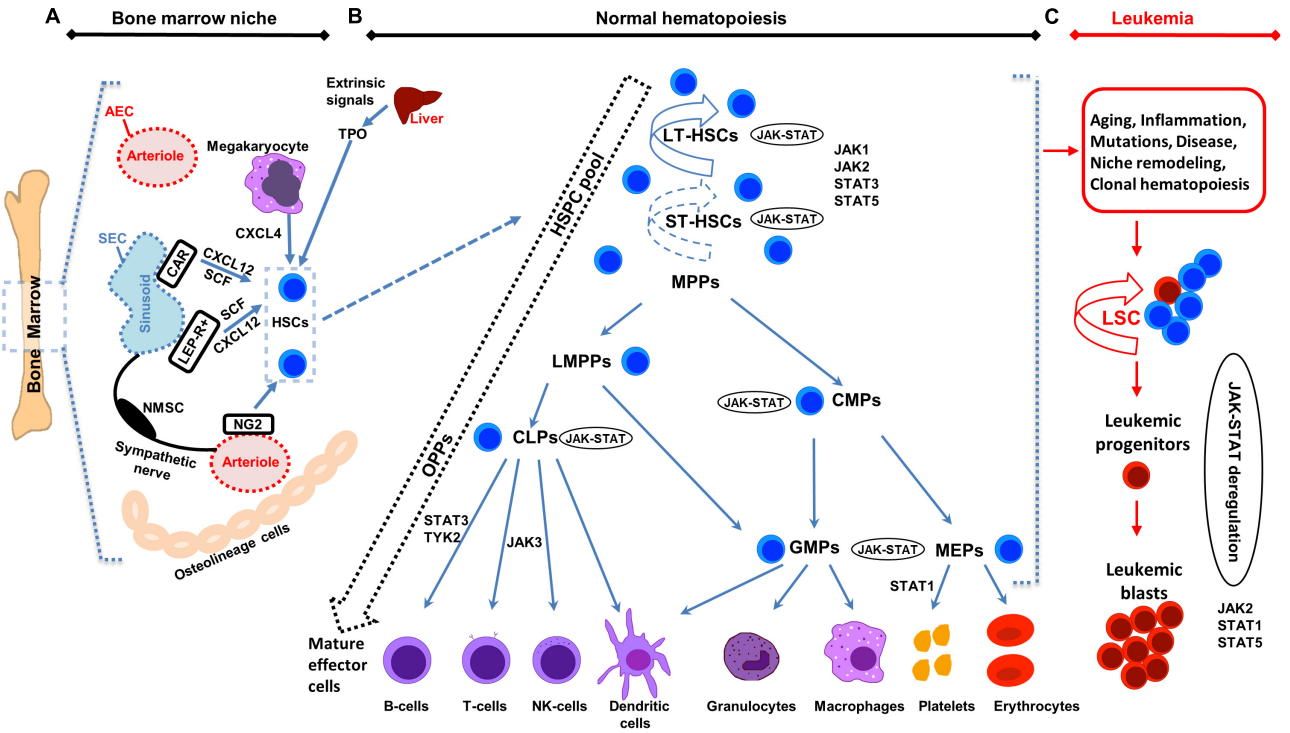

FIGURE 1 | Illustration of bone marrow niche, normal and leukemic hematopoiesis. **(A)** Schematic representation of the bone marrow (BM) niche summarizing key cell types and functional features. HSCs reside in the proximity of BM vessels (arterioles or sinusoids). Mesenchymal stem cell (MSC) populations include, among others, NG2 +, LEPR + and CAR cells and promote HSCs maintenance by releasing important factors (i.e., CXCL12, SCF). Endothelial cells (ECs) (arteriolar endothelial cells (AECs), surrounding the arterioles and sinusoidal endothelial cells (SECs), surrounding the sinusoids) also release important factors for HCSs maintenance. Sympathetic nerve fibers regulate HSCs migration through the sinusoids. MSC subpopulations, ECs, non-myelinating Schwann cells (NMSCs) and HSC progeny (i.e., megakaryocytes) contribute to the regulation of HSC homeostasis or regenerative hematopoiesis. Megakaryocytes produce CXCL4 to regulate HSCs. **(B)** Schematic representation of normal hematopoiesis. HSCs reside at the top of the hierarchy. Differentiation is considered today more of a continuum, than a step-by-step procedure, represented by the dashed arrow on the left. The HSC pool is heterogeneous in terms of self-renewal and differentiation properties. Self-renewal of HSCs is denoted by an arrow around the cells (solid arrow represents strong and dashed arrow weaker self-renewal potential). Hematopoietic Stem and Progenitor cells (HSPCs) pool contains long-term self-renewing HSCs (LT-HSCs), short-term self-renewing HSCs (ST-HSCs) and non-self-renewing multipotent progenitors (MPPs). Throughout differentiation, HSCs might first lose self-renewal capacity and then lineage potential as they commit to evolving to a mature functional cell of a specific lineage. MPPs, might have unilineage, bi- or trilineage potential. MPPs advance to oligopotent progenitors (OPPs), including the lymphoid-primed multipotent progenitors (LMPPs), the common lymphoid progenitors (CLPs) and the common myeloid progenitors (CMPs). The myeloid and lymphoid compartments remain associated in the hierarchy *via* the lymphoid-primed multipotent progenitors (LMPP). CMPs give rise to megakaryocyte/erythrocyte progenitors (MEPs) and granulocyte/macrophage progenitors (GMPs). LMPPs give rise to give GMPs and CLPs. The OPPs through the lineage-restricted progenitors give rise to the mature effector cells (B-cells, T-cells and NK-cells, dendritic cells, granulocytes, macrophages, platelets, and erythrocytes). **(C)** Schematic representation of leukemic hematopoiesis. Aging, mutations, disease, inflammation, niche dysfunction/alterations and clonal hematopoiesis can lead to the generation of a leukemic stem cell (LSC). LSC can differentiate into the hematopoietic lineage carrying the mutation/s or remain as immature progenitor cells, called blast cells. Not all intermediate cell stages are depicted and cells are not in scale. Main differentiation points where the JAK-STAT pathway, JAKs and STATs exert their roles are shown.

(LEPR) (Sugiyama et al., 2006; Ding et al., 2012; Sugiyama and Nagasawa, 2012; Ding and Morrison, 2013; Kunisaki et al., 2013). NG2^{+} pericytes, found spatially linked to arteriolar niches, have been confirmed to be important for the maintenance of HSC quiescence (Kunisaki et al., 2013). HSCs are localized predominantly in the perisinusoidal space and in close proximity to Leptin Receptor^{+}Cxcl12^{high} cells (Acar et al., 2015). Adipo-osteogenic progenitors have been also found essential for HSCs proliferation and maintenance in an undifferentiated state (Omatsu et al., 2010). Depletion of CXCL12-abundant reticular (CAR) cells *in vivo* has led to severe impairment of the adipogenic/osteogenic differentiation competency, and reduced SCF and CXCL12 production, resulting in decreased lymphoid and erythroid progenitors cycling (Omatsu et al., 2010). ECs and *Lepr*-expressing perivascular cells, through the expression of essential factors, such as SCF, maintain HSCs and *Scf* deletion from both endothelial and *Lepr*-expressing cells has led to HSCs depletion from the BM (Ding et al., 2012). *Foxc1* has been found significant for the development and maintenance of the mesenchymal niches, through enhancement of CAR cell development by upregulation of CXCL12 and SCF expression (Omatsu et al., 2014). Megakaryocytes have been also found to control HSCs quiescence by producing CXCL4 (Bruns et al., 2014). Single-cell RNA-seq has characterized in detail the mouse BM stroma in homeostasis and leukemia. Seventeen distinct cell populations have been defined, including MSCs, OLCs,

chrondrocytes, fibroblasts, pericytes, and EC subsets, together with new differentiation paths (Baryawno et al., 2019). The dynamic and diverse transcriptional landscape of vascular, perivascular, and osteoblast BM niche cell populations has been confirmed both at homeostasis and stress hematopoiesis (Tikhonova et al., 2019). Vascular-endothelial Delta-like Notch ligand 4 (*Dll4*) expression regulates HSC differentiation and lineage commitment. Under stress conditions transcriptional remodeling of the niche has been linked to an adipocytic skewing of perivascular cells and vascular *Dll4* absence has led to a premature skewing of HSPCs toward a myeloid transcriptional program (Tikhonova et al., 2019).

Together with perivascular MSCs, ECs control HSPCs maintenance and leukocyte trafficking by forming a network of blood vessel types with distinct permeability properties. Heterogeneity amongst the contribution of the EC subpopulations to the stem cell niches has been revealed. Deletion of *Scf* in AECs, but not in SECs, has led to a reduction of functional HSCs (Xu et al., 2018). The highly permeable SECs promote HSPCs activation and constitute the site for leukocyte trafficking to and from the BM. The high permeability, associated with high reactive oxygen species (ROS) levels, increases HSPCs migration and differentiation, while compromising their long-term repopulation and survival. The less permeable arterial blood vessels maintain HSPCs in low ROS levels (Itkin et al., 2016). The establishment of unique perivascular micro-niches has been moderated by divergent localization to sinusoidal and arteriolar surfaces of CAR cell subsets (Adipo-CAR and Osteo-CAR) that mainly function as cytokine-producing cells (Baccin et al., 2020). Furthermore, live imaging of LT-HSCs in the mouse native niche defined a subset of highly quiescent LT-HSCs, residing close to both sinusoidal blood vessels and the endosteal surface. MPPs have been mainly linked to transition zone vessels. Steady-state LT-HSCs showed limited motility in contrast with activated LT-HSCs exhibiting high motility or clonal expansion in spatially restricted domains. These domains include BM cavities with remodeling features, where HSCs expansion takes place, and cavities with low bone-resorbing activity, lacking HSCs expansion, where the microenvironment might differ (Christodoulou et al., 2020). In addition to the significance of the intrinsic BM signals, extrinsic factors are also critical for HSC maintenance, as shown for thrombopoietin (TPO) expressed by hepatocytes (Decker et al., 2018).

Changes in BM niche might directly affect myeloid vs. lymphoid output. The niche changes substantially during aging (Lee G.Y. et al., 2019) and plays a major regulatory role in malignancies, where either alterations in BM can promote leukemic transformation or create a favorable microenvironment for malignant proliferation, though BM remodeling by LSCs. For example, LSCs can upregulate CXCR4 expression (Pinho and Frenette, 2019; Mendez-Ferrer et al., 2020). Different leukemia types can be linked with induction of specific niche remodeling alterations. Remodeling of BM stromal cell subpopulations in AML has been confirmed by single-cell RNA-seq. These findings support a model where the malignant cells alter differentiation of the surrounding stromal cells and decrease the expression of signaling molecules regulating normal hematopoiesis. The malignant clone competes with the normal hematopoietic cells, creating a less supportive environment (Baryawno et al., 2019). Further characterization of the niche heterogeneity will provide additional insights on the control of HSC quiescence vs. proliferation in young, aged and malignant conditions.

JAK-STAT PATHWAY IN NORMAL HEMATOPOIESIS AND HEMATOLOGIC MALIGNANCIES

JAK-STAT in Early Hematopoiesis

The JAK-STAT is amongst the most conserved signaling pathways allowing communication between the extracellular environment and the nucleus. It can be activated by a plethora of cytokines, growth factors and hormones and regulates proliferation, differentiation, migration and cell survival depending on the cellular context and the environmental stimuli (Harrison, 2012). JAK-STAT is important in developmental and homeostatic processes including, stem cell maintenance, hematopoiesis and immune cell development. The JAK family of kinases includes JAK1, -2, -3, and TYK2 (Firmbach-Kraft et al., 1990; Krolewski et al., 1990; Wilks et al., 1991; Takahashi and Shirasawa, 1994). STAT protein family in mammals includes STAT1, -2, -3, -4, -5a, -5b, -6, which contain a conserved structure (Ihle, 1996, 2001; Darnell, 1997). Ligand binding to the receptor allows JAK phosphorylation and activation that leads to phosphorylation of the receptor, acting as a docking site for the STATs that are subsequently phosphorylated by JAKs. This leads to the formation of STAT homodimers or/and heterodimers that translocate to the nucleus and bind to DNA to regulate transcription.

JAK2 activation, by several hematopoietic and other cytokines, leads to phosphorylation of STATs (Bousoik and Montazeri Aliabadi, 2018), including STAT5 that regulates HSCs proliferation, survival and self-renewal (Wang and Bunting, 2013). JAK1 and JAK2 are essential for HSC homeostasis. Conditional *Jak1* deletion in HSCs *in vivo* reduced their self-renewal capacity and modified lympho-myeloid differentiation (Kleppe et al., 2017), whereas *Jak2* knock-out is embryonic lethal due to ineffective erythropoiesis (Neubauer et al., 1998; Parganas et al., 1998). Conditional *Jak2* knock-out leads to BM failure, increased apoptosis and loss of quiescence in HSC-enriched $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ cells, confirming its critical role for HSCs maintenance and function (Akada et al., 2014). JAK3 has been found essential for innate lymphoid cell development (Robinette et al., 2018) and TYK2 for B-lymphoid tumors regulation (Stoiber et al., 2004).

STAT1 plays an important role in megakaryopoiesis (Huang et al., 2007). Activated STAT3 has promoted HSC self-renewal, under stimulated but not homeostatic states, rendering STAT3 significant for hematopoietic regeneration (Chung et al., 2006). STAT3 phosphorylation is required for the IFN-β induced apoptosis in primary pro-B cells (Gamero et al., 2006). Selective activation of STAT5 confirmed its role in the self-renewal of normal and leukemic stem cells

(Kato et al., 2005). STAT5, through survival effects on HSCs, supports the hematopoietic reserve and promotes multilineage hematolymphoid development. STAT5A/5B-deficient mice show an impaired hematopoietic potential in diverse blood lineages (Snow et al., 2002). Induction of high STAT5A activity levels impaired myelopoiesis and induced erythropoiesis in $CD34^+$ cells, while intermediate levels resulted in maximum proliferation (Wierenga et al., 2008). Distinct cytokine responses in STAT5 phosphorylation at the single-cell level of leukemic and normal progenitors exist (Han et al., 2009). STAT5A and STAT5B possess distinct cell-growth-promoting properties that differentially affect the biological activity of HSPCs. STAT5A phosphorylation at Ser779/780 (mouse/human) controls proliferation and transformation/expansion of HSPCs with higher potency than STAT5B (Ghanem et al., 2017). Other STATs are also involved in normal and leukemic hematopoiesis. For instance, CD38 expression in the BM microenvironment of multiple myeloma cells is regulated by both STAT1 and STAT3 (Ogiya et al., 2020).

JAK-STAT in Hematologic Malignancies

Since the 1990s numerous studies have confirmed the association between activating mutations in JAK-STAT and hematologic disorders (Leonard and O'Shea, 1998; Levine et al., 2007; Jatiani et al., 2010). Such mutations leading to constitutive activation of JAK-STAT can occur upstream or within the molecular components of the pathway. These include mutations of the transmembrane receptors, the JAKs or other upstream oncogenes, the STATs and the autocrine/paracrine cytokine production, which collectively leads to STAT activation (O'Shea et al., 2015). Deregulated JAK/STAT signaling due to *JAK1* and *JAK3* somatic mutations has been observed in Cutaneous T-Cell Lymphoma (CTCL) (Perez et al., 2015). Translocations of the *JAK2* gene or the *JAK2*V617F mutation are underlying causes of hematological malignancies (Baxter et al., 2005; James et al., 2005; Jones et al., 2005; Levine et al., 2005). *JAK2*V617F is an activating point mutation resulting in increased JAK2 activity, leads to STAT5 activation (Levine et al., 2005), and has been described in the majority of patients with myeloproliferative neoplasms (MPNs). It has been detected in almost all patients with polycythemia vera (PV) and about 50% of the patients with essential thrombocytosis and primary myelofibrosis (Baxter et al., 2005; Passamonti and Maffioli, 2016). In PV patients the mutation occurs in HSCs and predisposes toward erythroid differentiation (Jamieson et al., 2006). Mouse models have contributed to the understanding of the mechanisms by which JAK-STAT or related mutations promote hematopoietic malignancies (Dunbar et al., 2017). Expression of *Jak2*V617F in BM progenitors resulted in a PV-like syndrome with myelofibrosis in a mouse BM transplant model (Wernig et al., 2006). Use of Pf4-Cre transgenic mice to drive *Jak2*V617F expression in megakaryocyte lineage-committed cells, augmented erythropoiesis and stimulated fibrosis, resulting in a myeloproliferative state. These findings confirmed that JAK/STAT activation in megakaryocytes induced myeloproliferation and is essential for MPN maintenance *in vivo* (Woods et al., 2019). Xenograft mouse models have also contributed to the understanding of JAK/STAT mechanisms in leukemia. For example, the importance of JAK/STAT in early T-cell precursor (ETP) acute lymphoblastic leukemia (ALL) has been confirmed when the JAK1/2 inhibitor ruxolitinib has been used in murine xenograft models leading to abrogation of the STAT5 activation in response to IL-7 (Maude et al., 2015).

STAT1, STAT3, and STAT5 have been found, since the 1990s, constitutively activated in cells from acute leukemias (Gouilleux-Gruart et al., 1996, 1997). STAT1 has been defined as a tumor promoter in leukemia development (Kovacic et al., 2006). STAT5 contributes to the development of malignancies influencing myeloid and lymphoid lineages. A constitutively activated STAT5A mutant, forming enhanced levels of stable tetramers has caused multilineage leukemias, with STAT5 tetramers to accumulate in excess to dimers in human leukemias (Moriggl et al., 2005). STAT5A Ser725 and 779 phosphorylation detected in human leukemic cell lines and primary patient samples has been found essential for hematopoietic cell transformation (Friedbichler et al., 2010). Additionally, the N-terminus of STAT5A/B is functionally important in B-lymphoid transformation (Hoelbl et al., 2006).

Myelodysplastic syndromes (MDS), a heterogeneous group of clonal disorders of HSCs with a risk of progression to AML (Sperling et al., 2017; Cazzola, 2020; Garcia-Manero et al., 2020), have complex molecular pathogenesis due to the high genomic heterogeneity (Awada et al., 2020). The development of AML is considered a multi-cause and -step process (Gruszka et al., 2017). Translocations and inversions including fusion oncogenes, that use the JAK-STAT pathway, have been involved. Initial activating mutations in receptor tyrosine kinases (e.g., FLT3) promote proliferation of hematopoietic progenitors and subsequently additional mutations targeting transcription factors and impairing differentiation and apoptosis are required to result in leukemic cells (Gilliland, 2002; Gruszka et al., 2017). *FLT3* is among the most commonly mutated genes in AML (Kiyoi et al., 2002; Ley et al., 2013). AML-specific *Flt3* mutations have induced STAT target genes (Mizuki et al., 2003) and *FLT3*-D835 mutation has led to constitutive activation of STAT5 (Taketani et al., 2004). Levels of CDC25A, a phosphatase important for proliferation and differentiation in AML expressing the *FLT3*-ITD mutation, are controlled by a complex STAT5/miR-16 transcription and translation pathway, confirming that *FLT3*-ITD/STAT5/miR-16/CDC25A interplay is important for AML cell proliferation and differentiation (Sueur et al., 2020). Furthermore, induced inflammatory response in the human AML niche leads to increased activity of the JAK/STAT pathway in AML blasts and BM stromal cells promoting leukemic proliferation (Habbel et al., 2020). An imatinib-upregulated lncRNA family has been identified as a negative regulator of Bcr-Abl-induced tumorigenesis, through suppression of the STAT5-CD71 pathway (Wang et al., 2019). STAT5B has been defined as more important than STAT5A in BCR/ABL-induced leukemia, explaining the high frequency of STAT5B mutations in hematopoietic malignancies (Kollmann et al., 2019). High activity levels of STAT5A and STAT5B variants in the hematopoietic system of transgenic mice can lead to a lethal condition resembling human peripheral T-cell lymphoma (PTCL) and

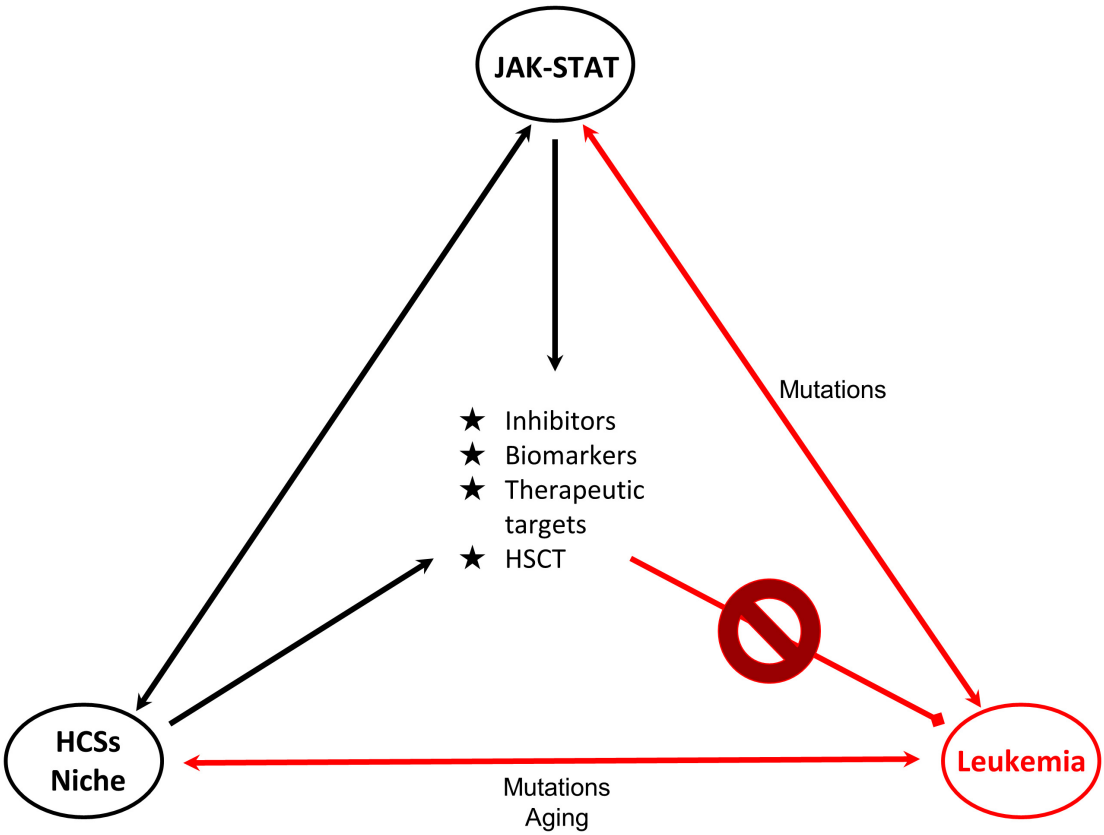

FIGURE 2 | Connections of JAK-STAT, HSCs and leukemia. Schematic representation of the connections of HSCs-bone marrow niche, JAK-STAT pathway and leukemia is shown. JAK-STAT pathway regulates HSCs proliferation, survival and self-renewal, and components of the BM microenvironment. Mutations linked to JAK-STAT and/or HSCs-niche can lead to leukemic transformation. Research findings on these connections provide opportunities (in the middle of the triangle) for the management and therapy of leukemia. Double arrows represent bidirectional connections. For example mutations in JAK-STAT can cause leukemia, but also in leukemia cells deregulated JAK-STAT pathway is observed. HSC: Hematopoietic stem cell, HSCT: Hematopoietic stem cell transplantation.

elevated expression of STAT5A/B has been detected in human PTCL samples. Both factors have been confirmed as oncogenes in PTCL, with STAT5B to be more transforming (Maurer et al., 2020). Mutations in *STAT3* (Koskela et al., 2012) and *STAT5B* genes have been detected in large granular lymphocytic (LGL) leukemia patients, with the *STAT5B*N642H mutation to be connected with unfavorable disease progression (Rajala et al., 2013). The same mutation has been commonly found in pediatric T-cell acute lymphoblastic leukemia (T-ALL) and is linked to a higher risk of relapse (Bandapalli et al., 2014). Recently, a key contributor to B-cell lymphopoiesis, Early B cell factor 1 (EBF1), has been shown to possess an inhibitory role in chronic lymphocytic leukemia (CLL) through inactivation of the STAT5 pathway (Wang et al., 2021).

These findings confirm the functional involvement of mutated/activated STATs, miRNAs, and lncRNAs in hematologic malignancies. Numerous studies have identified target genes regulated by STATs in normal and leukemic settings (Theodorou et al., 2013; Nanou et al., 2017). Developments in next-generation sequencing at the multi- and single-cell level have contributed to the acceleration of such identifications. Genes, lncRNAs, miRNAs targeted by STAT factors are useful in stratification strategies, management of leukemia and provision of novel therapeutic targets.

THERAPEUTIC IMPLICATIONS: HSCs TRANSPLANTATION AND JAK-STAT INHIBITORS

Hematopoietic stem cells are extensively utilized in advanced regenerative medicine therapies (Dessie et al., 2020). Cell damage in hematological malignancies can be restored by HSCs transplantation (HSCT). Advancements in transplant immunology led to decreased transplant-associated mortality and more effective HSCT. Efforts regarding allogeneic HSCT mainly focus on conditioning therapies, donor selection, and stem cell sources. The combination of graft-vs.-leukemia effector cells contained in the stem cell graft with advances on the human leukocyte antigen system allowed enhanced antitumor effect and improved donor selection (Juric et al., 2016). Alternative stem cell sources including granulocyte-colony stimulating factor-mobilized peripheral blood stem cells and cord blood cells have been also validated. Genetically modified T-cells

expressing chimeric antigen receptors (CARs) specific for a selected tumor antigen, such as CD19 in B-cell malignancies, have been also introduced as more effective antileukemic cell-based approaches. Gene-editing tools including transcription activator-like effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR) (Li et al., 2020) resulted in eliminated alloreactivity and decreased immunogenicity. However, further optimizations are needed, and many challenges still exist.

The JAK-STAT pathway constitutes a promising target for the development of various indirect and direct inhibitors for malignancies (Springuel et al., 2015; Brachet-Botineau et al., 2020). Indirect inhibitors focus on approaches using upstream tyrosine kinases targeting, natural and synthetic molecules, and drug repositioning. The understanding of the *JAK2*V617F mutation mechanism and the elaboration of the pseudokinase domain structure has provided the opportunity for the development of JAK2 inhibitors for MPN treatment targeting only the mutated kinase, as JAK2 is necessary for normal hematopoiesis. The first selective JAK inhibitor (JAKinib) to be tested and later approved in humans has been Tofacinitib, which targets JAK1, JAK2 and JAK3 (Kontzias et al., 2012; O'Shea et al., 2015). Ruxolitinib, the first JAKinib approved by the United States Food and Drug Administration (FDA), is a potent inhibitor of JAK1 and JAK2, used for primary myelofibrosis (O'Shea et al., 2015) and its effects have been also studied in MDS, AML, ALL, chronic myelomonocytic leukemia (CMML) and chronic myeloid leukemia (CML) (Eghtedar et al., 2012; Pemmaraju et al., 2015). JAKinibs might also ameliorate treatment by monoclonal antibody therapies for myeloma patients. This represents a novel therapeutic option, as Ruxolitinib inhibition of the JAK-STAT3 pathway has been shown to increase CD38 expression and anti-CD38 monoclonal antibody-mediated cytotoxicity (Ogiya et al., 2020). Another class of indirect inhibitors includes the first- and next-generation FLT3 inhibitors for AML. First-generation inhibitors lack specificity. Next-generation inhibitors have higher specificity, potency, lower toxicities and are under clinical investigation for AML (Daver et al., 2019). Recently an inhibitor targeting Aurora A (AKI604), has been shown to block the leukemic proliferation induced by STAT5, thus suggesting that the use of Aurora kinase inhibitors (AKIs) might be promising to overcome STAT-induced leukemic proliferation in AML (Wang et al., 2020).

Several natural and synthetic compounds exerting anti-tumor functions through their action on STAT3 and/or STAT5 signaling have been developed. These low toxicity compounds can synergize with other pharmacological agents to reverse chemoresistance. For example, the inhibitor 17f has been shown to selectively inhibit STAT5 signaling in CML and AML cells (Brachet-Botineau et al., 2019). Resveratrol, a naturally occurring plant compound, inhibited STAT5 activation in CML cell lines, providing a potential CML treatment (Li et al., 2018).

For drug repositioning, cell-based assays for high-throughput screening have been employed to identify compounds specifically inhibiting STAT3/5 transcriptional activity. For instance, pyrimethamine, an antimalaria drug, previously identified as a STAT3 signaling inhibitor, provided a potential AML treatment (Takakura et al., 2011; Sharma et al., 2016).

Direct inhibitors of STAT3/5 include molecules obstructing tyrosine phosphorylation, dimerization, nuclear translocation and/or DNA binding. Inhibitors targeting STAT3/5 domains or mRNAs have been developed (Brachet-Botineau et al., 2020). Nucleic acid based inhibition strategies include antisense oligonucleotides (ASO), siRNA, dominant-negative constructs, G-quartet oligonucleotides and decoy oligonucleotides (Sen and Grandis, 2012). AZD9150, an ASO targeting STAT3 mRNA, has decreased viability in leukemic cell lines (Shastri et al., 2018) and is now in phase 1/2 clinical trials (Brachet-Botineau et al., 2020).

CONCLUSION

Research on HSCs and the BM niche has shed light on normal and leukemic hematopoiesis, however, their molecular intricacies have not been fully delineated. The developments in the field of single-cell omics have enhanced the understanding of the cellular and molecular organization of the niche bringing us a step closer to a more detailed functional characterization to improve HSCT and to discover novel therapeutic strategies for leukemia. Applied induction of effector CAR immune cells from HSCs can produce large numbers of immune cells for clinical evaluation. Gene therapy using autologous HSCs overcame the major issue of donor compatibility and ongoing research will further optimize the therapeutic dosage control, the low cell targeting and the retention in malignancy sites, however, many challenges remain to fully treat leukemia and its relapse (Chu et al., 2020). Research findings on the interconnections between HSCs-niche and signaling pathways (i.e., JAK-STAT) will further contribute to new approaches in stem cell engineering, HSCT and combinations with pharmacological approaches to improve safety and efficacy.

The delineation of the role of the JAK/STAT pathway in hematologic malignancies rendered its components ideal candidates for the development of novel therapeutic strategies. STAT5, a significant signaling regulator in normal HSCs and LSCs constitutes an attractive candidate for innovative therapies. Combinations of JAKinibs with STAT inhibitors, monoclonal antibodies, growth factor support, hypomethylating agents, chemotherapy and allogeneic HSCT might be beneficial. Pyrimethamine, a direct inhibitor of activated STAT3, conjugated with histone deacetylase inhibitors, also known to inhibit STAT3 activation, has been used successfully in a breast cancer cell line for HDAC and STAT3 pathway inhibition (Wu et al., 2020). It cannot be excluded that conjugated inhibitors might also provide novel therapeutic solutions for hematologic malignancies. Targeting the communication between leukemia-initiating cells and their microenvironment together with the JAK-STAT pathway might be more effective and might overcome problems of inhibitor persistence and resistant subclones (Springuel et al., 2015). Furthermore, identification of genes, miRNAs, lncRNAs and other non-coding RNAs targeted by STATs will provide novel targets for therapies and useful biomarkers for monitoring of therapeutic strategies and patient stratification (**Figure 2**).

Although many new aspects and mechanisms of the hematologic malignancies have been revealed, further investigation is needed to define the role of JAK-STAT and the effects of BM niche in normal hematopoiesis, leukemia and aging. All the above will allow effective targeting of JAK-STAT and the development of personalized and accurate therapeutic management.

AUTHOR CONTRIBUTIONS

ESF and EK wrote and edited the manuscript. EK supervised manuscript preparation. Both authors contributed to the article and approved the submitted version.

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A Benchmark Side-by-Side Comparison of Two Well-Established Protocols for *in vitro* Hematopoietic Differentiation From Human Pluripotent Stem Cells

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The generation of transplantable hematopoietic stem cells (HSCs) from human pluripotent stem cells (hPSCs) remains challenging. Current differentiation protocols from hPSCs generate mostly hematopoietic progenitors of the primitive HSC-independent program, and it remains unclear what is the best combination of cytokines and hematopoietic growth factors (HGFs) for obtaining functional hematopoietic cells *in vitro*. Here, we have used the AND1 and H9 hESC lines and the H9:dual-reporter *RUNX1C*-GFP-*SOX17*-Cherry to compare the hematopoietic differentiation *in vitro* based on the treatment of embryoid bodies (EBs) with the ventral mesoderm inducer BMP4 plus HGFs in the absence (protocol 1) or presence (protocol 2) of stage-specific activation of Wnt/β-catenin and inhibition of Activin/Nodal. Despite a slight trend in favor of protocol 1, no statistically significant differences were observed between protocols at any time point analyzed throughout EB development regarding the frequency of hemogenic endothelial (HE) precursors; CD43+ CD45−, CD45+, and CD45 + CD34 + hematopoietic derivatives; or the output of clonogenic progenitors. Similarly, the kinetics of emergence throughout EB development of both *SOX17* + HE and *RUNX1C* + definitive hematopoiesis was very similar for both protocols. The expression of the early master mesendodermal transcription factors Brachyury, MIXL1, and KDR revealed similar gene expression kinetics prior to the emergence of *RUNX1C* + definitive hematopoiesis for both protocols. Collectively, the simpler protocol 1 is, at least, as efficient as protocol 2, suggesting that supplementation with additional morphogens/HGFs and modulation of Activin/Nodal and Wnt/β-catenin pathways seem dispensable for *in vitro* hematopoietic differentiation of hPSCs.

Keywords: HPSC, hematopoiesis, differentiation, cytokines, WNT/B-CATENIN

INTRODUCTON

Directed differentiation of human pluripotent stem cells (hPSCs) into specific cell types would enable the generation of large numbers of patient- or donor-derived cells for regenerative medicine and the implementation of unique *in vitro* models for studying developmental biology, disease modeling, and drug screening (Menendez et al., 2006). In the hematopoietic setting, the generation of transplantable hematopoietic stem cells (HSCs) from hPSCs remains challenging because both the primitive and definitive developmental programs are intermingled, and current hPSC differentiation protocols generate mostly hematopoietic progenitors of the primitive HSC-independent program (Medvinsky et al., 2011). However, multiple studies have reported the generation of distinct hematopoietic cell types from hPSCs *in vitro*, either by co-culturing them with stromal cell layers such as OP9 cells or by directing their differentiation through embryoid body (EB) development with specific morphogens and hematopoietic growth factors (HGFs) (Demirci et al., 2020).

Hematopoietic development from hPSCs arises from early VEGF receptor (KDR) + CD34−CD31− mesodermal progenitors, and it transitions through CD34 + CD31 + CD45− hemogenic endothelium (HE) precursors (Chadwick et al., 2003; Wang et al., 2004). The expression of glycophorin A (CD235) is used as a surrogate marker to identify the primitive (CD235+) or definitive (CD235−) hematopoietic potential (Sturgeon et al., 2014). HE precursors further differentiate toward CD43 + CD45− and then CD45 + hematopoietic cells (Chadwick et al., 2003; Menendez et al., 2004; Wang et al., 2004, 2005). Early protocols for the successful EB-based *in vitro*-directed differentiation of hPSCs into hematopoietic cells relied on the use of the master early ventral mesoderm inducer BMP4 and different cocktails of HGFs including the early acting hematopoietic cytokines SCF and FLT3L (Chadwick et al., 2003; Wang et al., 2004, 2005; Ledran et al., 2008; Ditadi et al., 2017). Further studies over the last decade suggested that Activin/Nodal and Wnt/β-catenin pathways regulate primitive vs. definitive *in vitro* hematopoietic specification from hPSCs (Sturgeon et al., 2014; Ditadi and Sturgeon, 2016; Ditadi et al., 2017). These studies suggest that the specification of definitive hematopoiesis requires early stage-specific activation of Wnt/β-catenin and inhibition of Activin/Nodal signaling pathways, which is efficiently achieved by treatment with the GSK-3 inhibitor CHIR99021, a Wnt agonist, and the Activin/Nodal inhibitor SB-431542, respectively (Bendall et al., 2007; Kennedy et al., 2012). Although many studies have investigated early hematopoietic development by interrogating the role of instructive transcription factors, it remains unclear what is the best combination of morphogens, cytokines, and HGFs to be used for obtaining functional hematopoietic cells *in vitro*. Here, we have compared the hematopoietic differentiation *in vitro* of two well-established protocols which rely on EB treatment with BMP4 plus a different cocktail HGFs in the absence or presence of stage-specific activation of Wnt/β-catenin and inhibition of Activin/Nodal.

MATERIALS AND METHODS

Maintenance of hPSC Lines

Human embryonic stem cell (hESC) lines, including the dual reporter $SOX17^{mCHERRY/W}$ $RUNX1C^{GFP/W}$ H9 cells [kindly provided by Prof. Andrew Elefanty (Murdoch Children's Research Institute, Monash University, VIC, Australia) and Dr. Andrea Ditadi (Ospedale San Raffaelo, Milan, Italy)], were maintained undifferentiated in T25 flasks on a layer of irradiated murine embryonic fibroblasts in complete Dulbecco's modified Eagle's medium (DMEM) containing 20% knockout (KO) serum replacement and 8 ng/ml basic fibroblast growth factor (bFGF) as extensively described (Chadwick et al., 2003; Ramos-Mejia et al., 2014; Bueno et al., 2019). The medium was changed daily and cells were passaged weekly by dissociation with 1:1 collagenase type IV:dispase (Invitrogen, Carlsbad, CA, United States). Cultures were visualized daily by phase contrast microscopy. Approval for the hESC work was obtained from our local health authorities and the Spanish National Pluripotent Ethical Committee (0336E/14973/2017).

Hematopoietic Differentiation From hPSCs by EB Formation

On the day of passage, undifferentiated hESCs at confluence in T25 culture flasks (∼8 × 10^6 alive cells) were first treated with collagenase type IV:dispase for 1 h at 37°C, and dispersed cells were transferred to six-well low-attachment plates (∼1 × 10^6 alive cells/well/condition; alive cells were measured by trypan blue exclusion) and incubated overnight in differentiation medium (DM; KO-DMEM supplemented with 20% fetal bovine serum, 1% non-essential amino acids, 1 mmol/L L-glutamine, and 0.1 mmol/L β-mercaptoethanol). Media changes and supplementation with BMP4, different HGFs, and inhibitors were performed as in **Figure 1A**. Concentrations used were as follows: 3 μM CHIR99021, 3 μM SB-431542, 25 ng/ml BMP4, 300 ng/ml stem cell factor (SCF), 300 ng/ml FMS-like tyrosine kinase 3 ligand (Flt3L), 10 ng/ml interleukin (IL)-3, 10 ng/ml IL-6, 50 ng/ml granulocyte-colony stimulating factor (G-CSF), 15 ng/ml VEGF, 10 ng/ml basic fibroblast growth factor 2 (FGF2), 25 ng/ml insulin-like growth factor-1 (IGF1), 30 ng/ml thrombopoietin (TPO), and two IU erythropoietin (EPO) (all from R&D Systems, Minneapolis, MN, United States) (Chadwick et al., 2003; Sturgeon et al., 2014; Ditadi and Sturgeon, 2016). In the serum-free experiments, the basal media SFD composed of IMDM and Ham's F12 (Gibco) supplemented with L-glutamine (2 mM), ascorbic acid (1 mM), monothioglycerol (MTG, 4 × 10^{-4} M; Sigma), transferrin (150 μg/ml), N2 (100×), B27 (100×), and bovine serum albumin (BSA) 0.1% was used.

Embryoid bodies were dissociated at different time points during development using collagenase B and enzyme-free cell dissociation buffer (Invitrogen). Dissociated cells were stained with anti-CD34-PE, anti-CD31-FITC, anti-CD45-APC or anti-CD34-PE-Cy7, CD31-BV510, anti-glycophorin A-PE, anti-CD43-FITC, and anti-CD45-APC antibodies and 7-actinomycin D (7AAD) for exclusion of dead cells and analyzed using a FACS Canto flow cytometer (BD Biosciences). The emergence of alive

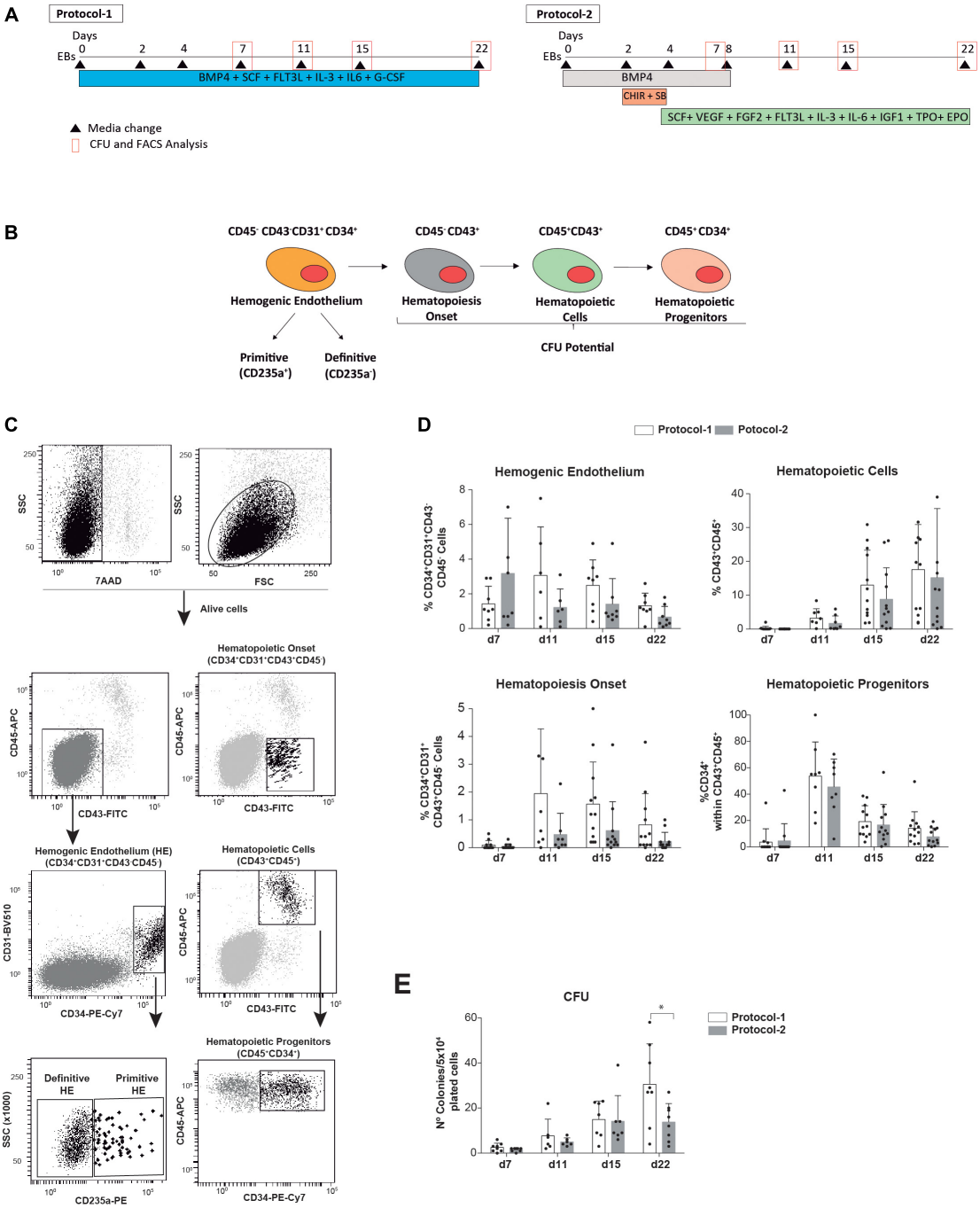

FIGURE 1 | Side-by-side comparison of two well-established EB-based directed hematopoietic differentiation protocols. **(A)** Schematic of the differentiation protocols (protocol 1 and protocol 2) and time-point analyses. **(B)** Cartoon depicting the hematopoietic cell fate specification through the formation of hEBs. **(C)** Representative FACS analysis and identification of the indicated alive hematopoietic cell populations analyzed during hematopoietic commitment of hPSCs. **(D)** Frequency of alive (7AAD–) HE precursors (CD45–CD43–CD34 + CD31 +), CD43 + CD45– and CD45 + hematopoietic cells, and hematopoietic progenitors (CD45 + CD34 +) analyzed at the indicated time points throughout EB development (n = 8). **(E)** Clonogenic progenitors detected by hematopoietic CFU assays were analyzed at the indicated time points (n = 8). Each individual dot represents the value for each independent *in vitro* differentiation experiment. Data were plotted as mean ± SD. n.s., not significant. *p value < 0.05.

(7AAD−) *SOX17* + and *RUNX1C* + cells during EB development was analyzed using Cherry and GFP reporters, respectively. Colony-forming unit (CFU) assays were performed at different time points along EB differentiation by plating 5 × 10^4 EB-derived cells onto serum-free methylcellulose H4435 (Stem Cell Technologies, Vancouver, BC, Canada). Colonies were scored

after 12 days using standard CFU scoring assays (Chadwick et al., 2003; Ramos-Mejia et al., 2014; Bueno et al., 2019).

Real-Time Reverse Transcriptase-Polymerase Chain Reaction

DNAse-treated total RNA was extracted from EBs using the Maxwell RSC simplyRNA Cells Kit (Promega, Madison, WI, United States). Two hundred and fifty nanograms of RNA was retrotranscribed with random hexamers using the Superscript III first-strand synthesis kit (Thermo Fisher Scientific, Waltham, MA, United States). The resulting cDNA was diluted 1:2 and analyzed for differential gene expression using the PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, United States) on a Bio-Rad CFX384 qPCR Platform (Bio-Rad, Hercules, CA, United States). Relative expression of the mesoderm markers was calculated with the $\Delta\Delta$Ct method using *RPL19* as the housekeeping gene. Primer sequences were as follows: *KDR*: Fw-CCACTGGTATTGGCAGTTGGA, Rev-CACAAGGGTATGGGTTTGTCAACT (80 bp, exons 10–11); *T/BRACHYURY*: Fw-ATGAGCCTCGAATCCACATAGT, Rev-TCCTCGTTCTGATAAGCAGTCA (108 bp, exon 3); *MIXL1*: Fw-GGATCCAGGTATGGTTCCAG, Rev-GGAGCACAGTGGTTGAGGAT (130 bp, exons 1–2); and *RPL19*: Fw-GCGGAAGGGTACAGCCAAT, Rev-AGCAGCCGGCGCAAAATCC (78 bp, exon 4). The absence of genomic DNA contamination was confirmed by running RT controls.

Statistical Analyses

Data are plotted as mean ± standard deviation (SD). *p* values were calculated using paired two-tailed Student's *t* test for each time point using the Prism software version 8.0 (GraphPad Prism Software Inc., San Diego, CA, United States).

RESULTS

We have compared whether two extensively employed EB-based directed differentiation protocols influence hPSC-derived hematopoietic differentiation *in vitro*. The differentiation protocols exclusively differed in the cocktail of morphogens and HGFs used during EB development (**Figure 1A**). The simpler protocol was developed by Bhatia's lab in 2003 and used the ventral mesoderm inducer BMP4 plus the HGFs SCF, FLT3L, IL-3, IL-6, and G-CSF throughout the entire 22-day differentiation protocol (Chadwick et al., 2003; Menendez et al., 2004; Wang et al., 2004, 2005) (termed as protocol 1, **Figure 1A**). The other differentiation protocol was developed later on by Keller's lab and employed a more complex setup of morphogens and HGFs suggested to more efficiently promote EB differentiation toward the definitive hematopoietic program (Kennedy et al., 2012; Ditadi and Sturgeon, 2016; Ditadi et al., 2017). It includes an 8-day treatment with BMP4 and a concomitant stage-specific (day 2 to day 4 of EB development) Wnt/β-catenin activation with the GSK-3 inhibitor CHIR99021 and Activin/Nodal inhibition with the inhibitor SB-431542 followed by treatment with SCF, VEGF, FGF2, FLT3L, IL-3, IL-6, IGF1, TPO, and EPO from day 4 of EB development onward (termed as protocol 2, **Figure 1A**). The *in vitro* efficiency of both protocol 1 and protocol 2 was compared side-by-side using the hESC lines AND1 and H9 ($n = 8$ independent experiments). The frequency of HE precursors (CD45−CD43−CD34 + CD31+), hematopoietic cells (both CD43 + CD45− and CD43 + CD45 +), and hematopoietic progenitors (CD45 + CD34 +) as well as the output of clonogenic progenitors detected by hematopoietic CFU assays was analyzed at indicated time points throughout EB development (**Figures 1B–E**). Overall, although a slight trend in favor of protocol 1 was consistently observed, no statistically significant differences were observed in the frequency of either HE precursors; CD43 + CD45−, CD45+, or CD45 + CD34 + hematopoietic derivatives (**Figure 1D**); or the output of clonogenic progenitors (**Figure 1E**) at any time point analyzed (from day 7 to day 22 of EB development). Of note, ∼95% of the HE precursors were CD235− regardless of the protocol employed (**Figure 1C**).

We next took advantage of the *RUNX1C*-GFP-*SOX17*-Cherry dual reporter H9 hESC line to track the endothelial (*SOX17* +) to definitive hematopoietic (*RUNX1C*+) transition (Ng et al., 2016). Our data showed that the protocol employed (protocol 1 vs protocol 2) had no impact on the kinetics of emergence of either *SOX17* + HE or *RUNX1C* + definitive hematopoiesis throughout the 22 days of EB development ($n = 4$, **Figures 2A,B**). The limited impact of the early stage-specific activation of Wnt/β-catenin and inhibition of Activin/Nodal pathways in definitive hematopoietic development *in vitro* was further confirmed at the molecular level. Real-time reverse transcriptase-PCR-based expression of the early master mesendodermal transcription factors Brachyury, MIXL1, and KDR revealed very similar gene expression kinetics prior to the emergence of *RUNX1C* + definitive hematopoiesis ($n = 4$, **Figure 2C**).

Protocol 1 has been historically used in the presence of serum and protocol 2 in the absence of serum. To assess whether the presence of serum masks the differences between both protocols, the *RUNX1C*-GFP-*SOX17*-Cherry H9 hESC line was differentiated ($n = 2$) for 22 days in serum-free medium with the morphogens and HGFs of protocol 1 vs. protocol 2. As shown in **Figures 2D–E**, no significant differences were observed either in frequencies or in absolute numbers between protocols for either *RUNX1C* + or *SOX17* + cells, indicating that the lack of significant differences in the hematopoietic output between both protocols is not attributable to (masked by) the presence of serum. Collectively, our *in vitro* data using different hESC lines demonstrate that the simpler protocol 1 is, at least, as efficient as protocol 2, suggesting that the supplementation with additional morphogens/HGFs and modulation of Activin/Nodal and Wnt/β-catenin pathways seem dispensable for *in vitro* hematopoietic differentiation of hPSCs.

DISCUSSION

Human PSCs have long been postulated as an unprecedented model for studies on human development and disease modeling

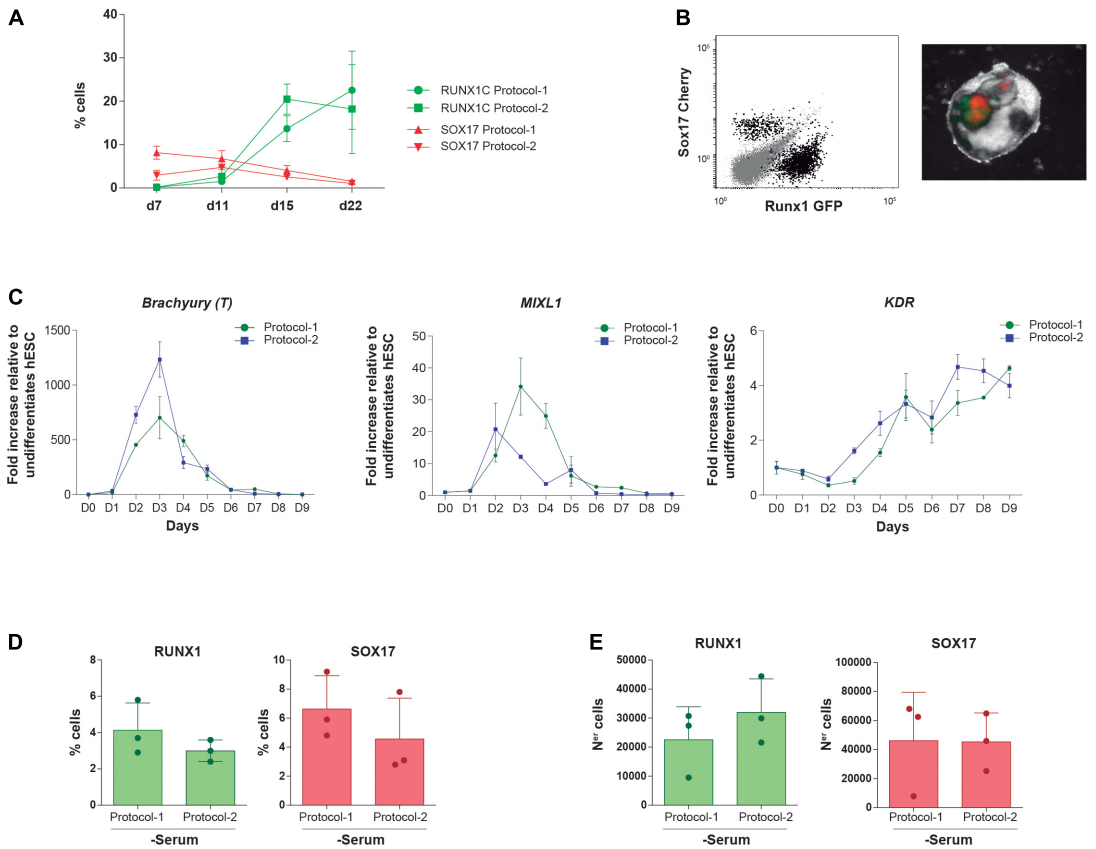

FIGURE 2 | Impact of both differentiation protocols on the emergence kinetics of *SOX17* + HE and *RUNX1C* + definitive hematopoietic cells. **(A)** Frequency of alive (7AAD–) *SOX17*:Cherry + and RUNX1C:GFP + cells along EB differentiation with protocol 1 and protocol 2 (n = 4). **(B)** *Left panel*, representative flow cytometry plots displaying how (SOX17/Cherry +) and definitive hematopoietic cells (RUNX1/GFP +) are identified. The right panel shows mCherry and GFP expression within differentiating EBs by fluorescence microscopy. **(C)** Gene expression kinetics of the mesendodermal transcription factors Brachyury (T), MIXL1, and KDR during mesoderm induction and early hematopoietic differentiation with protocol 1 and protocol 2 (n = 4). **(D,E)** Frequency **(D)** and absolute numbers **(E)** of RUNX1/GFP + definitive hematopoietic cells and SOX17/Cherry + HE cells at endpoint (day 22) of *in vitro* differentiation using protocol 1 or protocol 2 in a serum-free media. Data were plotted as mean ± SD. n.s., not significant.

and for drug testing (Menendez et al., 2006). Many studies have investigated early hematopoietic development by interrogating the role of instructive transcription factors (Real et al., 2012, 2013; Ramos-Mejia et al., 2014; Ayllón et al., 2015; Bueno et al., 2019). Nonetheless, it remains unclear regarding the best combination of cytokines and growth factors, the timing of treatment, and the methodology to be used for obtaining fully functional hematopoietic cells *in vitro*. There are a large number of published *in vitro* protocols for differentiation of hPSCs toward hematopoietic cells. These protocols largely differ in many aspects including (i) the use of several EB systems (spin and non-spin EBs) vs. stromal cells such as OP9, MSCs, AGM feeders, fetal liver feeders, etc.; (ii) the absence or presence of sera from different mammalian sources; and (iii) a never-ending combination of morphogens and HGFs, among others. However, none of the protocols used so far render hPSC hematopoietic derivatives capable of reconstituting the hematopoietic system in immune-deficient mice.

Two well-established, widely used *in vitro* hematopoietic differentiation protocols exist. The first protocol, initially developed by Bhatia's lab (termed as protocol 1 in this study), relies on EB treatment with the ventral mesoderm inducer BMP4 together with the HGFs SCF, FLT3L, IL-3, IL-6, and G-CSF for further blood specification for 22 days (Chadwick et al., 2003; Menendez et al., 2004; Wang et al., 2004, 2005). The other protocol developed by Keller's lab employs BMP4 together with a concomitant stage-specific (days 2–4 of EB development) Wnt/β-catenin activation with the GSK-3 inhibitor CHIR99021 and Activin/Nodal inhibition with the inhibitor SB-431542 followed by treatment with SCF, VEGF, FGF2, FLT3L, IL-3, IL-6, IGF1, TPO, and EPO from day 4 of EB development onward (termed as protocol 2 in this study) (Kennedy et al., 2012; Sturgeon et al., 2014; Ditadi and Sturgeon, 2016; Ditadi et al., 2017). The GSK-3 inhibitor CHIR99021 and the Activin/Nodal inhibitor SB-431542 were selected based on previous reports showing that WNT3A exposure during mesoderm patterning of hESCs suppresses primitive hematopoiesis (Gertow et al., 2013) and that WNT agonists or ACTIVIN antagonists may support definitive

hematopoiesis from hPSCs (Kennedy et al., 2012; Sturgeon et al., 2014). Both inhibitors are used between day 2 and day 4 of EB differentiation, the period during which HOX gene expression is initiated (Ramos-Mejia et al., 2014; Ng et al., 2016).

In this study, we have used several wild-type and reporter hPSC lines to compare the hematopoietic differentiation *in vitro* based on the treatment of EBs with BMP4 plus HGFs in the absence (protocol 1) or presence (protocol 2) of stage-specific activation of Wnt/β-catenin and inhibition of Activin/Nodal. We demonstrate that the simpler protocol 1 is, at least, as efficient as protocol 2, suggesting that the supplementation with additional morphogens/HGFs and modulation of Activin/Nodal and Wnt/β-catenin pathways seem dispensable for yielding a higher number of hematopoietic (progenitor) derivatives for subsequent downstream *in vitro* applications. Furthermore, preliminary data suggest that a similar trend of hematopoietic differentiation was observed in serum-free conditions indicating that the lack of significant differences in the hematopoietic output between both protocols is not masked by the presence of serum. Further side-by-side *in vitro* comparisons should comprehensively investigate the hematopoietic output of the multiple combinations of morphogens and HGFs used in parallel or sequentially in EB-based hematopoietic differentiation protocols reported from multiple laboratories.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Approval for hESC work was obtained from the ISCIII-Comisión Nacional de Garantías (0336E/14973/2017).

AUTHOR CONTRIBUTIONS

FG-A designed and performed the experiments and analyzed the data. VR-C and PP performed the experiments. CB and PM conceived the study, designed the experiments, analyzed the data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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KIT Is Required for Fetal Liver Hematopoiesis

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In the mouse embryo, endothelial cell (EC) progenitors almost concomitantly give rise to the first blood vessels in the yolk sac and the large vessels of the embryo proper. Although the first blood cells form in the yolk sac before blood vessels have assembled, consecutive waves of hematopoietic progenitors subsequently bud from hemogenic endothelium located within the wall of yolk sac and large intraembryonic vessels in a process termed endothelial-to-hematopoietic transition (endoHT). The receptor tyrosine kinase KIT is required for late embryonic erythropoiesis, but KIT is also expressed in hematopoietic progenitors that arise via endoHT from yolk sac hemogenic endothelium to generate early, transient hematopoietic waves. However, it remains unclear whether KIT has essential roles in early hematopoiesis. Here, we have combined single-cell expression studies with the analysis of knockout mice to show that KIT is dispensable for yolk sac endoHT but required for transient definitive hematopoiesis in the fetal liver.

Keywords: KIT, hemogenic endothelium, erythromyeloid progenitors, yolk sac, fetal liver

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INTRODUCTION

Several consecutive waves of hematopoietic progenitors arise and contribute blood and immune cells to the growing vertebrate embryo in close spatiotemporal proximity to developing blood vessels (Hoeffel and Ginhoux, 2018). The first primitive hematopoietic precursors originate in the yolk sac blood islands between E7.0 and E8.25 in the mouse and differentiate into embryonic erythrocytes and yolk sac macrophages (Ginhoux and Guilliams, 2016; Hoeffel and Ginhoux, 2018; Canu and Ruhrberg, 2021). These yolk sac macrophages colonize the embryo and differentiate into tissue-resident macrophages. The transient definitive wave of hematopoietic precursors arises when a subset of ECs in the yolk sac specializes into hemogenic endothelium to undergo an endothelial-to-hematopoietic transition (endoHT) between E8.5 and E9.5 in the mouse (Hoeffel and Ginhoux, 2018). This process generates erythromyeloid progenitors (EMPs), which leave the yolk sac after the onset of blood flow and colonize the liver (Lux et al., 2008; Frame et al., 2013; McGrath et al., 2015; Hoeffel et al., 2015; Hoeffel and Ginhoux, 2018). In the liver, EMPs give rise to both transient definitive erythrocytes and monocyte precursors for tissue macrophages. It is thought that the liver EMP-derived macrophages replace the initial pool of yolk sac-derived macrophages in all organs, with the exception of the tissue-resident macrophages of the central nervous system, termed microglia (Hashimoto et al., 2013; Hoeffel et al., 2015). Finally, the definitive wave of hematopoietic precursors emerges via endoHT in the aorta-gonad-mesonephros (AGM) region

(Sanchez et al., 1996; Yokomizo and Dzierzak, 2010) as a continuum of pro-, pre-, and definitive hematopoietic stem cells (HSCs) that seed the liver from E10.5 in the mouse before colonizing the bone marrow just before birth (Taoudi et al., 2008; Rybtsov et al., 2014, 2011; Azzoni et al., 2018).

Hemogenic ECs are induced by retinoic acid signaling, which upregulates the expression of the receptor tyrosine kinase KIT (Dejana et al., 2017), whereby KIT cell surface expression is often used as a distinguishing feature from non-blood-forming ECs (Goldie et al., 2008). Moreover, KIT is also used as a key marker for the progeny of hemogenic endothelium, including EMPs (Gomez Perdiguero et al., 2015; Hoeffel et al., 2015; McGrath et al., 2015) and HSCs (Sanchez et al., 1996; Gomez Perdiguero et al., 2015). Genetic defects that disrupt KIT signaling reduce the number of late embryonic HSCs (Ikuta and Weissman, 1992), affect lymphopoiesis in the adult (Waskow et al., 2002), and cause severe macrocytic anemia and thus late embryonic or perinatal lethality (Bernex et al., 1996; Broudy, 1997; Ding et al., 2012). The anemic phenotype was ascribed to an erythroid differentiation block in the fetal liver after E13.5 (Chui and Russell, 1974; Chui and Loyer, 1975; Chui et al., 1978; Broudy, 1997) that could be rescued by wild-type HSCs (Fleischman and Mintz, 1979) or by erythropoietin overexpression (Waskow et al., 2004). Subsequent studies with function-blocking antibodies further suggested that hematopoietic waves originating before E12.5 depend less on KIT than later embryonic waves (Ogawa et al., 1993). However, it has not been directly addressed whether KIT is required for yolk sac endoHT, EMP formation, or EMP function.

Here, we have combined confocal imaging, single-cell transcriptomic analyses, and flow cytometry to identify the cellular profile of *Kit* expression during early hematopoiesis in the mouse. Our functional studies further show that the hematopoietic requirement for KIT does not comprise yolk sac hematopoiesis, including the generation of EMPs. Instead, we find that KIT is required for EMP expansion and the EMP-dependent process of transient-definitive hematopoiesis that takes place in the fetal liver and includes the generation of fetal erythroid cells. KIT is therefore required for erythropoiesis earlier than previously reported. These conclusions agree with those in prior studies of mice lacking the KIT ligand KITL, also known as stem cell factor (SCF) (Azzoni et al., 2018).

RESULTS

KIT Marks EMPs but Is Dispensable for endoHT and Hematopoiesis in the Yolk Sac

Whole-mount immunostaining of E9.5 yolk sacs localized KIT to small clusters of cells within the CDH5$^+$ KDR$^+$ endothelium that appeared rounder and smaller than neighboring ECs, consistent with imminent budding into the vascular lumen (**Figures 1A,B**). These observations support the idea that KIT is expressed by EMPs generated by hemogenic ECs undergoing endoHT and corroborate that KIT immunostaining distinguishes hemogenic from non-blood-forming ECs. We further found that CDH5 was concentrated at adherens junctions at cell–cell contacts between KIT$^-$ ECs, consistent with its key role in joining ECs into vascular channels (Giannotta et al., 2013). Notably, KIT$^+$ EMPs budding from the hemogenic endothelium had more intracellular CDH5 staining than neighboring ECs (**Figures 1B,C**, full arrowhead), and EMPs already released into the vessel lumen appeared negative for CDH5 by immunostaining (**Figure 1C**, empty arrowhead). These findings suggest that CDH5 internalization precedes EMP budding, presumably as a prerequisite for EMPs to break contact with the endothelial monolayer.

To identify EMPs with flow cytometry, KIT surface expression together with low levels of CD45 staining (CD45^{low}) has previously been combined with *Csf1r-iCre*-mediated lineage tracing (Gomez Perdiguero et al., 2015; Hoeffel et al., 2015) or with CD41 co-expression (McGrath et al., 2015; Frame et al., 2016). Using *Csf1r-iCre*-mediated lineage tracing with the recombination reporter *Rosa*^{Yfp} or *Rosa*^{tdTom}, we found that more than 80% of CD45^{low} YFP$^+$ or TdTomato$^+$ cells were also KIT$^+$ in E9.5 yolk sacs (**Figure 1D**). Analysis of KIT surface expression with both CD41 and CD45 identified a KIT$^-$ cell population composed of CD45$^+$ CD41$^-$ cells (**Figure 1E**) that likely correspond to myeloid cells (MCs) (McGrath et al., 2015; Frame et al., 2016) and CD45$^-$ CD41$^+$ cells (**Figure 1E**) that likely correspond to megakaryoblasts (McGrath et al., 2015; Frame et al., 2016; Cortegano et al., 2019). By contrast, the KIT$^+$ population (**Figure 1E**) included CD41^{low} cells, which were mostly CD45$^+$. This cell population likely contains progenitors with definitive erythroid, myeloid, and lymphoid potential, including the previously described *Csf1r*-lineage traced CD45^{low} KIT$^+$ EMPs (Gomez Perdiguero et al., 2015; Hoeffel et al., 2015) and CD45$^+$ CD41$^+$ KIT$^+$ EMPs (McGrath et al., 2015; Frame et al., 2016), as well as lymphomyeloid progenitors (Boiers et al., 2013). The smaller proportion of KIT$^+$ CD41^{low} progenitors that did not express CD45 (**Figure 1E**) may include EMPs that are CD45$^-$ (McGrath et al., 2015), possibly because they are less mature, as well as other KIT$^+$ CD41$^+$ CD45$^-$ hematopoietic precursors that have recently been described (Yamane, 2018).

To understand whether KIT promotes EMP formation and/or affects the differentiation potential of EMPs, we performed flow cytometry analysis of yolk sacs from mice lacking KIT. For this experiment, we used mice homozygous for the *Kit*^{CreERT2} knock-in allele, in which the *Cre* recombinase gene is inserted into the endogenous *Kit* locus (Klein et al., 2013) to generate a true *Kit* null allele (Heger et al., 2014). These mice are hereafter referred to as *Kit*$^{-/-}$ mutants. Notably, KIT loss in E9.5 yolk sacs (**Figure 2A**) did not significantly alter the proportion or the number of the CD45$^-$ CD41$^-$ TER119$^+$ erythroblasts (Frame et al., 2016), the CD45$^-$ CD41$^+$ population that includes megakaryoblasts, the CD41^{low} progenitor population that includes both CD45$^+$ and CD45$^-$ EMPs, or the CD45$^+$ CD41$^-$ population corresponding to differentiating MCs (**Figures 2B,C**). Moreover, functional CFU-C assays with E9.5 yolk sac cells identified progenitors with either mixed erythro-myeloid (GEMMk: granulocyte, erythroid, monocyte/macrophage, megakaryocyte) or a more myeloid committed potential (GM: granulocyte, monocyte/macrophage; GMo: granulocyte, monocyte) in similar proportions in

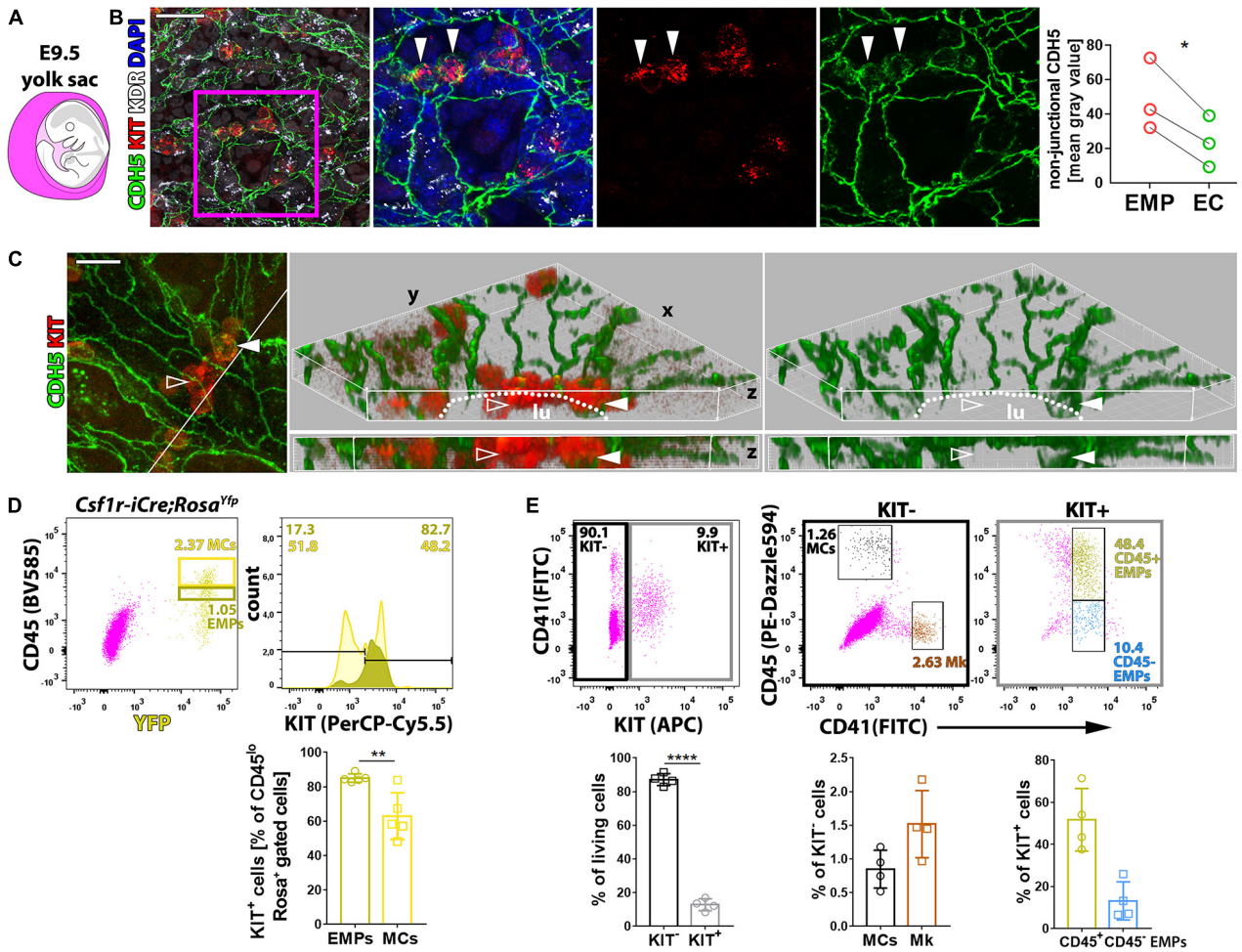

FIGURE 1 | KIT marks EMPs budding from hemogenic endothelium. **(A)** Schematic representation of an E9.5 mouse embryo surrounded by the yolk sac, shown in purple. **(B,C)** Whole-mount labeling of E9.5 mouse yolk sacs with the indicated markers ($n = 3$). The dot plot represents the staining intensity (mean gray value) for CDH5 in the cytoplasm of KIT$^+$ EMPs and KIT$^-$ ECs; each data point represents the average value from at least seven cells in $\geq$2 fields of view from one yolk sac; $^*p < 0.05$ (paired Student's *t*-test). The white line in the left panel in **(C)** marks the clipping plane for the 3D-rendered lateral views in the center and right panels; white arrowheads indicate KIT$^+$ CDH5$^+$ budding EMPs, empty arrowheads indicate KIT$^+$ CDH5$^-$ EMPs in the vessel lumen; the dotted lines indicate the lumen (lu); scale bars: 50 μm **(B)**, 20 μm **(C)**. **(D)** Flow cytometry analysis of single living cells from E9.5 *Csf1r-iCre;Rosa*^{Yfp} *or Rosa*^{tdTom} mouse yolk sacs with the indicated markers ($n = 5$). The dark and bright yellow boxes in the scatter plot indicate the gates used to quantify the percentage of KIT$^+$ cells in the populations containing putative EMPs (CD45^{low} YFP$^+$ or TdTomato$^+$) and myeloid cells (MCs, CD45^{high} YFP$^+$ or TdTomato$^+$; a presentative experiment is shown for *Rosa*^{Yfp}), respectively. Pooled data for *Rosa*^{Yfp} or *Rosa*^{tdTom} experiments are shown as mean ± SD; each data point represents the value from one embryo; $^{**}p < 0.01$ (paired Student's *t*-test). **(E)** Flow cytometry analysis of single living cells from E9.5 mouse yolk sacs with the indicated markers ($n = 4$). The black and gray boxes in the scatter plot on the left-hand side indicate the gates used to quantify the proportion of KIT$^+$ and KIT$^-$ cells, which were further analyzed, as shown in scatter plots in the center and on the right-hand side, respectively. The color-coded annotation indicates the gates used to determine the percentage of the KIT$^-$ MCs and megakaryoblasts (Mk) as well as the KIT$^+$ CD45$^+$ and CD45$^-$ putative EMPs.

KIT-deficient and littermate control embryos (**Figure 2D** and **Supplementary Figure 1**). By contrast, the number of functional progenitors in the mutants appeared reduced, albeit not at statistically significant levels (**Figure 2D**). Moreover, the colonies that grew from the mutant progenitors contained significantly fewer cells than those of wild-type littermates (**Figure 2D**), which indicates that the progenitors have reduced proliferative capacity.

Taken together, the analysis of KIT-deficient mice at E9.5 suggests that KIT is dispensable for primitive yolk sac hematopoiesis and for EMP formation from hemogenic endothelium in the yolk sac, but that it is required cell autonomously in EMPs for their expansion.

KIT Is Dispensable for Yolk Sac-Derived Macrophage Colonization of Embryonic Organs

Yolk sac-born macrophages give rise to microglia (Schulz et al., 2012; Kierdorf et al., 2013; Gomez Perdiguero et al., 2015; Hoeffel et al., 2015). They also contribute to the initial pool of tissue-resident macrophages in several other developing organs,

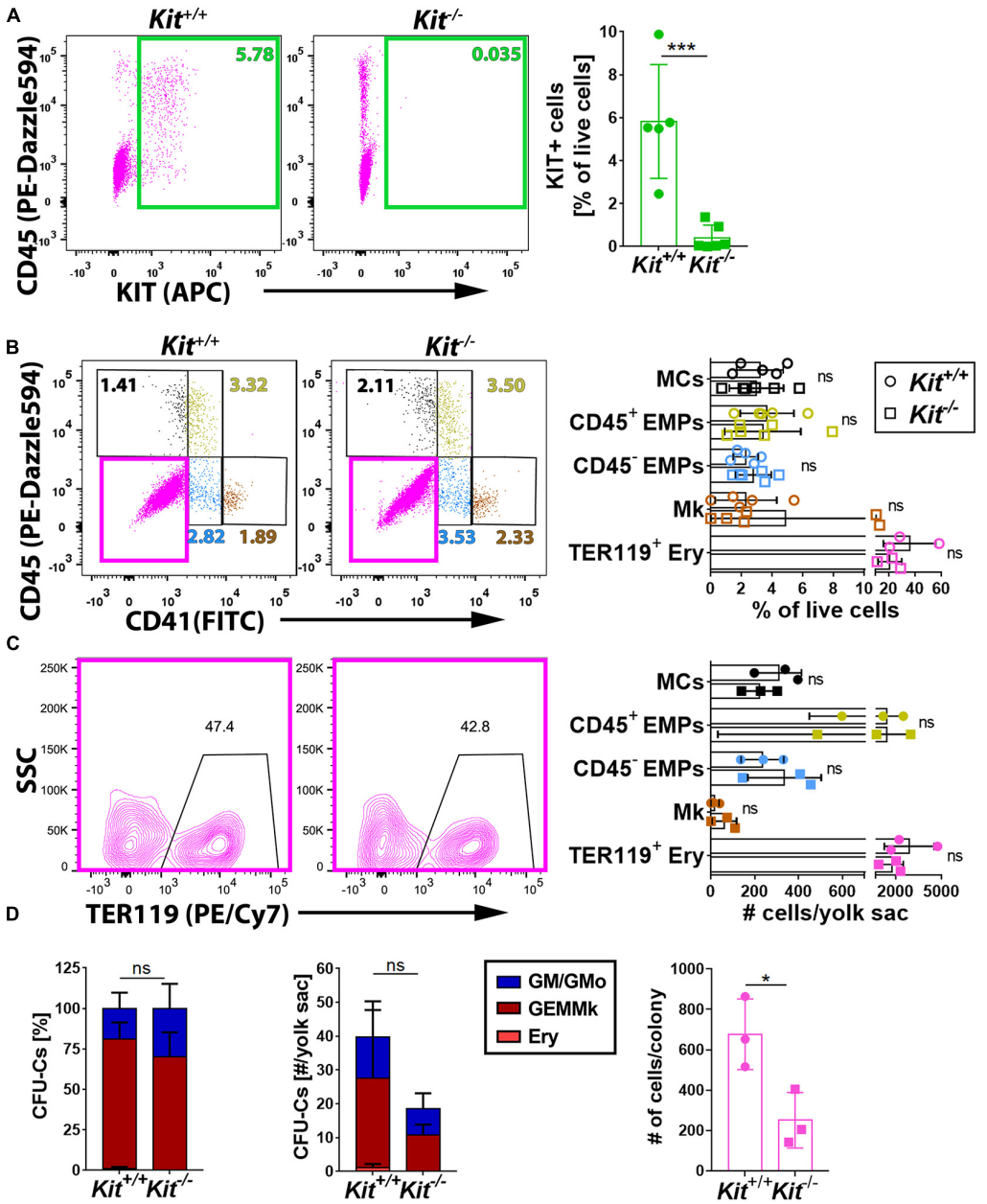

FIGURE 2 | KIT is dispensable for yolk sac hematopoiesis. **(A)** Flow cytometry analysis of single living cells from E9.5 $Kit^{+/+}$ and $Kit^{-/-}$ littermate mouse yolk sacs (n = 5 each) with the indicated markers. The green boxes in the scatter plots indicate the gates used to determine the percentage of KIT^{+} cells. Data are shown as mean ± SD; each data point represents the value from one embryo; ***$p < 0.001$ (unpaired Student's t-test). **(B,C)** Flow cytometry analysis of single living cells from E9.5 $Kit^{+/+}$ and $Kit^{-/-}$ ($n \geq 3$ each) littermate mouse yolk sacs with the indicated markers. The color-coded boxes or annotation in the scatter plots indicate the gates used to determine the percentage (top bar graph) as well as the embryo equivalent number (bottom bar graph) of cell populations defined by CD45, CD41, or TER119 expression levels. Data are shown as mean ± SD; each data point represents the value from one embryo; non-significant (two-way ANOVA followed by Sidak's multiple comparisons test). **(D)** Quantification of the percentage (left panel), number (center panel), and colony cell number of CFU-C (right panel) in $Kit^{+/+}$ versus $Kit^{-/-}$ littermate E9.5 yolk sacs (n = 3 each). Each data point represents the value from one embryo; stacked bar graph data are shown as mean ± SEM; ns, non-significant (two-way ANOVA); bar graph data are shown as mean ± SD; *$p < 0.05$ (unpaired Student's t-test).

including lung, liver, and skin, where they constitute the main macrophage population at E12.5 (Hoeffel et al., 2015) before being complemented by monocytes derived from liver EMPs (Ginhoux and Guilliams, 2016). These yolk sac-derived tissue macrophages can be distinguished by high F4/80 and CSF1R expression from the monocyte-derived macrophages derived from liver EMPs, which have low F4/80 levels (Schulz et al., 2012; Hoeffel et al., 2015). CSF1R immunofluorescence analysis showed that the number of microglia was similar between $Kit^{+/+}$ and $Kit^{-/-}$ hindbrains at E12.5 (**Figures 3A–C**). Moreover,

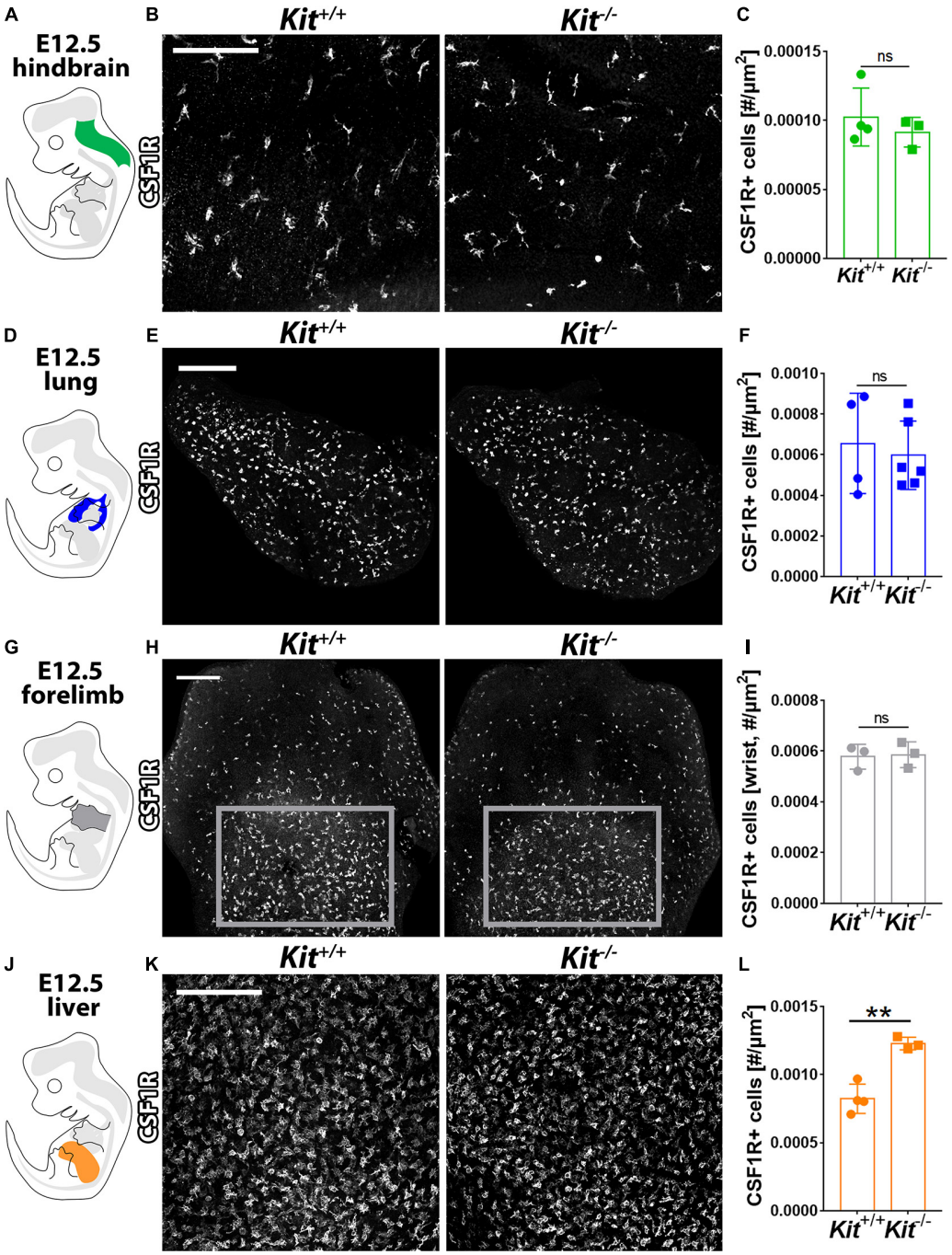

FIGURE 3 | KIT loss does not affect yolk sac-derived macrophage colonization of embryonic brain, lung, and limb but promotes their expansion in the liver. E12.5 mouse hindbrain **(A–C)**, lung **(D–F)**, forelimb **(G–I)**, and liver **(J–L)** from $Kit^{+/+}$ versus $Kit^{-/-}$ littermates were whole mount stained for CSF1R, imaged by confocal microscopy, and shown in gray scale. Scale bars: 200 µm. In **(H)**, the areas indicated with gray rectangles indicate the regions used for the quantification of CSF1R + macrophages in the wrist area of the forelimb. Quantification of CSF1R + macrophages: $n = 4$ wild-type and 3 mutant hindbrains from three litters **(C)**, $n = 4$ wild-type and 6 mutant lungs from three litters **(F)**, $n = 3$ each for forelimbs from two litters **(I)**, and $n = 4$ wild-type and 3 mutant livers from three litters **(L)**. Bar graph data are shown as mean ± SD; each data point represents the value from one embryo; ns, non-significant; $^{**}p < 0.01$ (unpaired Student's *t*-test).

CSF1R immunostaining showed a similar number of tissue macrophages in the E12.5 lung and forelimb of *Kit*-null embryos and their wild-type littermates (**Figures 3D–I**). By contrast, the number of tissue macrophages in the E12.5 liver (termed Kupffer cells) appeared significantly increased in mutants compared to wild-type controls (**Figures 3J–L**). KIT is therefore not required for the formation of yolk sac-derived macrophages (**Figure 2**) or their colonization of embryonic organs (**Figure 3**).

KIT Is Required for Transient-Definitive Fetal Liver Hematopoiesis

Starting from E10.5 onward, EMPs leave the yolk sac and colonize the fetal liver (Lux et al., 2008; Frame et al., 2013; Hoeffel et al., 2015; McGrath et al., 2015; Hoeffel and Ginhoux, 2018). To define the KIT$^+$ cell populations in the fetal liver, we analyzed the E12.5 mouse liver transcriptome by scRNA-seq (detailed characterization in a manuscript in preparation). Consistent with the liver being the major hematopoietic organ at E12.5, our annotation identified several hematopoietic cell types in a UMAP continuum. Thus, a cluster of hematopoietic stem and progenitor cells (HSPCs, *Myb*$^+$), composed mostly of EMPs and also the first HSCs, branched into several cell trajectories defined by markers of granulocytes (G, *Ly6g*$^+$), monocytes and Kupffer cells (Mo/KC, *Fcgr1*$^+$), megakaryocytes (Mk, *Pf4*$^+$), or erythroid-committed progenitors such as burst-forming unit erythroid (BFU-E) cells (Ery, *Klf1*$^+$ *Rhd*$^-$) and erythroblasts (Eryb, *Klf1*$^+$ *Rhd*$^+$) (**Figures 4A,B**). As expected, we also identified distinct clusters of yolk sac-derived primitive orthochromatic erythroblasts (EryP, *Hba-x*$^+$) as well as hepatoblasts (Hepa, *Alb*$^+$) and ECs (*Cldn5*$^+$) (**Figure 4B**).

We next examined which liver cell types expressed *Kit*. Hepatoblasts and fetal liver ECs did not contain *Kit* transcripts or the hemogenic endothelium marker *Runx1* (**Figures 4C,D**). These findings support the idea that the fetal liver, even though it harbors recruited hematopoietic progenitors (Lux et al., 2008; Frame et al., 2013; Hoeffel et al., 2015; McGrath et al., 2015; Hoeffel and Ginhoux, 2018), lacks hemogenic endothelium. *Kit* transcripts were also not detected in primitive erythroblasts, differentiating megakaryocytes, or differentiated MCs (**Figures 4C,D**). By contrast, *Kit* transcripts were abundant in the HSPC population that includes EMPs (**Figures 4C,D**). *Kit* transcripts were also abundant in erythroid-committed progenitors (BFU-E), but the number of *Kit*$^+$ cells gradually decreased in erythroid-committed progenitors as they differentiated toward an erythroblast phenotype, with barely any *Kit*$^+$ cells present in the erythroblast cluster (**Figures 4C,D**).

Liver EMPs can also be identified by flow cytometry in the E12.5 *Csf1r-iCre;Rosa*^{tdTom} liver as CD45$^+$ tdTomato$^+$ cells lacking the differentiated MC marker CD11b. In agreement with the scRNA-seq data, we found that ∼90% of EMPs in wild-type liver were KIT$^+$ (**Figure 4E**). In contrast, CD45$^+$ tdTomato$^+$ CD11b$^+$ cells mostly lacked KIT (**Figure 4E**), agreeing with the finding that *Kit* transcripts were not identified in differentiated MCs (**Figures 4C,D**). Moreover, KIT$^+$ cells were also detected in the CD45$^-$ population, both within the TER119$^+$ erythroid cells and the TER119$^-$ cells that likely include erythroid progenitors (**Supplementary Figure 2**). Immunostaining confirmed that KIT is expressed in large and round TER119$^-$ cells that likely represent hematopoietic progenitors and only a few of the smaller TER119$^+$ erythroblasts (**Figure 4F**), most likely proerythroblasts (Azzoni et al., 2018). This expression pattern is compatible with a selective role for KIT in hematopoietic progenitor populations that include EMPs and their erythroid-committed progeny during fetal liver erythropoiesis.

We next examined how KIT loss affected hematopoietic cells including EMP progeny in the liver (**Figures 4G–I**). Overall, organ cellularity was similar in the liver of E12.5 KIT-deficient and wild-type mice (**Figure 4G**). Nevertheless, both the proportion and total number of TER119$^+$ cells were significantly decreased in mutants (**Figure 4H**). By contrast, both the proportion and total number of CD45$^+$ cells were significantly increased (**Figure 4G**). Additionally, the proportion of CD41$^+$ megakaryocytes (**Figure 4H**) and both the proportion and total number of CD45$^+$ F4/80$^+$ macrophages (**Figure 4I**) were significantly increased. The increase in non-erythroid hematopoietic cells may explain why organ cellularity was not decreased in KIT-deficient livers, despite impaired erythropoiesis.

To better understand the origin of this hematopoietic imbalance, we compared the clonogenic potential of hematopoietic progenitors in the E12.5 liver from KIT-deficient and littermate control embryos. In both genotypes, we observed similar proportions of mixed erythro-myeloid progenitors (GEMMk) and progenitors with a more lineage-committed potential (GM; GMo; GMMk: granulocyte, monocyte/macrophage, megakaryocyte; Ery: erythroid) (**Figure 4J** and **Supplementary Figure 1**). Finding that the clonogenic potential of liver EMPs is preserved despite KIT deficiency implies that KIT does not play a role in fate decisions made by EMPs as they differentiate into committed progenitors in the fetal liver. Nevertheless, CFU-C assays revealed a significant decrease in the absolute number of clonogenic progenitors across all lineages in *Kit*-null livers, with virtually no erythroid or mixed erythro-myeloid colonies (**Figure 4J**). Moreover, the colonies produced by the remaining progenitors from mutant livers contained significantly fewer cells (**Figure 4J**). These results identify an essential role for KIT in progenitor expansion after their recruitment into the liver.

DISCUSSION

In recent years, it has become clear that EMPs form in the yolk sac but, starting from E10.5, they migrate from the yolk sac into the fetal liver (Palis and Yoder, 2001), where they expand and differentiate (Ginhoux and Guilliams, 2016). Our analysis showed KIT localization to a cluster of hematopoietic progenitors that appeared to bud from yolk sac ECs at E9.5 and therefore likely represent EMPs (**Figure 1**). Contrary to prior *ex vivo* findings, which concluded that KIT is required for hemogenic endothelial cell (EC) specification and function *ex vivo* (Marcelo et al., 2013), our flow cytometry analysis of KIT null mutants showed that KIT is dispensable for the generation of CD41^{low} progenitors, which include both CD45$^+$ and CD45$^-$ KIT$^+$ EMPs (**Figure 2**). Furthermore, we found that KIT is dispensable for the formation of differentiated hematopoietic cells in the E9.5 yolk sac (**Figure 2**), which are generated by both primitive progenitors and the earliest wave of EMPs (Hoeffel and Ginhoux, 2018). These findings, in turn, suggest that hemogenic endothelium can form and function even in the absence of KIT to generate EMPs.

EMPs arising in E8.5 and E9.5 yolk sacs exhibit erythroid and broad myeloid, but not lymphoid potential (Frame et al., 2013; Ginhoux and Guilliams, 2016). Notably, we observed a diminished proliferative capacity for E9.5 yolk sac EMPs in

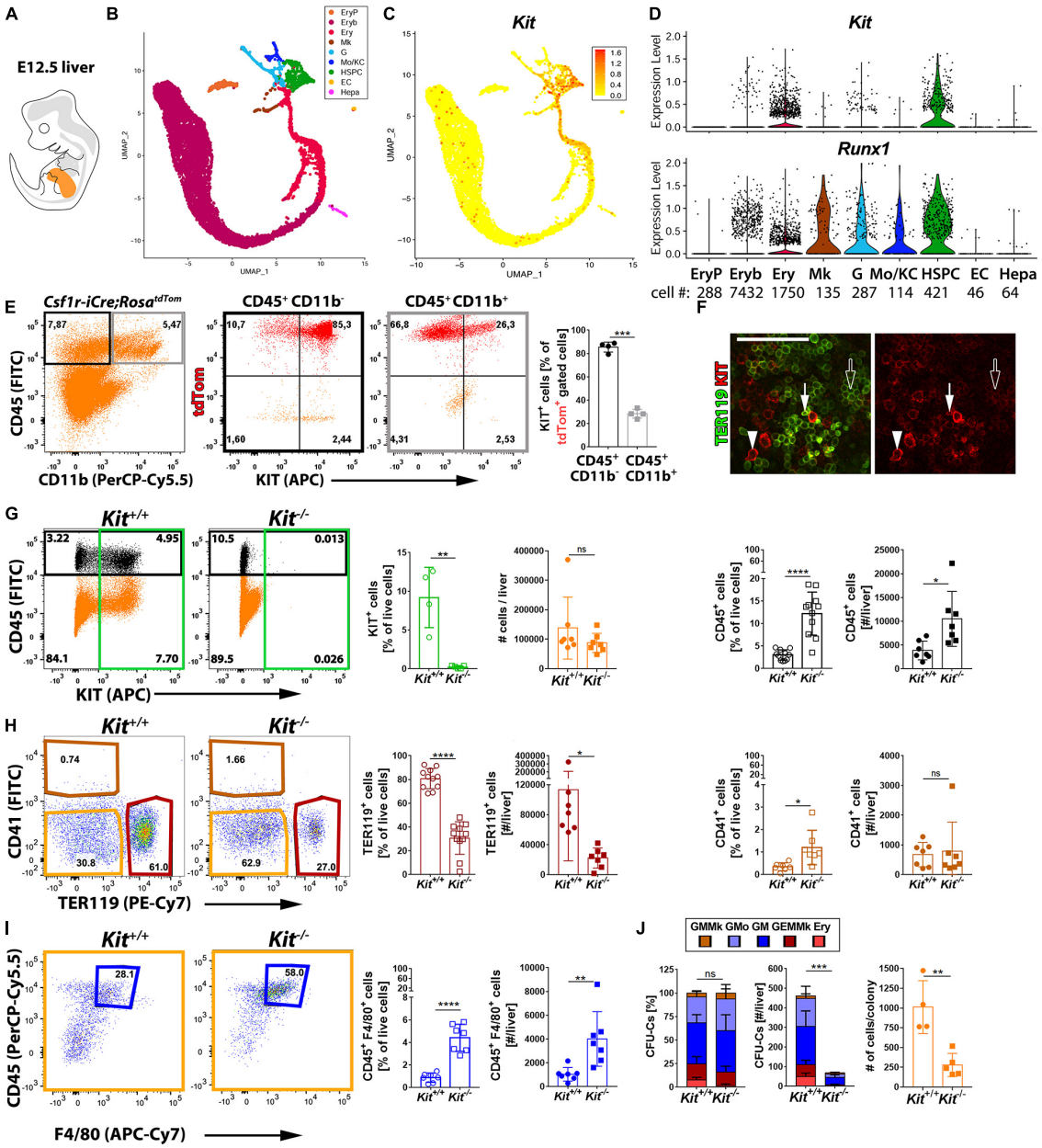

FIGURE 4 | *Kit* expression profiling and requirement during fetal liver hematopoiesis. **(A)** Schematic representation of an E12.5 mouse embryo with the fetal liver shown in orange. **(B–D)** scRNA-seq analysis of E12.5 mouse liver. UMAP plots were used to visualize clusters of distinct cell types **(B)** and *Kit* transcript levels in each cell **(C)**. Violin plots **(D)** illustrate *Kit* and *Runx1* single-cell transcript levels in each cluster; the number of cells in each cluster is indicated below the violin plots. **(E)** Flow cytometry analysis of single living cells from E12.5 *Csf1r-iCre;Rosa*^{tdTom} fetal mouse livers with the indicated markers. The boxes in the left-hand scatter plot indicate the gates used to generate the scatter plots of cells expressing CD45 only (black frame) or CD45 and CD11b (gray frame). The bar graph illustrates that the proportion of KIT$^+$ tdTomato$^+$ cells is enriched in the CD45$^+$ CD11b$^-$ compared to the CD45$^+$ CD11b$^+$ population (n = 4 livers). Data are shown as mean ± SD; each data point represents the value from one liver. ***$p < 0.001$ (paired Student's t-test). **(F)** Whole-mount labeling of E12.5 mouse liver with the indicated markers (n = 2). White arrowheads indicate KIT$^+$ TER119$^-$ hematopoietic progenitors, arrows denote KIT$^+$ TER119$^+$ proerythroblasts, and empty arrows indicate KIT$^-$ TER119$^+$ erythroblasts; scale bar: 100 μm. **(G–I)** Flow cytometry analysis of single living cells from E12.5 *Kit*$^{-/-}$ and *Kit*$^{+/+}$ littermate mouse livers with the indicated markers. The color-coded boxes in the scatter plots indicate the gates used to analyze specific cell populations, shown in corresponding colors in the bar charts. Data are shown as mean ± SD; each data point represents the value from one liver; ns, non-significant, *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ (unpaired Student's t-test). **(G)** Loss of KIT$^+$ cells in *Kit*-null mutants (green; n = 4 wild types and n = 5 mutants), total cell number (orange: n = 7 each) and percentage as well as the number of CD45$^+$ cells in each liver (black; n = 11 and n = 7 each, respectively). **(H,I)** Quantification of percentage as well as the number of TER119$^+$ CD41$^-$ erythroid cells (red; n = 11 and n = 7 each, respectively) and CD41$^+$ TER119$^-$ megakaryocytes (brown; n = 7 each). The double-negative population (orange) was further analyzed to identify CD45$^+$ F4/80$^+$ macrophages (blue; n = 7 each). **(J)** Quantification of the percentage (left panel), number (center panel), and colony cell number (right panel) of CFU-C in *Kit*$^{+/+}$ versus *Kit*$^{-/-}$ littermate E12.5 livers (n = 4 wild types and n = 5 mutants). Each data point represents the value from one embryo; stacked bar graph data are shown as mean ± SEM; ns, non-significant; ***$p < 0.001$ (two-way ANOVA); bar graph data are shown as mean ± SD; **$p < 0.01$ (unpaired Student's t-test).

KIT-deficient mice, together with a trend for fewer clonogenic progenitors overall (**Figure 2**). This impairment became statistically significant in fetal liver of the E12.5 KIT-deficient mice, which showed a major decrease in the number of functional hematopoietic progenitors as well as their proliferative capacity (**Figure 4**). Our observations agree with a recent study of mice lacking the KIT ligand KITL (also known as stem cell factor, SCF), which is expressed by yolk sac and fetal liver ECs as well as fetal liver stromal cells (Azzoni et al., 2018). Specifically, this prior study reported the normal emergence of EMPs in the yolk sac at E9.5 but a striking decrease in their number in the fetal liver (Azzoni et al., 2018). This reduction in liver EMPs was ascribed to reduced EMP proliferation in the yolk sac after E9.5, which resulted in a decreased number of circulating EMPs and reduced their colonization, proliferation, and survival in the liver (Azzoni et al., 2018).

Our single-cell transcriptomic analysis of E12.5 fetal liver demonstrated that KIT transcripts are not detectable in ECs at this stage (**Figure 4**). Lack or low levels of *Kit* expression in E12.5 liver ECs is unexpected, because *Kit* is abundantly expressed in adult hepatic sinusoidal ECs (Mansuroglu et al., 2009); see also Tabula Muris database[1] (Tabula Muris Consortium et al., 2018). Our findings therefore suggest that robust *Kit* expression in liver ECs is acquired later on during development, perhaps after the liver vasculature has specialized into the sinusoids that connect the portal triads to the central veins (Strauss et al., 2017). Instead, KIT transcripts were enriched in liver hematopoietic progenitors, including EMPs and erythroid progenitors such as the BFU-E (**Figure 4**). This expression pattern agrees with the finding that KIT is required for EMP expansion, which starts in the E9.5 yolk sac downstream of EMP formation from the yolk sac hemogenic endothelium (**Figure 2**) and continues in the E12.5 liver (**Figure 4**). Moreover, *Kit* expression in liver erythroid progenitors is consistent with a KIT requirement for EMP-dependent transient-definitive erythropoiesis taking place in the fetal liver (**Figure 4**), a process that is required to sustain embryonic life until birth (Soares-da-Silva et al., 2021). Our study therefore provides new insights into the precise requirement of KIT in specific progenitor populations for developmental erythropoiesis. Specifically, our study refines previous work based on $Kit^{W/W}$ and $Kitl^{Sl/Sl}$ spontaneous mutants (Russel et al., 1968; Chui and Russell, 1974; Chui et al., 1978) and neutralizing anti-KIT antibodies (Ogawa et al., 1993), which showed an erythroid differentiation block in the fetal liver after E13.5 without identifying the specific progenitor population involved. Accordingly, we have identified EMP expansion and erythroid differentiation from EMP-derived progenitors in the fetal liver as the first events in which KIT acts to prevent severe anemia.

We further show that KIT is dispensable for the generation of tissue-resident macrophages in the E12.5 brain, lung, and limb bud (**Figure 3**). This finding agrees with KIT being dispensable for the generation of MCs in the yolk sac (**Figure 2**), because tissue macrophages in early embryonic organs are known to differentiate from hematopoietic progenitors that arise in the yolk sac before the birth of the liver-colonizing EMPs (Ginhoux and Guilliams, 2016; Hoeffel and Ginhoux, 2018). Our observations also agree with findings in mice lacking KITL (Azzoni et al., 2018), in which the proportion of yolk sac-derived tissue macrophages ($F4/80^{hi}$ $CD11b^{lo}$) in the brain, lungs, and limb buds are unaffected at E14.5 (Azzoni et al., 2018). However, we have also observed a notable difference between the analyses of KIT- and KITL-deficient mice. Thus, KIT-deficient mice have an increased population of yolk sac-derived macrophages in the E12.5 liver compared to wild-type littermates (**Figure 3**), but the liver of E11.5 or E14.5 KITL-deficient mice does not (Azzoni et al., 2018). It is not known whether this difference in ligand and receptor mutant mice is due to the different embryonic stages examined (E12.5 vs. 11.5 and E14.5) or whether defective liver hematopoiesis caused by KIT deficiency indirectly allows for early tissue macrophage expansion, whereas KITL deficiency does not.

Notably, the population of $F4/80^{lo}$ $CD11b^{hi}$ monocyte-derived macrophages, which are derived from fetal liver EMPs, is significantly reduced in the E14.5 liver lacking KITL (Azzoni et al., 2018). This observation is consistent with the reduced number of clonogenic fetal liver progenitors across all lineages and their reduced expansion, a phenotype that is observed for both E11.5 KITL-deficient mice (Azzoni et al., 2018) and E12.5 *Kit*-null mice (**Figure 4**). While we have not specifically examined whether the population of E14.5 monocyte-derived macrophages is affected downstream of the E12.5 EMP defect in *Kit*-null mice, it stands to reason that the overall increase in macrophage numbers in the liver of *Kit* mutants at E12.5 might be explained by a KIT deficiency-induced mechanism that promotes the self-renewal of yolk sac-derived macrophages to compensate for the reduction in liver EMP and EMP-derived cells, which are mostly erythroblasts at this stage.

The central finding of our study is the KIT requirement downstream of EMP formation in the yolk sac, with a key role in EMP expansion and regulating transient-definitive fetal liver hematopoiesis across several cell lineages to affect erythroid, myeloid, and megakaryocyte generation (see working model, **Supplementary Figure 3**). At E12.5, KIT loss has a major impact on fetal liver erythropoiesis, which normally peaks at E12.5 (McGrath et al., 2011; Iturri et al., 2021). By contrast, the reduction in myeloid output from liver EMPs in the mutants cannot yet be appreciated at E12.5, when the tissue macrophage population is still mostly of yolk sac origin (Hoeffel et al., 2015). Moreover, the local expansion of the yolk sac-derived macrophage population (**Figure 3**) would likely mask any deficiency in myeloid output from liver EMPs (**Supplementary Figure 3**, model). In agreement with our observation that KIT promotes erythroid development, gain-of-function mutations in the *Kit* coding sequence have been described to trigger clonal expansion of malignant pro-erythroblasts in murine erythroleukemia (Kosmider et al., 2005). Moreover, compatible with KIT regulating the expansion of EMPs with their intrinsic myeloid potential, gain-of-function mutations have been associated with human adult and pediatric core binding factor acute myeloid leukemia (CBF-AML), for which KIT mutations are poor prognostic factors (Cairoli et al., 2006; Krauth et al., 2014; Chen et al., 2018). It should therefore be

[1] https://tabula-muris.ds.czbiohub.org/

investigated whether *KIT* mutations also contribute to human erythroleukemia.

METHODS

See **Supplementary Material**.

DATA AVAILABILITY STATEMENT

Fetal liver scRNAseq data can be found here: [https://www.ncbi.nlm.nih.gov/geo/, GSE180050]. The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Welfare Ethical Review Body (AWERB) and UK Home Office.

AUTHOR CONTRIBUTIONS

AF and CR contributed to the conception, design of the study, and co-wrote the manuscript. AF, CT, CR, and LD performed mouse experiments. AF and CT analyzed data. EV and EC performed bioinformatic analyses. All authors read and approved the submitted manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.648630/full#supplementary-material

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