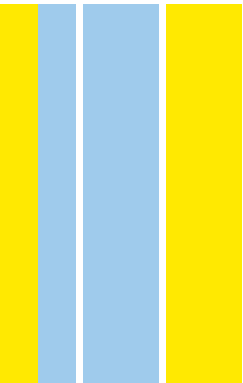
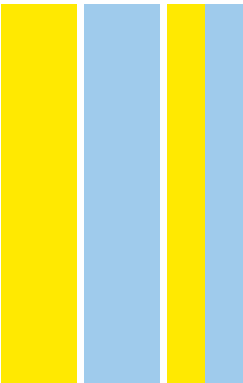


Environmental Signals Driving the Onset of Hematopoiesis in the Fetal Liver
Francisca Soares da Silva

D
2020



Environmental Signals Driving the Onset of Hematopoiesis in the Fetal Liver
Francisca Soares da Silva



FRANCISCA CASTRO GOMES SOARES DA SILVA

ENVIRONMENTAL SIGNALS DRIVING THE ONSET OF HEMATOPOIESIS IN THE FETAL LIVER

Tese de Candidatura ao grau de Doutor em
Biologia Básica e Aplicada;

Programa Doutoral da Universidade do Porto
(Instituto de Ciências Biomédicas Abel Salazar,
Faculdade de Ciências e Faculdade de Medicina)

Orientadora – Doutora Perpétua Pinto do Ó
Categoria – Investigadora Principal
Afiliação – Instituto de Investigação e Inovação em
Saúde, Instituto de Engenharia Biomédica; Instituto
de Ciências Biomédicas Abel Salazar da
Universidade do Porto;

Coorientadora – Doutora Ana Cumano
Categoria – Investigadora Principal
Afiliação – Institut Pasteur, Paris

Coorientadora – Doutora Tatiana de Pinho Resende
Categoria* – Investigadora Pós-Doutorada
Afiliação* – Instituto de Investigação e Inovação em
Saúde, Instituto de Engenharia Biomédica
*Categoria Atual – Investigadora Associada Sénior
*Afiliação Atual – FairJourney Biologics, Porto

Ph.D. program and institutions involved in the development of this thesis work:



Funding:

This work was financed by FEDER - Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020 - Operacional Programme for Competitiveness and Internationalisation (POCI), Portugal 2020, and by Portuguese funds through FCT/MCTES in the framework of the project "POCI-01-0145-FEDER-032656" granted to Perpétua Pinto-do-Ó, and by the Institut Pasteur, INSERM, ANR (grant Twothyme and grant Epi-Dev), REVIVE Future Investment Program and Pasteur-Weizmann Foundation through grants to Ana Cumano. Francisca Soares da Silva was a recipient of the grant PD/BD/114156/2016.

Cofinanciado por:



We shall not cease from exploration
And the end of all our exploring
Will be to arrive where we started
And know the place for the first time.

– *T.S. Eliot, Four Quartets*

ACKNOWLEDGMENTS

I would like to thank the coordinating committee of my doctoral program GABBA, for the high-quality scientific program that was assembled for the first-year lectures and courses. More than providing the funding for the Ph.D. thesis, this program allowed me to have close contact with the top scientist in a variety of fields before choosing a project to pursue. It is this transversal higher education, together with the community of alumni, that marks the excellence of the program. I am sincerely thankful for the opportunity to have enrolled in such a remarkable program.

I am deeply grateful to my supervisors, for the guidance and support throughout the last five years, and for honoring the role of women in science.

To my supervisor Perpétua, first and foremost, thank you for the opportunity to pursue this project and re-start the chapter on hematopoiesis in the lab. Thank you for your support and far-fetched thinking, and for always fighting for a dignified career in science. I will always carry what I have learned from you.

To my supervisor Ana, thank you for the opportunity of joining your lab and pursuing this project. The time I spent with you taught me to be an assertive, confident, and pragmatic scientist. Thank you for teaching me not to be afraid to share my ideas. More than an academic supervisor, you have truly been a mentor.

To my supervisor Tatiana, thank you for the advice, the good example of a researcher, and for teaching me that there's more to life than work.

To our collaborators, a special thanks to Elisa for the availability, insightful discussions, and advice.

To the scientific platforms both at i3S and at Institut Pasteur, thank you for the availability and for always being keen on helping with any unexpected problem. A special thanks to Catarina Meireles at i3S, and Sophie Novault, Sandrine Schmutz, and Sebastien Megharba at Institut Pasteur.

To Marie-Christine, a very special thanks for always making the transitions to Institut Pasteur and all the bureaucracies less difficult. For being such a good help on anything related to reagent orders and allowing us to do the science. For the smile you always show when someone asks for help.

To all the members of Perpétua's lab, present and past, for always creating a good environment and provide valuable scientific input. A very special thanks to Diana, for the

great example of a rising leader, for the help and advice. A very special thanks to Ana Freire, who although was not present during the time of this thesis, prepared me just as right for what I would be facing. A very special thanks to Márcia, for being my right (and sometimes left) hand, for allowing me to tutor you, and for being a friend. A very special thanks to Elsa for always being there, and for the fun moments we shared. A special thanks to all others with whom I spent more time, Vasco, Tiago, Ana Silva, Daniel, and Rita, for always making me feel as if I never left.

To all the members of Ana's lab, present and past, for always making me feel included, all the help and lively discussions. A very special thanks to António for the advice, teaching me not to jump into conclusions, not be "precipitada como as mulheres", and for the friendship. A very special thanks to Odile, for teaching me so much about embryo dissection and mice manipulation, and for all the beautiful flowers on my birthday. I will always cherish these moments. A very special thanks to Marie-Pierre, for being the perfect teamwork colleague and making me improve my French! A very special thanks to Ramy, for all the discussion and support, being my conference comrade, and for being a friend. A special thanks to all others with whom I spent more time, Paulo, Pablo, Rachel, Françoise, Junjie, Thibaut, Sylvain, Nathan, Elsa B.

On a more personal and informal note, I would like to thank all the people with whom I had fun all these years. Those in Porto, especially Andreia, Telmo, Elsa and Vânia, for keeping close even when the distance was long, those in Paris, especially Ana Antão and Lucie, for making me feel at home, and those both in Porto and in Paris, especially Mariana V., Mariana T. and Ana Cat for the easy friendship that warms the heart.

Por fim, à minha família que sempre me encorajou em todos os momentos, à minha mãe, ao meu pai e ao meu irmão pelo apoio incondicional e amor infindável.

RESUMO

A hematopoiese embrionária é caracterizada por uma sobreposição no tempo e em diferentes localizações anatómicas de diversos progenitores hematopoiéticos. Os progenitores recém-gerados migram para o fígado fetal, onde proliferam e/ou diferenciam, tornando o fígado fetal o maior órgão hematopoiético durante o desenvolvimento. Inicialmente considerava-se que as ondas hematopoiéticas transientes do saco vitelino eram necessárias para os primeiros estádios do desenvolvimento embrionário, mas substituídas pelas células estaminais hematopoiéticas após o seu aparecimento e colonização do fígado fetal. Estudos recentes demonstraram que o saco vitelino tem a capacidade de produzir células que persistem durante a vida adulta, como é o caso da microglia.

Nesta tese, análise de *lineage tracing* de progenitores eritromielóides do saco vitelino identificou progenitores eritroides altamente proliferativos no fígado fetal que são os maiores contribuidores para o compartimento eritroide no embrião. Para além disso, diferentes modelos animais de *lineage tracing* mostraram que, ao contrário do que era previamente considerado, as células estaminais hematopoiéticas não contribuem para a produção de eritrócitos até ao nascimento, indicando que no embrião, a diferenciação de células estaminais hematopoiéticas é enviesada. Nós demonstramos que, no fígado fetal, progenitores eritroides derivados de progenitores eritromielóides necessitam de concentrações de eritropoietina dez vezes mais baixas para uma diferenciação eritroide eficiente, quando comparados com os mesmos progenitores derivados de células estaminais hematopoiéticas. Propomos que os níveis baixos de eritropoietina no fígado fetal conferem uma vantagem seletiva aos progenitores eritróides com origem no saco vitelino, uma vez que são mais sensíveis a esta hormona.

O papel do microambiente no destino dos progenitores hematopoiéticos está bem estabelecido na medula óssea no adulto, mas subavaliado no fígado fetal. O compartimento não-hematopoiético, que representa cerca de 3% do número total de células do fígado fetal a E14.5, foi sistematicamente caracterizado neste trabalho. A análise de hepatoblastos e células endoteliais, perivasculares e mesoteliais demonstrou que todas as populações expressam padrões distintos, mas relevantes de citocinas hematopoiéticas. A quimiocina *Cxcl12*, produzida pelas células perivasculares, é o fator mais expresso ao longo do desenvolvimento do fígado, enquanto outras citocinas como *Epo* e *Il7* são expressas a níveis baixos pelos hepatoblastos. As células mesoteliais, que não tinham sido previamente analisadas, expressam níveis de *Kitl* relevantes, tal como os hepatoblastos. No conjunto, o trabalho desenvolvido nesta tese sugere que a hematopoiese no fígado fetal

é influenciada por diferentes células do nicho que cooperam para fornecer os sinais necessários para expansão e/ou diferenciação de células estaminais ou progenitores hematopoiéticos.

Palavras-chave: hematopoiese embrionária, fígado fetal, saco vitelino, progenitores eritromielóides, células estaminais hematopoiéticas, eritrócitos, nicho, eritropoietina

ABSTRACT

Embryonic hematopoiesis is characterized by an overlap of hematopoietic progenitors in time and distinct anatomical locations. As newly generated hematopoietic progenitors migrate to the fetal liver, where they proliferate and/or differentiate, the fetal liver becomes the major hematopoietic organ during development. Despite the established paradigm that transient yolk sac hematopoietic waves were required for early embryonic development, and readily replaced by hematopoietic stem cells after these emerge and colonize the fetal liver, recent evidence endows the yolk sac with the capacity to generate cells that persist throughout adulthood, such as the case of microglia.

Here, lineage tracing analysis of yolk sac erythromyeloid progenitors identified highly proliferative erythroid progenitors in the fetal liver that are the major contributors to the erythroid compartment in the embryo. Furthermore, distinct lineage tracing mouse models showed that, opposite to previous views, hematopoietic stem cells do not contribute to red blood cell production until birth, indicating that in the embryo hematopoietic stem cell differentiation is skewed. We demonstrate that erythromyeloid-derived erythroid progenitors in the fetal liver require ten-fold lower concentrations of erythropoietin for efficient erythroid production when compared to their hematopoietic stem cell-derived counterpart. We propose that low levels of erythropoietin in the fetal liver confer a selective advantage to erythroid progenitors of yolk sac origin that are highly sensitive to this hormone.

The role of the microenvironment on the fate of hematopoietic progenitors is well established in the adult bone marrow but remains to elucidate in the fetal liver. Here, the non-hematopoietic compartment, which represents only around 3% of total fetal liver cells by E14.5, was systematically characterized. Analysis of hepatoblasts and endothelial, perivascular and mesothelial cells showed that all subsets express relevant but distinct patterns of known hematopoietic cytokines. The chemoattractant *Cxcl12*, produced by perivascular cells, is the most expressed factor across liver development, while other cytokines such as *Epo* and *Il7* are expressed at low levels by hepatoblasts. Mesothelial cells, which had not been previously analyzed, express relevant levels of *Kitl*, together with hepatoblasts. Altogether, our work supports the premise that fetal liver hematopoiesis is influenced by distinct niche cells that cooperate to provide the signals required for expansion and/or differentiation of hematopoietic stem and progenitor cells.

Keywords: embryonic hematopoiesis, fetal liver, yolk sac, erythromyeloid progenitors, hematopoietic stem cells, erythrocytes, niche, erythropoietin

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	III
RESUMO	V
ABSTRACT	VII
CHAPTER I – OBJECTIVES, THESIS OUTLINE AND LIST OF CONTRIBUTIONS.....	1
OBJECTIVES	1
THESIS OUTLINE	1
LIST OF CONTRIBUTIONS IN THE SCOPE OF THIS THESIS	2
<i>Publications</i>	2
<i>Communications</i>	2
<i>Awards</i>	4
<i>Organization of conferences</i>	4
CHAPTER II – INTRODUCTION	5
HEMATOPOIESIS	6
<i>Embryonic Hematopoiesis</i>	8
<i>A Look into Erythropoiesis</i>	16
SITES FOR HEMATOPOIETIC CELLS DURING DEVELOPMENT	20
<i>Yolk Sac</i>	20
<i>Placenta</i>	21
<i>AGM</i>	22
<i>Fetal Liver</i>	23
<i>Fetal and Adult Bone Marrow</i>	24
THE FETAL LIVER STROMA AS A NICHE FOR HEMATOPOIESIS	28
<i>Law of attraction: what brings hematopoietic progenitors to the developing liver?</i>	28
<i>Populations present in the fetal liver during embryogenesis</i>	30
<i>Signals provided by the fetal liver stroma</i>	36
OPEN QUESTIONS IN THE FIELD.....	41
<i>Ex vivo expansion of HSCs</i>	41
CHAPTER III – NON-HEMATOPOIETIC POPULATIONS IN THE FETAL LIVER AT THE PEAK OF HSC EXPANSION	44
FOREWORD	45
CHARACTERIZATION BY FLOW CYTOMETRY	46
<i>Optimization protocol for analysis of stromal cell populations: Elimination of hematopoietic cells</i>	46
<i>Optimization protocol for analysis of stromal cell populations: Obtaining single cell suspensions</i>	48
<i>Panel design to identify stromal populations</i>	50
CHARACTERIZATION BY GENE EXPRESSION	51
<i>RNA-Seq analysis identifies P2 cells as being affiliated to the hematopoietic lineage</i>	52
DISCUSSION	55
MATERIALS AND METHODS	56
SUPPLEMENTARY INFORMATION	58

<i>Supplementary figures</i>	58
<i>Supplementary tables</i>	62
CHAPTER IV – THE FETAL LIVER AS A HUB FOR YOLK SAC ERYTHROPOIESIS THROUGHOUT EMBRYO DEVELOPMENT	69
FOREWORD.....	70
A UNIQUE POPULATION OF KIT ⁺ CELLS REPRESENTS THE MAJORITY OF FL TER119 ⁻ CD45 ⁻ CELLS	71
P1 AND P2 CELLS IN THE FL HAVE AN ERYTHROID PROGENITOR SIGNATURE	72
P1, P2 AND P3 CELLS REPRESENT INCREASINGLY MATURE STAGES WITHIN THE ERYTHROID LINEAGE ..	75
P2 AND P3 CELLS ARE COMMITTED ERYTHROID PROGENITORS WHEREAS P1 RETAIN RESIDUAL MYELOID POTENTIAL	77
P1/P2 PROGENITORS REQUIRE C-MYB EXPRESSION	79
P1/P2 CELLS ORIGINATE FROM YOLK SAC AND ARE MAJOR CONTRIBUTORS TO EMBRYONIC ERYTHROPOIESIS.....	81
HSCs DO NOT CONTRIBUTE TO ERYTHROPOIESIS UP UNTIL BIRTH.....	84
DISCUSSION	88
MATERIALS AND METHODS	91
SUPPLEMENTARY INFORMATION	94
<i>Supplementary figures</i>	94
<i>Supplementary tables</i>	99
CHAPTER V – HEMATOPOIETIC CYTOKINE-EXPRESSING CELLS IN THE DEVELOPING LIVER	102
FOREWORD.....	103
HEMATOPOIETIC CYTOKINES ARE PRODUCED BY DISTINCT STROMAL POPULATIONS.....	104
<i>Characterization of the stromal subsets</i>	104
<i>Hematopoietic cytokine-expressing cells</i>	105
IN SITU ANALYSIS OF STROMAL SUBSETS	108
<i>Hepatoblasts and endothelial cells</i>	108
<i>Mesothelial and sub-mesothelial cells</i>	108
<i>Mesenchymal cells</i>	110
IN SITU LOCALIZATION OF HEMATOPOIETIC CELLS	112
FUNCTIONAL ANALYSIS OF STROMAL SUBSETS.....	115
DISCUSSION	116
MATERIALS AND METHODS	118
SUPPLEMENTARY INFORMATION	121
<i>Supplementary figures</i>	121
<i>Supplementary tables</i>	123
CHAPTER VI – GENERAL DISCUSSION AND PERSPECTIVES	129
REFERENCES	133
ANNEX I	159
ANNEX II	179
ANNEX III	214

LIST OF FIGURES

Figure 1 Models of hierarchical hematopoiesis.....	7
Figure 2 Ontogeny of the hematopoietic system.....	9
Figure 3 Contributions of the murine model for hematopoiesis.....	13
Figure 4 Erythroid differentiation in the mouse	17
Figure 5 Model of erythroid ontogeny in the mammalian embryo	19
Figure 6 Extraembryonic tissues in the developing mouse embryo.....	20
Figure 7 Endothelial-to-hematopoietic transition in the dorsal aorta.....	23
Figure 8 Circulation of HSPCs in the embryo	24
Figure 9 Adult bone marrow niche	27
Figure 10 Mechanisms of fetal liver colonization	29
Figure 11 Ontogeny of the fetal liver	32
Figure 12 Fetal liver niche.....	37
Figure 13 Depletion of hematopoietic cells	47
Figure 14 Digestion of fetal liver.....	49
Figure 15 T-SNE analysis of E14.5 Ter119 ⁺ CD45 ⁺ fetal liver cells	50
Figure 16 Gene expression analysis of E14.5 fetal liver cells.....	51
Figure 17 RNA-Seq analysis of E14.5 fetal liver cells.....	53
Figure 18 A population of Kit ⁺ cells unique to FL represents the majority of Ter119 ⁺ CD45 ⁺ cells and has an erythroid progenitor transcriptional signature	73
Figure 19 P1, P2 and P3 cells represent increasingly maturation stages within the erythroid lineage	76
Figure 20 P2 and P3 cells are committed erythroid progenitors whereas P1 retain residual myeloid potential.....	78
Figure 21 P1/P2 progenitors require c-Myb expression.....	80
Figure 22 P1/P2 cells originate from yolk sac and are major contributors to embryonic erythropoiesis	83
Figure 23 HSCs do not contribute to erythropoiesis up until birth.....	86
Figure 24 P1/P2 cells have higher sensitivity to erythropoietin than HSC derived progenitors	87
Figure 25 Flow cytometry analysis of Ter119 ⁺ CD45 ⁺ Kit ⁺ CD71 ⁺ E14.5 fetal liver cells.....	104
Figure 26 Gene expression analysis of the stromal subsets.....	105
Figure 27 Cytokine expression in FL stromal subsets	106
Figure 28 Single-cell gene expression analysis of E14.5 FL stromal subsets	107
Figure 29 Hepatoblasts and endothelial cells	109
Figure 30 Mesothelial and sub-mesothelial cells	110
Figure 31 Mesenchymal cells.....	111
Figure 32 Distribution of hematopoietic cells in fetal liver	112
Figure 33 Imaging mass cytometry of the FL at E16.5, E18.5 and P7.....	114

LIST OF SUPPLEMENTARY FIGURES

Supplementary Figure 1 Flow cytometry profiles of E14.5 Ter119-CD45- cells obtained by depletion with dynabeads or MACS columns	58
Supplementary Figure 2 Flow cytometry profiles of E15.5 fetal liver cells obtained by mechanical or enzymatic digestion.....	59
Supplementary Figure 3 Manual gating identifying populations obtained by unsupervised analysis of E14.5 fetal liver cells	60
Supplementary Figure 4 ARCH4 tissue and cell lines	61
Supplementary Figure 5 Phenotype of E12.5, E14.5 and E18.5 fetal liver and adult bone marrow populations.....	94
Supplementary Figure 6 Gating strategies used to determine YFP labeled hematopoietic populations in the $Csf1^{MeriCreMer}Rosa26^{YFP}$ lineage tracing model (related to Figure 22).....	95
Supplementary Figure 7 Gating strategies used to determine YFP labeled hematopoietic populations in the $CDH5^{CreERT2}Rosa26^{YFP}$ fate-mapping model (related to Figure 23).....	96
Supplementary Figure 8 Gating strategies used to determine YFP labeled hematopoietic populations in the $Flt3^{Cre}Rosa26^{YFP}$ fate-mapping model (related to Figure 23)	97
Supplementary Figure 9 Gating strategies used to determine YFP labeled hematopoietic populations in the $Cdh5^{CreERT2}Rosa26^{YFP}$ fate-mapping model (related to Figure 23).....	98
Supplementary Figure 10 Cytokine expression in FL stromal subsets	121
Supplementary Figure 11 Flow cytometry analysis of E14.5 FL stroma.....	122
Supplementary Figure 12 RFP expression in ALB-Cre Rosa26-RFP mice	122

LIST OF TABLES

Table 1 Site of origin and lineage potential of embryonic hematopoietic waves.....	12
Table 2 Markers used for identification of hematopoietic progenitors.....	15
Table 3 Fetal liver cell types, gene expression and markers used for isolation.....	35
Table 4 Cytokine signaling in fetal liver.....	39
Table 5 Expression of surface markers in fetal liver cells.....	48

LIST OF SUPPLEMENTARY TABLES

Supplementary Table 1 List of submitted genes for enrichr analysis – CD31 ⁺	62
Supplementary Table 2 List of submitted genes for enrichr analysis – P2.....	64
Supplementary Table 3 List of submitted genes for enrichr analysis – CD324 ⁺	65
Supplementary Table 4 List of antibodies used for flow cytometry.....	67
Supplementary Table 5 List of taqman probes used.....	68
Supplementary Table 6 List of antibodies for flow cytometry.....	99
Supplementary Table 7 List of primers for qRT-PCR.....	100
Supplementary Table 8 List of taqman probes for single-cell multiplex gene expression.....	101
Supplementary Table 9 List of antibodies used for flow cytometry.....	123
Supplementary Table 10 List of primers for qRT-PCR.....	124
Supplementary Table 11 List of taqman probes for single-cell multiplex gene expression.....	125
Supplementary Table 12 List of antibodies for IHC.....	126
Supplementary Table 13 List of antibodies used for imaging mass cytometry.....	128

LIST OF ABBREVIATIONS

AGM – aorta-gonads-mesonephros
BA – bile acid
BFU-E – burst forming unit-erythroid
BM – bone marrow
CAR - CXCL12-abundant reticular cell
CB – cord blood
CFU-E – colony-forming unit-erythroid
CFU-F – colony-forming unit-fibroblast
CFU-G – colony-forming unit-granulocyte
CFU-GEMM – colony-forming unit-erythroid, myeloid and megakaryocyte
CFU-GM – colony-forming unit-granulocyte and macrophage
CFU-M – colony-forming unit-macrophage
CFU-Mk – colony-forming unit-megakaryocyte
CLP – common lymphoid progenitor
CMP – common myeloid progenitor
CXCL12 – CXC chemokine ligand 12, also named stromal cell-derived factor-1 or SDF-1
drHSC – developmentally restricted hematopoietic stem cell
E – embryonic day
ECM – extracellular matrix
EdU - 5-ethynyl-2'-deoxyuridine
EHT – endothelial-to-hematopoietic transition
EMP – erythromyeloid progenitor
Epo – erythropoietin
EryP-CFC – primitive erythroid progenitor
FBM – fetal bone marrow
FL – fetal liver
FLT3L – FMS-like tyrosine kinase 3 ligand
Fn – fibronectin
G-CSF – granulocyte-colony stimulating factor
GM-CSF – granulocyte/macrophage-colony stimulating factor
GMP – granulocyte-monocyte progenitor
GvHD - graft-versus-host disease
HS – heparan sulfate
HSC – hematopoietic stem cell

HSPC – hematopoietic stem and progenitor cell
 imHSC – immature hematopoietic stem cell
 KIT – KIT proto-oncogene receptor tyrosine kinase, also known as c-Kit or CD117
 KITL – kit ligand, also named stem cell factor or SCF
 LIF – leukemia inhibitory factor
 LMPP – lympho-myeloid primed progenitor
 LSK – Lin⁻Sca1⁺Kit⁺ cell
 LT-HSC – long-term HSC
 LT_i – lymphoid tissue inducer
 LTR – long-term reconstitution
 Mac-CFC – monopotent macrophage precursor
 MC – mesothelial cell
 MEP – megakaryocyte-erythroid progenitor
 MMP – multipotent progenitor
 MSC – mesenchymal stem cell
 OH-TAM - 4-hydroxytamoxifen
 OPN – osteopontin
 PSp – para-aortic splanchnopleure
 RBC – red blood cell
 SCF – stem cell factor, also named Kit ligand or KITL
 SDF-1 – stromal cell-derived factor-1, also named CXC chemokine ligand 12 or CXCL12
 SEC – sinusoidal endothelial cell
 SPP1 - secreted phosphoprotein 1
 ST-HSC – short-term HSC
 STM – septum transversum mesenchyme
 Sub-MC – sub-mesothelial cell
 TF – transcription factor
 TGF- β – transforming growth factor- β
 TPO – thrombopoietin
 trOPN - thrombin-cleaved osteopontin
 VLA – very late antigen
 Wpc – weeks post-conception
 YS – yolk sac

CHAPTER I – OBJECTIVES, THESIS OUTLINE AND LIST OF CONTRIBUTIONS

OBJECTIVES

Hematopoiesis occurs in specialized organs and the role of the environment in the behavior of hematopoietic progenitors is increasingly recognized. There is compelling evidence that the major stromal cell types in the bone marrow are endothelial and mesenchymal stromal cells. In contrast, little information exists on the stromal compartment in the fetal liver. Here, we aimed at characterizing the microenvironment hematopoietic stem cells encounter in the fetal liver and the role of the distinct populations in supporting embryonic hematopoiesis. We focus on the fetal liver as it is the major hematopoietic organ during embryonic development and the only site where hematopoietic stem cell expansion occurs.

The specific aims of this thesis were to:

1. Isolate and characterize, in fetal liver, the stromal compartment that conditions hematopoietic development and its role in lineage commitment
2. Identify the environmental signals that drive self-renewal versus differentiation

THESIS OUTLINE

This thesis is divided into six chapters, comprising a literature overview, three chapters containing the experimental work and a general discussion with concluding remarks.

Chapter I comprises the main objectives of this thesis work, the thesis outline and the list of contributions.

Chapter II introduces an overview of the topics presented and discussed in this thesis: embryonic hematopoiesis and the environments where it occurs. It introduces the theme of layered hematopoiesis in the embryo, with distinct anatomical and timed origins, and explores the role of the microenvironments where hematopoietic cells proliferate and/or differentiate, with a focus on the fetal liver.

Chapter III investigates the non-hematopoietic compartment of the fetal liver, with optimization of cell isolation techniques, identification of cell surface markers and characterization of gene expression. From this exploratory study, it was possible to identify a population that corresponds to around 70% of the non-hematopoietic compartment, and although does not express conventional hematopoietic markers, belongs to the erythroid lineage.

Chapter IV is dedicated to understanding the novel population identified in **Chapter III**. Analysis of gene expression, lineage potential *in vitro* and *in vivo*, and lineage tracing studies allowed the identification of these cells as erythroid progenitors of yolk sac origin that are responsible for maintaining hematopoiesis until birth. The advantage of these over hematopoietic stem cell-derived cells appears to be due to a higher sensitivity to erythropoietin, which is low in the fetal liver microenvironment.

Chapter V re-addresses the non-hematopoietic compartment of the fetal liver after the identification and characterization of the previously unknown cells examined in **Chapter IV**. Analysis of gene expression allowed the identification of four cell subsets that produce distinct hematopoietic cytokines. These subsets were further characterized by their localization *in situ* in the fetal liver.

Chapter VI provides a general discussion, concisely correlating results from **Chapters III-V**, and finishes with the main conclusions and perspectives for future research.

LIST OF CONTRIBUTIONS IN THE SCOPE OF THIS THESIS

PUBLICATIONS

- Soares-da-Silva F, Freyer L, Elsaid R, Burlen-Defranoux O, Iturri L, Sismeiro O, Pinto-do-Ó P, Gomez-Perdiguero E, Cumano A (2020) **Yolk sac, but not HSC-derived progenitors, sustain erythropoiesis throughout murine embryonic life** bioRxiv doi: 10.1101/2020.02.27.968230, *Under review*
- Soares-da-Silva F*, Peixoto M*, Cumano A, Pinto-do-Ó P (2020) **Crosstalk between the hepatic and hematopoietic systems during embryonic development**. Front. Cell Dev. Biol. 8:612. doi: 10.3389/fcell.2020.00612. *these authors contributed equally (Annex I)
- Elsaid R*, Soares-da-Silva F*, Peixoto M, Amiri D, Mackowski N, Pereira P, Bandeira A, Cumano A. (2020) **Hematopoiesis: a layered organization across species**. Front. Cell Dev. Biol. doi: 10.3389/fcell.2020.606642 *these authors contributed equally (Annex II)
- Elsaid R, Meunier S, Burlen-Defranoux O, Soares-da-Silva F, Perchet T, Iturri L, Freyer L, Vieira P, Pereira P, Golub R, Bandeira A, Gomez Perdiguero E, Cumano A (2020) **A wave of bipotent T/ILC-restricted progenitors shapes the embryonic thymus microenvironment in a time-dependent manner**. Blood; doi: 10.1182/blood.2020006779 (Annex III)

COMMUNICATIONS

Oral Communications

- Soares-da-Silva F, Freyer L, Elsaid R, Burlen-Defranoux O, Iturri L, Sismeiro O, Pinto-do-Ó P, Gomez-Perdiguero E, Cumano A. **Yolk sac drives embryonic erythropoiesis**, GDR Stem Cells Annual Meeting, Paris, France. Nov 2020

- Soares-da-Silva F, Peixoto M, Burlen-Defranoux O, Resende TP, Schmutz S, Novault S, Vassilevskaia T, Talaei N, Chang Q, Ornatsky O, Pinto-do-Ó P & Cumano A. **The mouse fetal liver stroma: Signals for HSC Expansion**, 11th International Meeting of the Portuguese Society for Stem Cells and Cell Therapies (SPCE-TC), Lisbon, Portugal. Oct 2019
- Soares-da-Silva F, Pinto-do-Ó P & Cumano A. **The mouse fetal liver stroma: Signals for HSC Expansion**, Young Scientist Seminars, Porto, Portugal, Jun 2019
- Soares-da-Silva F, Peixoto M, Burlen-Defranoux O, Resende TP, Schmutz S, Novault S, Vassilevskaia T, Talaei N, Chang Q, Ornatsky O, Pinto-do-Ó P & Cumano A. **The mouse fetal liver stroma: Signals for HSC Expansion**, ISSCR 2019 Annual Meeting, Los Angeles CA, USA, Jun 2019
- Soares-da-Silva F, Pinto-do-Ó P & Cumano A. **The interplay between liver development and the onset of hematopoiesis**, Journées Départementales d'Immunologie – Institut Pasteur, Pornic, France, Oct 2018
- Soares-da-Silva F, Resende TP, Cumano A & Pinto-do-Ó P. **The fetal liver microenvironment: driving the onset of hematopoiesis**, 6th i3S Annual Meeting, Póvoa de Varzim, Portugal, Nov 2017
- Soares-da-Silva F, Pinto-do-Ó P, Cumano A. **Environmental signals driving the onset of hematopoiesis in the fetal liver**, 6th Annual Revive Consortium Meeting, Château de Montvillargenne, France, Jan 2017

Poster Communications

- Soares-da-Silva F, Burlen-Defranoux O, Elsaid R, Iturri L, Freyer L, Sismeiro O, Gomez-Perdiguerro E, Pinto-do-Ó P & Cumano A. **A unique population of yolk-sac derived erythroid progenitors sustains erythropoiesis during mouse fetal life**. PhDay, Porto, Portugal, Sep 2019
- Mackowski N, Soares-da-Silva F, Cumano A. **Hematopoietic Stem Cell Expansion in the Fetal Liver**. Hydra XIV: European Summer School on Stem Cell Biology and Regenerative Medicine 2019, Hydra, Greece, Sep 2019
- Soares-da-Silva F, Peixoto M, Burlen-Defranoux O, Resende TP, Schmutz S, Novault S, Vassilevskaia T, Talaei N, Chang Q, Ornatsky O, Pinto-do-Ó P & Cumano A. **The mouse fetal liver stroma: Signals for HSC Expansion**, ISSCR 2019 Annual Meeting, Los Angeles CA, USA, Jun 2019
- Soares-da-Silva F, Resende TP, Schmutz S, Novault S, Burlen-Defranoux O, Vassilevskaia T, Talaei N, Chang Q, Ornatsky O, Pinto-do-Ó P & Cumano A. **Decoding the role of fetal liver microenvironment in hematopoiesis**, The Stem Cell Niche Conference, Copenhagen, Denmark, May 2018
- Soares-da-Silva F, Hryshkina A, Resende TP, Pinto-do-Ó P & Cumano A. **The fetal liver microenvironment: driving the onset of hematopoiesis**, 10th International

Meeting Of The Portuguese Society For Stem Cells And Cell Therapies (SPCE-TC), Covilhã, Portugal, Oct 2017

- Soares-da-Silva F, Hryshkina A, Resende TP, Pinto-do-Ó P & Cumano A. **Setting the tone for hematopoiesis in the fetal liver**, EMBO Conference: Advances in Stem Cells and Regenerative Medicine, Heidelberg, Germany, May 2017
- Soares-da-Silva F, Pinto-do-Ó P, Cumano A. **Environmental signals driving the onset of hematopoiesis in the fetal liver**, 20th Annual GABBA Scientific Meeting, Porto, Portugal, Dec 2016

AWARDS

2019: FSSCR/BeSSCR Travel Grant, ISSCR 2019 Annual Meeting, Los Angeles CA, USA, Jun 2019

2018: Best Poster Award for the work “Decoding the role of fetal liver microenvironment in hematopoiesis”, 2nd Stapa International Retreat, Athens, Greece, Jun 2018

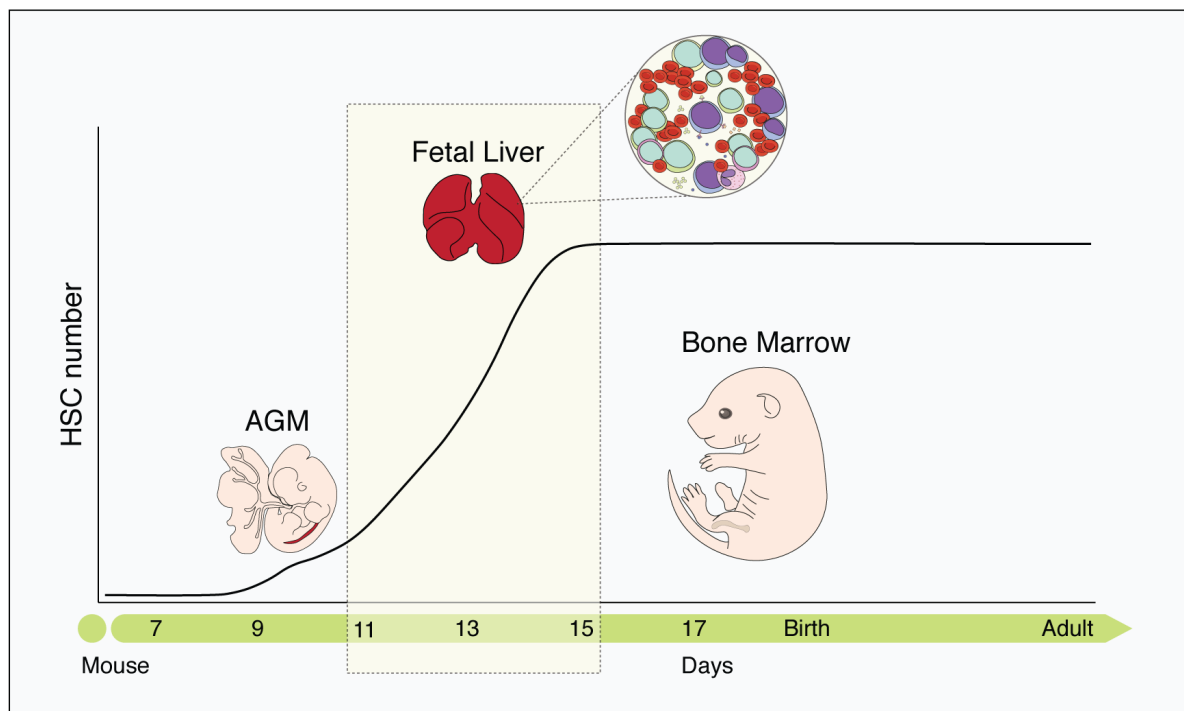
2017: EMBO Travel Grant, “EMBO Conference: Advances in Stem Cells and Regenerative Medicine, Heidelberg, Germany, May 2017

ORGANIZATION OF CONFERENCES

- Young Scientist Seminars, i3S, Porto, Portugal **2018-2019**
- Maria de Sousa Summer Research Program **2017-2018**
- Breaking boundaries in Science, GABBA Symposium, Porto, Portugal **2016**

CHAPTER II – INTRODUCTION

The adult hematopoietic system relies on a robust process whereby hematopoietic stem cells divide and differentiate generating all mature blood lineages. In physiological conditions, this process takes place in the bone marrow in both humans and mice. Even though adult hematopoiesis occurs in the bone marrow, this is merely the end-site of an otherwise thrilling journey through different anatomic locations. Embryonic hematopoiesis is characterized by an overlap in time and space of distinct waves of hematopoietic progenitors with distinct origins and lineage potential that converge to the fetal liver. This organ is the major hematopoietic site during embryogenesis and the only location where hematopoietic stem cell expansion occurs, generating the pool of HSCs that are maintained throughout adulthood.



Parts of this chapter are included in the following publications:

Soares-da-Silva F*, Peixoto M*, Cumano A, Pinto-do-Ó P (2020) **Crosstalk between the hepatic and hematopoietic systems during embryonic development.** Front. Cell Dev. Biol. 8:612. doi: 10.3389/fcell.2020.00612. *these authors contributed equally

Elsaid R*, **Soares-da-Silva F***, Peixoto M, Amiri D, Mackowski N, Pereira P, Bandeira A, Cumano A. (2020) **Hematopoiesis: a layered organization across species.** Front. Cell Dev. Biol. doi: 10.3389/fcell.2020.606642 *these authors contributed equally

HEMATOPOIESIS

Hematopoiesis is the process by which all blood lineages are generated. The adult hematopoietic system, composed of erythro-megakaryocytic, myeloid and lymphoid lineages, develops from multipotent hematopoietic stem cells (HSCs) (**Figure 1**). This concept of a stem cell that is able to generate the entire blood system was firstly described in the 19th century (**Pappenheim, 1896**), but direct evidence of HSC existence was only reported in the 60s, with the work of James Till, Ernest McCulloch, and others (**Till et al., 1961, 1964; Becker et al., 1963**). Subsequent studies pointed to a lineage relationship between HSCs and their derivatives, with a hematopoietic tree where HSCs are placed at the apex of the hierarchy and more committed progenitors lie downstream (**Kondo et al., 1997; Metcalf, 1999; Akashi et al., 2000; Reya et al., 2001**). HSCs can be defined by their ability to provide long-term multilineage hematopoietic reconstitution (LTR) when transplanted to lethally irradiated mice (**Morrison et al., 1995**) and further repopulate secondary recipients. These cells can be found within the Lin⁻CD45⁺Sca1⁺Kit⁺ (LSK) compartment and be further divided according to their reconstitution ability in long-term (LSK CD150⁺CD48⁻ LT-HSC) or short-term (LSK CD150⁻CD48⁻ ST-HSC) reconstituting cells (**Kiel et al., 2005**).

In the classical hierarchical model of hematopoiesis, multipotent progenitor (MPP) are downstream of ST-HSCs as they are still multipotent but have lost self-renewal capacity. MPPs then branch into common myeloid progenitors (CMP) or common lymphoid progenitors (CLP). Megakaryocyte-erythroid progenitors (MEP) and granulocyte-monocyte progenitors (GMP) differentiate from CMP (**Figure 1A**). This model of hematopoiesis has suffered modifications along the years with the introduction of other surface markers that allowed a better characterization of the system, such as the identification of a common lymphoid and myeloid progenitor (LMPP) downstream of MPP (**Adolfsson et al., 2005**), an early branching of the megakaryocyte lineage from HSCs (**Yamamoto et al., 2013; Sanjuan-Pla et al., 2013**), and a subdivision of the MPP compartment into distinct populations (**Cabezas-Wallscheid et al., 2014; Pietras et al., 2015**) (**Figure 1B**).

Both models described above depict stepwise, discrete differentiation steps to obtain mature blood cells. However, recent single-cell studies point to a continuous differentiation process, with a collection of heterogeneous compartments organized hierarchically in which hematopoiesis occurs in an orchestrated manner, with suppression of cell-division related genes and upregulation of lineage-specific transcripts (**Figure 1C**) (**Velten et al., 2017; Karamitros et al., 2018; Buenrostro et al., 2018; Briggs et al., 2018; Farrell et al., 2018**). Moreover, the classical hierarchical model has been devised based on lineage potential and functions of HSCs in colony or transplantation assays rather than steady-state, unperturbed settings. New technologies to assess the lineage output of single HSCs in native

hematopoiesis, such as lineage tracing relying on barcodes, revealed that in contrast to transplantation approaches where HSCs differentiate and replenish the mature blood cell compartment, it is the MPPs that contribute predominantly to mature blood cell formation (**Sun et al., 2014; Busch et al., 2015; Rodriguez-Fraticelli et al., 2018**).

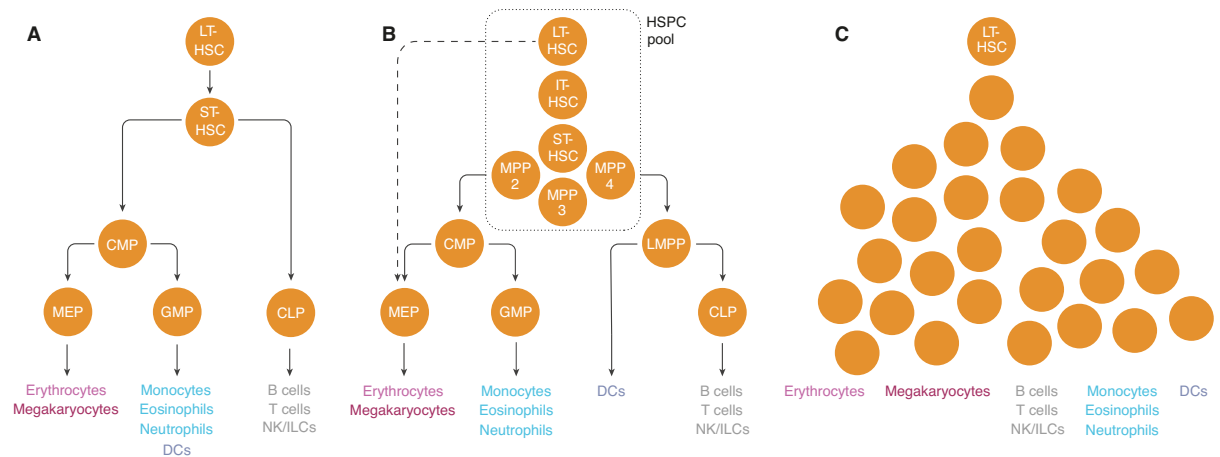


FIGURE 1 MODELS OF HIERARCHICAL HEMATOPOIESIS

Model of hierarchical trees. Adapted from (**Laurenti et al., 2018**).

The analysis of HSC cell fate in unperturbed conditions also showed that the stem cell pool is composed of a heterogeneous population, with HSC subsets already primed for specific lineages (**Rodriguez-Fraticelli et al., 2018**), a feature previously observed upon transplantation (**Dykstra et al., 2007; Challen et al., 2010; Sanjuan-Pla et al., 2013; Carrelha et al., 2018**). The causes of HSC heterogeneity are not known and are probably the result of a combination of different factors, including cell-intrinsic factors such as DNA mutations, epigenetic alterations or asymmetric segregation of cell fate determinants during division; stochastic processes; or cell-extrinsic factors due to microenvironment constraints.

Different bone marrow (BM) niche factors such as HSC localization or proximity to niche cells, cytokines or extracellular matrix (ECM) composition and biomechanical properties can affect adult HSC heterogeneity (reviewed in (**Crane et al., 2017; Haas et al., 2018; Pinho et al., 2019**)). A heterogeneous HSC compartment can be already identified in the embryo, at the fetal liver (FL) stage (**Benz et al., 2012**). Fetal and adult HSCs share both the multilineage potential and self-renewal ability, although fetal HSCs cycle at higher rates and the adult counterpart is mostly quiescent (**Passegué et al., 2005; Nakamura-Ishizu et al., 2014**). Although adult hematopoietic stem and progenitor cells (HSPCs) generate the majority of hematopoietic cells present in the adult organism, specific populations that persist in the adult are generated during embryonic development (**Ikuta et al., 1990; Eberl et al., 2004; Haas et al., 2012; Gomez-Perdiguero et al., 2015; Hoeffel et al., 2015**).

EMBRYONIC HEMATOPOIESIS

During embryogenesis, successive waves of hematopoietic progenitors contribute to the formation of erythroid, myeloid and lymphoid lineages (**Figure 2**). 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, in structures named blood islands. These structures and the order of events that resulted in their formation led embryologists to hypothesize that both hematopoietic and endothelial cells had a common origin, designated hemangioblast (**His, 1900; Sabin et al., 1920; Maximow, 1924; Murray, 1932**). Later, Moore and Owen showed that the 7-day chick YS generates mostly erythroid but also myeloid and lymphoid progeny after transplantation into irradiated embryos, pointing to a YS origin of HSCs (**Moore et al., 1967**). Similar results were observed in mammals in 1970 by Moore and Metcalf, with the first assays probing the existence of multilineage progenitors, by culturing single mouse YS hematopoietic progenitors shortly after beginning of circulation (**Moore et al., 1970**). Subsequent studies using chick-quail or chick-chick chimeras challenged such hypothesis where YS was appointed as the source of HSCs and showed that long-lasting hematopoietic potential was only found in intraembryonic progenitors (**Dieterlen-Lievre, 1975; Lassila et al., 1978**). In mammals, because embryonic development takes place *in utero*, such chimeras were not possible. Instead, culture of YS versus the intraembryonic aorta-gonad-mesonephros (AGM) region before circulation was established, demonstrated that HSCs are exclusively originated intra-embryonically (**Cumano et al., 1996; Medvinsky et al., 1996**) and it is now commonly accepted that 3 waves of hematopoiesis exist in the embryo. The first blood cells are generated early in development, as gastrulation is initiated, in the YS blood islands. Soon after, a second set of progenitors emerges in the YS vascular plexus. These two hematopoietic waves are responsible for generating erythrocytes responsible for providing the oxygen needed to sustain embryo survival, megakaryocytes and myeloid subsets important for tissue remodeling and hemostasis. Later on, HSCs are produced in the great vessels of the embryo and expand in the FL generating the pool of HSC that will sustain hematopoiesis during adult life. Alongside producing erythroid and myeloid cells, HSCs also generate the lymphoid lineage.

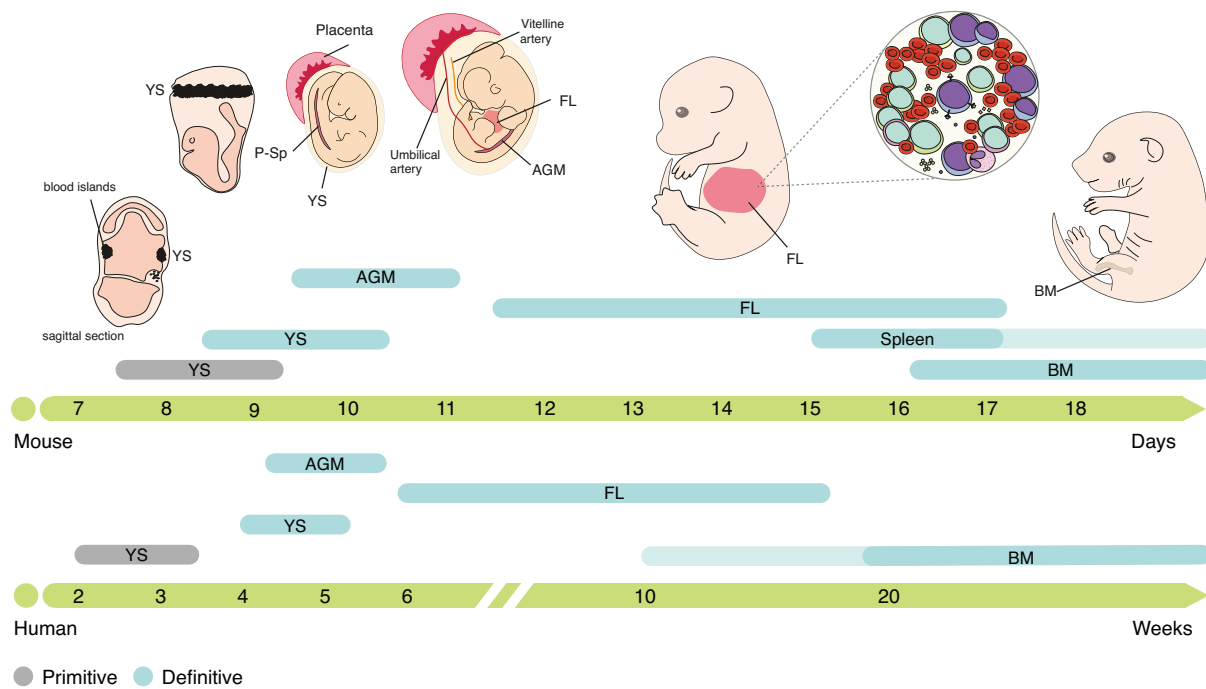


FIGURE 2 ONTOGENY OF THE HEMATOPOIETIC SYSTEM

Embryonic hematopoiesis is established in three distinct waves. The first hematopoietic cells emerge in YS blood islands, generating primitive erythrocytes, macrophages and megakaryocytes, constituting the primitive hematopoietic wave. The second hematopoietic wave is initiated in the vascular plexus of the YS, generating erythromyeloid progenitors (EMPs) that produce definitive erythrocytes, megakaryocytes, macrophages, neutrophils, granulocytes and mast cells, but lacks HSC activity. EMPs are the origin of tissue-resident macrophages that can persist throughout life. HSCs emerge in the AGM region and migrate to the fetal liver, the major embryonic hematopoietic organ. In FL, HSCs expand and differentiate into all mature blood lineages. Small numbers of hematopoietic progenitors also colonize the fetal spleen and are still found a few weeks after birth. Migration to the BM, where HSCs reside during adulthood, occurs as a continuous process and, in humans, can take several weeks. YS – yolk sac; P-Sp – para-aortic splanchnopleura; FL – fetal liver; AGM – aorta-gonads-mesonephros; BM – bone marrow. Reproduced from (Soares-da-Silva et al., 2020).

Sites of Origin and Lineage Potentials

In the mouse, the first hematopoietic cells arise around embryonic day (E) 7.5 in extra-embryonic structures named blood islands, in the YS (Moore et al., 1970). Blood islands are clusters of primitive blood cells lined by an endothelial layer. These structures are formed by migration of mesodermal cells already committed into the hematopoietic or endothelial lineages to the YS (Huber et al., 2004). Bipotent erythroid/megakaryocyte (Xu et al., 2001; Tober et al., 2007) and macrophage (Palis et al., 1999) progenitors within blood islands have been identified by colony-forming assays. Primitive erythroid progenitors (EryP-CFC) are exclusively present in the YS from E7.5 to E8.5 (Palis et al., 1999) and generate primitive erythrocytes that oxygenate the developing embryo. This wave is also termed primitive as the resulting erythrocytes have a larger size than their BM counterparts, a specific globin profile (Wong et al., 1986) and particularly because they maintain their nucleus for several days, similar to non-mammalian species, although they eventually enucleate (Kingsley et al.,

2004) . These cells undergo a similar maturation process as described for BM erythrocytes and can still be found in circulation around birth (**Kingsley et al., 2004**) . The primitive wave also generates megakaryocytes, as their erythroid/megakaryocyte bipotent progenitor can be identified in the yolk sac from E7.5 through E9.5 by the *in vitro* formation of erythroid and megakaryocyte colonies expressing the embryonic associated globin β H1 and the megakaryocyte marker GP1b β (**Xu et al., 2001; Tober et al., 2007**) . Macrophages are also present at this stage. Opposite to what is found in the adult BM, primitive macrophages do not differentiate through a monocyte stage, but directly from monopotent macrophage precursors (Mac-CFC/YS-Mp) (**Takahashi et al., 1989; Bertrand et al., 2005b**) .

The second hematopoietic wave starts one day later, at E8.25-E8.5, with erythro-myeloid progenitors (EMPs) emerging from hemogenic endothelium in the recently formed vascular bed of the YS, in a process called endothelial-to-hematopoietic (EHT) transition (**Bertrand et al., 2005b; Goldie et al., 2008; McGrath et al., 2015; Frame et al., 2016; Kasaai et al., 2017**) . For the following 48 hours these progenitors proliferate extensively, being found in the bloodstream shortly after (E9) and colonizing the hepatic primordium by E10.5 (**Palis et al., 1999**) . This second wave of hematopoiesis is responsible for the generation of definitive erythroid progenitors (burst-forming unit-erythroid – BFU-E and colony-forming unit-erythroid – CFU-E) and the first mast-cell and bipotent macrophage-granulocyte progenitors (**Palis et al., 1999, 2001a**) . Opposite to primitive erythroid progenitors, BFU-E do not mature in the bloodstream and cells at the different maturation steps are found in the FL (**Palis et al., 1999**) . Definitive erythrocytes can be found in circulation around E11.5-E12.5 and can be identified by their smaller size (similar to adult erythrocytes) and absence of nucleus. It is from E8.25 onwards that myeloid progenitors with neutrophil, mast-cell and granulocyte-macrophage potential are found. The kinetics of these progenitors is similar to that of BFU-E, with an expansion in the YS followed by a transit through circulation and are finally found in the FL (**Palis et al., 1999**) . Of note, it is during this second hematopoietic wave that most tissue-resident macrophages are generated (**Gomez-Perdiguero et al., 2015; Hoeffel et al., 2015**) . These cells seed the developing tissues and acquire specific signatures and functions. Some of these populations have self-renewal ability, maintaining the population throughout adult life (**Merad et al., 2002; Ajami et al., 2007; Jenkins et al., 2011**) .

The third and last wave of hematopoietic cell generation starts around E10.5, with HSC emergence in the embryo AGM region through EHT (**de Bruijn et al., 2002; Zovein et al., 2008; Boisset et al., 2010**) . Opposite to the two first hematopoietic waves, HSC generate the lymphoid lineage (**Ogawa et al., 1988; Cumano et al., 1996**) . These cells are generated exclusively in intraembryonic structures, namely in the AGM region (**Müller et al., 1994; Godin et al., 1995; Cumano et al., 1996; Medvinsky et al., 1996**) , although some authors claim

HSCs can also be generated in extraembryonic structures, namely in the YS (**Huang et al., 1993**) and placenta (**Gekas et al., 2005; Ottersbach et al., 2005; Rhodes et al., 2008a**). After generation, HSCs migrate to the FL (**Houssaint, 1981**) where they mature (**Kieusseian et al., 2012**), proliferate (expanding in numbers by more than 30-fold (**Ema et al., 2000**)) and differentiate into erythroid, myeloid and lymphoid lineages. Emerging immature HSCs (imHSCs) lack LTR activity (**Cumano et al., 2001; Bertrand et al., 2005a**). Only after undergoing maturation in FL, CD31⁺Kit⁺CD45⁻ imHSCs acquire an adult HSC phenotype (LSK CD150⁺CD48⁻) and develop LTR activity in Rag2^{-/-} or conventional mice as they upregulate MHC class I molecules (**Kieusseian et al., 2012**). HSCs in the FL undergo a proliferative stage, generating the final number of HSC that will home to the BM and maintain the pool throughout adult life. In the adult, all blood lineages arise from differentiation of HSCs and although adult and embryonic HSCs have similar lineage potentials, some lymphoid lineages are only produced during embryonic development, namely dendritic epidermal T cells (**Ikuta et al., 1990**), lymphoid tissue-inducer cells (**Eberl et al., 2004**), and a subset of IL-17-producer $\gamma\delta$ T cells (**Haas et al., 2012**).

During embryonic development, two sites of hematopoietic cell production exist, an extraembryonic site, the YS, and an intraembryonic site, the AGM. Cells that are formed in the YS have erythroid and myeloid potential, and cells formed in the AGM have both erythroid and myeloid, but also lymphoid potential. AGM vs YS hematopoiesis is distinguishable as AGM-derived HSCs are able to repopulate adult irradiated mice and self-renew as shown by secondary transplantations. Nonetheless, YS hematopoiesis is extremely important as it serves as the source of red blood cells that provide oxygen at early stages and myeloid cells that remodel newly formed tissues before HSCs are functional. A summary of the site of origin and lineage potential of the three hematopoietic waves can be found in **Table 1**.

A Layered Hematopoietic System – Identification and Contribution of Hematopoietic Progenitors in Time and Space

The three hematopoietic waves described above overlap in time and embryonic organs/regions, creating the need to accurately identify the distinct progenitors and derivatives according to their origin. Seminal studies have identified primitive YS hematopoietic progenitors based on single-cell *in vitro* differentiation potential (**Moore et al., 1970; Palis et al., 1999**). Although there is no defined surface signature for these primitive YS progenitors, they generate Kit^{low}CD41^{low} EryP (**Ferkowicz et al., 2003; Isern et al., 2012**), and CD45⁻Kit⁺ YS-Mp (**Bertrand et al., 2005b**). YS EMPs at early timepoints (E8.5) can also be identified based on the surface expression of Kit and CD41, and further divided by the expression of CD16/32, that by E9.5 is present in around 90% of the cells (**McGrath et al., 2015**). EMPs in the E10.5 FL can also be identified as Kit⁺CD41⁺CD16/32⁺ (**McGrath et al., 2015**). Emerging

HSCs in the AGM at E10 have both hematopoietic and endothelial markers and can be defined as CD45⁻CD41⁺Kit⁺AA4.1⁺Runx1⁺ (Jordan et al., 1990, 1995; North et al., 2002; Matsubara et al., 2005) . As they mature and migrate to FL, HSCs downregulate CD41 and upregulate CD45 and Sca1 expression (Table 2) (de Bruijn et al., 2002; Bertrand et al., 2005a; Kiel et al., 2005; Matsubara et al., 2005) .

TABLE 1 SITE OF ORIGIN AND LINEAGE POTENTIAL OF EMBRYONIC HEMATOPOIETIC WAVES

Hematopoietic Wave	Site of Origin	Lineage Potential	References
First or Primitive wave	Yolk Sac Blood Islands	Erythro-Megakaryocyte (primitive erythrocytes and megakaryocytes) Macrophages	(Palis et al., 1999; Xu et al., 2001)
Second or EMP wave	Yolk Sac Vascular Plexus	Erythro-Megakaryocyte (definitive erythrocytes, megakaryocytes) Myeloid (macrophages, granulocytes, mast cells)	(Palis et al., 1999)
Third or HSC wave	Embryo Proper AGM	Erythro-Megakaryocyte (definitive erythrocytes, megakaryocytes) Myeloid (all cell types) Lymphoid (all cell types)	(Cumano et al., 1996; Medvinsky et al., 1996)

The understanding of the dynamics of each wave output and contribution to embryonic and adult hematopoiesis is limited if one relies solely on the identification/isolation of precursors and their *in vitro* or post-transplantation *in vivo* potential (Figure 3). Gene-targeting experiments have offered important contributions to the understanding of hematopoietic lineage emergence and maintenance. Studying genes that affect definitive but not primitive hematopoiesis, such as *Runx1* (Okuda et al., 1996; Wang et al., 1996a; Castilla et al., 1996), *c-Myb* (Mucenski et al., 1991), *Cbfβ* (Wang et al., 1996b), *Ikaros* (Georgopoulos et al., 1994; Nichogiannopoulou et al., 1999), *Lhx2* (Porter et al., 1997) and *Rel* (Grossmann et al., 1999), provides important information about the dynamics of the first wave. For example, *c-Myb* mutant mice appear normal by E13.5 but are severely anemic by E15.5, showing a reduced hematocrit and a high percentage of nucleated erythrocytes, indicating that up until this stage, primitive hematopoiesis is able to sustain embryonic life (Mucenski et al., 1991). The contribution of primitive *versus* definitive erythropoiesis during embryonic development was also analyzed in unperturbed mice using primitive epsilon globin gene *Hbb-y* reporters (Dyer et al., 2001; Kingsley et al., 2006; Isern et al., 2008).

The first blood cells generated in the YS are erythrocytes responsible for carrying oxygen throughout the developing embryo, macrophages that play important roles in tissue

development and remodeling, and megakaryocytes that produce platelets responsible for clotting processes. The idea that these YS populations are only produced and required for embryonic development has been recently challenged. Two major recent contributions using inducible lineage-tracing mouse models directly showed that distinct tissue-resident macrophage populations are generated during embryonic development, specifically from YS progenitors and persist throughout adulthood (**Gomez-Perdiguero et al., 2015; Hoeffel et al., 2015**). Using $\text{Runx1}^{\text{MerCreMer}}\text{Rosa26}^{\text{EYFP}}$ embryos pulsed with 4-hydroxytamoxifen (OH-TAM) at E7.5 that labels YS progenitors, Hoeffel and colleagues showed that around 30% of microglia (resident macrophages of the brain) was labeled in adult mice (**Hoeffel et al., 2015**). Using two distinct lineage-tracing models $\text{Csf1r}^{\text{MerCreMer}}\text{Rosa26}^{\text{YFP}}$ pulsed with OH-TAM at E8.5 and $\text{Tie2}^{\text{MerCreMer}}\text{Rosa26}^{\text{YFP}}$ pulsed with tamoxifen at E7.5 or E8.5, Gomez-Perdiguero and colleagues showed that most tissue-resident macrophages (microglia from brain, Kupffer cells from liver, Langerhans cells from skin and alveolar macrophages from lung) originate from a Tie2 expressing EMP progenitor (**Gomez-Perdiguero et al., 2015**). While the majority of these cells are replaced by HSC-derived progeny with time, microglia cells are only marginally replaced (**Schulz et al., 2012; Gomez-Perdiguero et al., 2015**). These studies have challenged the dogma where HSCs are the source of all hematopoietic lineages in the adult, providing evidence for the existence of a “layered” developmental system.

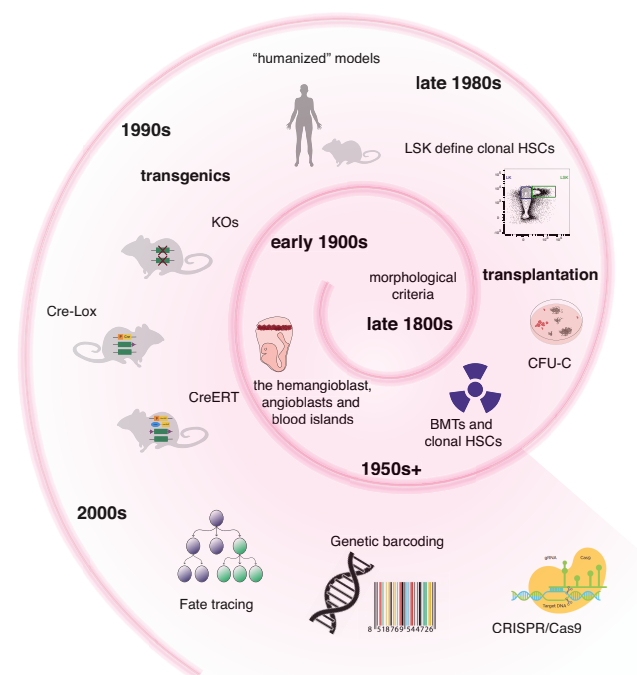


FIGURE 3 CONTRIBUTIONS OF THE MURINE MODEL FOR HEMATOPOIESIS

A spiral timeline of major contributions the mouse system has made to our understanding of hematopoiesis. From early transplantation and clonal analyses to transgenic technology and future advancements, the mouse system has served as a critical model for hematopoietic biology. Adapted from (**Schmitt et al., 2014**)

The “layered immune system” model was initially proposed by Herzenberg et al, with the identification of functionally distinct “lineages” of B cells that are generated at different times during ontogeny (**Herzenberg et al., 1989**). B-1a cells, which reside mainly in the peritoneal cavity and secrete natural antibodies in a T-cell independent manner, can only be obtained by embryonic precursors, and contribute to innate-like immunity (reviewed in (**Montecino-Rodriguez et al., 2012; Herzenberg, 2015**)). Other innate-like cells only produced during embryogenesis include LT_i (lymphoid tissue inducer cells) and V γ 5⁺ T cells (**Ikuta et al., 1990; Yoshida et al., 2001; Eberl et al., 2004**). This layered organization has been recently exploited to the thymus, where two waves of distinct hematopoietic precursors colonize the developing organ (**Ramond et al., 2014; Elsaid et al., 2020a**). Further analysis of the function and the differentiation potential of the first wave of early thymic progenitors unveiled that they produce mainly innate-like lymphocytes and innate lymphoid cells both known to be required for the maturation of thymic epithelial cells (**Elsaid et al., 2020a**). This sequence of events highlights the necessity of an immune stratification to establish a healthy and diverse immune system (**Elsaid et al., 2020b**).

The identification of cell populations only produced during embryonic development led to the hypothesis that these cells could derive from distinct progenitors of those found in the adult. The observations that fetal and neonatal HSCs could generate B1-a cells, demonstrated that HSCs are the origin of these cells and that this ability may be lost during ontogeny (**Kikuchi et al., 2006; Barber et al., 2011**). To better understand this shift in HSC state, the Yuan group used cellular barcoding coupled with transplantation to show that FL HSCs have B-1a but also B-2 and granulocyte potential that is lost on a per-cell basis during development (**Kristiansen et al., 2016**). On the other hand, recent studies investigated the existence of distinct lineages of HSCs during embryonic development as has been proposed in the late 1980s (**Herzenberg et al., 1989**). Beaudin and colleagues used the FlkSwitch model and showed that innate-like B and T cells originate from a developmentally restricted HSC (drHSC) (**Beaudin et al., 2016**). These cells are exclusively found in fetal and neonatal stages and do not give rise to adult HSCs, suggesting that they most likely represent two distinct lineages.

Embryonic hematopoiesis is thus characterized by the partial overlap of hematopoietic waves, each serving specific needs for the time of development of the embryo and set the ground for an efficient assembly of the innate and adaptive immune system before birth.

TABLE 2 MARKERS USED FOR IDENTIFICATION OF HEMATOPOIETIC PROGENITORS

	Primitive	EMPs			HSCs				References
	E8.5 YS	E8.5 YS	E9.5 YS	E10.5 FL & Blood	E10.5 (AGM)	E11-E12.5 AGM	E12.5-E16.5 FL	Adult	
AA4.1		+	+		+	+	+		(Jordan et al., 1990, 1995; Bertrand et al., 2005a; Gomez-Perdiguero et al., 2015)
Kit	low/+	+	+	+	+	+	+	+	(Sánchez et al., 1996; Yoder et al., 1997; Bertrand et al., 2005b; Isern et al., 2012)
CD11b							+	-	(Morrison et al., 1995)
CD150							+	+	(Kiel et al., 2005; Kim et al., 2006)
CD16/32		+/-	+	+					(McGrath et al., 2015)
CD31					+	+		-	(Baumann et al., 2004)
CD34	+				+	+	+		(Sánchez et al., 1996; Yoder et al., 1997; Ema et al., 2006)
CD41	low	+	+	+	+	+/-	-	-	(Corbel et al., 2002; Ferkowicz et al., 2003; Mikkola et al., 2003a; Bertrand et al., 2005a)
CD45	-				-	+	+	+	(North et al., 2002; Bertrand et al., 2005b; Matsubara et al., 2005)
CD48							-	-	(Kiel et al., 2005; Kim et al., 2006)
Csf1r		+	+						(Gomez-Perdiguero et al., 2015)
Runx1					+	+	+	+	(North et al., 2002)
Sca1					-	+/-	+	+	(Morrison et al., 1995; de Bruijn et al., 2002; Matsubara et al., 2005)
VE-Cadherin	+				+	+	+/-	-	(Fraser et al., 2002; North et al., 2002; Taoudi et al., 2005; Kim et al., 2005; Ema et al., 2006)

A LOOK INTO ERYTHROPOIESIS

In the adult, the production of erythrocytes is the largest output of the hematopoietic system, with around 2×10^{11} red blood cells (RBCs) being produced daily in the BM and released into circulation (**Palis, 2014**). This production rate can be adjusted depending on requirement. Distinct pathways are involved in the control of steady-state or stress-induced erythropoiesis, namely oxygen sensors, cytokines or other factors, such as iron metabolism (**Hattangadi et al., 2011; Oburoglu et al., 2016; Liang et al., 2016**). Erythropoiesis is characterized by the movement of lineage-committed cells through progenitor, precursor and RBC compartments (**Figure 4**). The most immature compartment consists of progenitors that can be detected by their ability to form erythroid colonies *in vitro* and are named BFU-E and CFU-E (**Iscoe, 1977; Gregory et al., 1978**). BFU-Es are the most immature hematopoietic progenitor that is already committed to the erythroid lineage and although there is a continuous differentiation process between BFU-Es and CFU-Es, their growth factor requirements are completely different. While BFU-Es require stem cell factor/Kit ligand (SCF/KITL) (**Emerson et al., 1985**) and other hematopoietic growth factors for proliferation and differentiation, CFU-Es are highly dependent on erythropoietin (EPO) (**Iscoe et al., 1974, 1978**). The subsequent phase of erythroid maturation involves the differentiation of the nucleated precursors from proerythroblasts through distinct stages of erythroblasts (basophilic, polychromatophilic and orthochromatic/acidophilic). Enucleation of the orthochromatic erythroblast gives rise to the reticulocyte which finally matures into the RBC. The distinct cell types along the erythroid differentiation pathway can be identified by specific surface marker expression (**Figure 4**) (**McGrath et al., 2017**). This differentiation process occurs in extravascular spaces, in anatomic niches known as erythroblastic islands (**Bessis, 1958**). These structures are composed of a central macrophage that is surrounded by up to 30 erythroid cells at varying degrees of maturation (**Mohandas et al., 1978; Lee et al., 1988**). The central macrophage serves as an anchor maintaining erythroblasts in close proximity to provide the cellular interactions necessary for erythroid proliferation and differentiation (**Hanspal et al., 1994, 1998; Rhodes et al., 2008b**). These macrophages are also responsible for the phagocytosis of the extruded nucleus from terminally differentiating erythroblasts (**Seki et al., 1965; Skutelsky et al., 1972**).

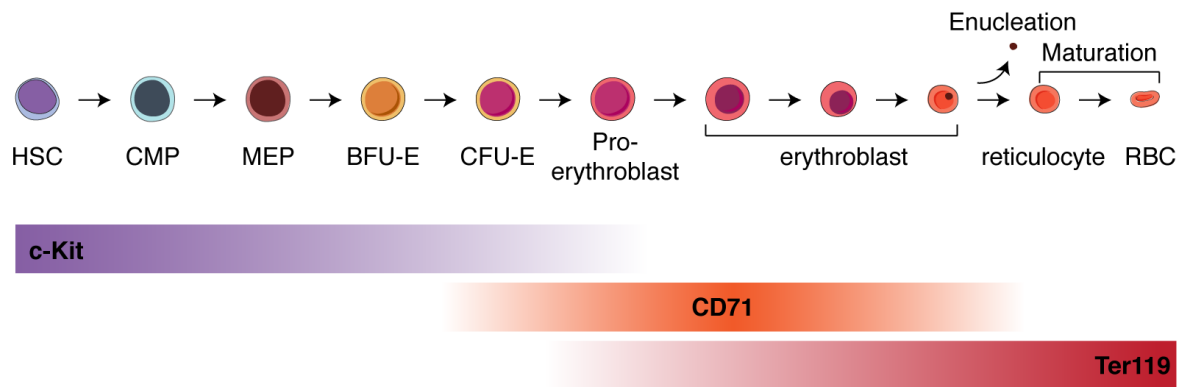


FIGURE 4 ERYTHROID DIFFERENTIATION IN THE MOUSE

Adult erythropoiesis derives from a series of commitment steps that lead to the generation of the most immature erythroid progenitor (BFU-E) that differentiates into CFU-E and pro-erythroblast. This differentiation is accompanied by changes in the cell surface of the key markers Kit, CD71 and Ter119. Erythroblasts undergo a series of maturation that result in enucleation and formation of the red blood cell. Adapted from (Dzierzak et al., 2013).

Erythropoiesis at the transcriptional level

Multiple transcription factors (TFs) are involved in the regulation of RBC production. The core erythroid network is composed of the DNA-binding transcription factors GATA-1, TAL-1, and KLF1, and the non-binding transcription factors LDB1 and LMO2 (Cantor et al., 2002). During erythropoiesis, there is a sequential but overlapping expression of *Gata1* and *Gata2*. *Gata2* is expressed by hematopoietic progenitors and regulates their maintenance and proliferation (Tsai et al., 1994), while *Gata1* is necessary for the survival and terminal differentiation of erythroid progenitors (Pevny et al., 1991, 1995; Fujiwara et al., 1996). Crosstalk between the two factors occurs at the transcriptional level, as they can inhibit each other's transcription (Weiss et al., 1994). It is the fine balance between GATA-1 and GATA-2 that drives the expression of target genes essential for erythroid differentiation and maturation, and β -globin gene expression (reviewed in (Moriguchi et al., 2014)). Transcriptional regulators that can form complexes with GATA-1 include TAL-1, LDB1, and LMO2. These complexes can activate the expression of terminally expressed erythroid-specific genes, and their targeted disruption can lead to marked defects in erythropoiesis (Warren et al., 1994; Shivdasani et al., 1995; Li et al., 2010). TAL-1 is also a target of GATA-1 and is required for the specification of all hematopoietic lineages (Shivdasani et al., 1995). Even though it is critical for stem cell generation, it is not required for HSC survival, multipotency or long-term repopulation in mice. However, megakaryocyte and erythroid differentiation are severely hampered in the absence of this TF (Mikkola et al., 2003b). KLF1, also known as EKLF, is a direct target of GATA-1 (Crossley et al., 1994). Its expression is critical for the activation of β -globin transcription, and mice lacking this TF die *in utero* (Donze et al., 1995; Nuez et al., 1995; Perkins et al., 1995). Moreover, KLF1 directly controls the majority of heme synthesis

and iron procurement pathways in maturing erythroid cells, being crucial for the establishment of functional erythrocytes (Tallack et al., 2010).

Signaling pathways involved in erythropoiesis

One of the most well-studied cytokines in erythropoiesis is EPO. EPO promotes cell survival by retarding DNA breakdown and preventing programmed death in erythroid progenitors (Koury et al., 1990a). CFU-Es require EPO for survival during *in vitro* cell culture, and for forming colonies in methylcellulose media (Iscoe et al., 1974, 1978). EPO and EPO receptor knockout mice die at E13 due to failure of FL erythropoiesis (Wu et al., 1995b; Lin et al., 1996). CFU-E and BFU-E erythroid progenitors can still be found in the FL of these mice, indicating that this signaling pathway is not required for erythroid lineage commitment nor the proliferation or differentiation of BFU-E to CFU-E (Wu et al., 1995b).

Another important signaling pathway is that of KITL. KITL, by signaling through the Kit receptor, potentiates erythropoietin signaling and plays an important role in proliferation of definitive erythroid precursors. Mice lacking Kit signaling have reduced CFU-E progenitors in the FL and die of severe anemia between E14.5 and E16.5 (Russel et al., 1968; Nocka et al., 1989). Moreover, KITL can indirectly phosphorylate the EPO receptor to induce further proliferation and maturation of CFU-Es (Wu et al., 1995a). The role of EPO and Kit signaling in erythropoiesis is extensively reviewed in (Munugalavada et al., 2005).

Embryonic erythropoiesis

Red blood cells are the first hematopoietic population that can be identified in the developing embryo. The embryonic RBC compartment is constituted by erythroid cells from all three hematopoietic waves (Figure 5). EryP-CFC are capable of generating compact red colonies composed of large erythroid cells that express both embryonic and adult globins (Palis et al., 1999). EryP-CFC are exclusively present in the YS from E7.5 to E8.5 and do not possess a surface signature that allows purification, as they share expression of CD41 (Ferkowicz et al., 2003) and Kit (Isern et al., 2012) with other hematopoietic progenitors (see Table 2). To overcome this, genetically engineered mice with enzymatic or fluorescent reporters have been devised, namely based on the lineage-restricted expression of *Hbb-y* (Dyer et al., 2001; Isern et al., 2008). These transgenic mice also allowed a thorough analysis of the dynamics of primitive erythrocytes. Primitive erythrocytes can be detected in circulation as soon as E9 and can still be found 3 weeks post-birth (Kingsley et al., 2004; Fraser et al., 2007; Isern et al., 2012). These cells have been named as primitive as they are larger than the adult counterparts and are the only mammalian erythroid cells that maintain their nucleus when they enter the circulation, eventually enucleating after E12.5 (Fraser et al., 2007).

Definitive erythrocytes originate from the second and third wave of hematopoiesis. EMPs migrate to the FL where they differentiate to originate the first definitive erythrocytes. EMPs in early FL stages can be identified by co-expression of Kit, CD16/32 and CD41 (**McGrath et al., 2015**). EMP-derived definitive erythrocytes are first detected in the blood around E12 (**Kingsley et al., 2004; McGrath et al., 2011**). HSCs colonize the FL starting at E10 (**Houssaint, 1981**), where they also differentiate and contribute to the definitive erythroid cell pool. Opposite to primitive erythropoiesis, definitive erythroid cells associate with macrophages to form erythroblastic islands where they expel their nuclei before entering circulation (**Sasaki et al., 2000**). Another key difference between primitive and definitive erythropoiesis is transcription factor requirements. Mice lacking c-Myb or Runx1 fail to generate definitive erythrocytes but have normal numbers of primitive RBCs (**Mucenski et al., 1991; Okuda et al., 1996**). Disruption of Gfi-1b or Klf1 also causes a disruption in definitive erythroid formation, without major defects in the primitive erythroid compartment (**Nuez et al., 1995; Perkins et al., 1995; Saleque, 2002**).

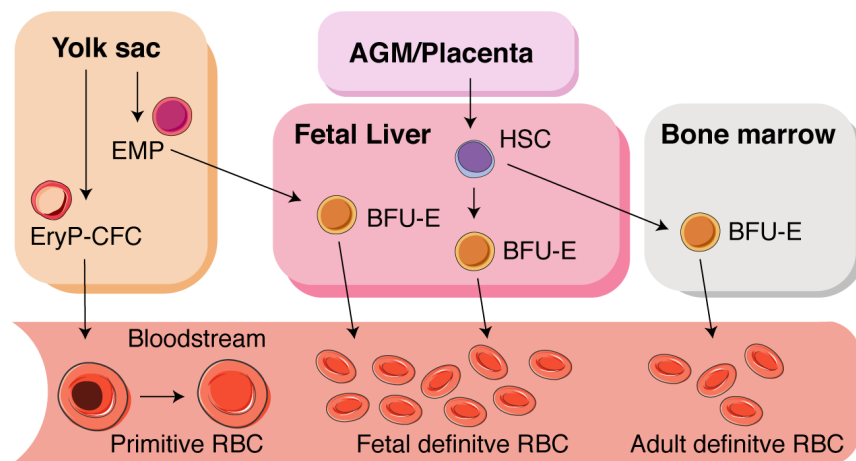


FIGURE 5 MODEL OF ERYTHROID ONTOGENY IN THE MAMMALIAN EMBRYO

Primitive red blood cells originate from yolk sac EryP-CFC and mature in circulation. In the embryo, definitive erythrocytes have two sources: the yolk sac EMP and the AGM HSC. Adult definitive erythrocytes derive from bone marrow erythroid progenitors. Adapted from (**McGrath et al., 2008**).

SITES FOR HEMATOPOIETIC CELLS DURING DEVELOPMENT

YOLK SAC

The visceral YS is an extraembryonic structure that forms during gastrulation, a process that starts at E6.5 in the mouse embryo. It consists of two layers, a visceral endoderm layer derived from the primitive endoderm and an extraembryonic mesoderm layer derived from epiblast cells (**Figure 6**). This association of cells that derive from the endoderm and the mesoderm germ layers is designated splanchnopleure. The proper function of the YS is critical for normal embryonic development: the endoderm layer transports and metabolizes maternally-derived molecules and synthesizes serum proteins, and the mesoderm layer produces the first hematopoietic cells (reviewed by (**Palis et al., 2001b; Moore, 2009**)).

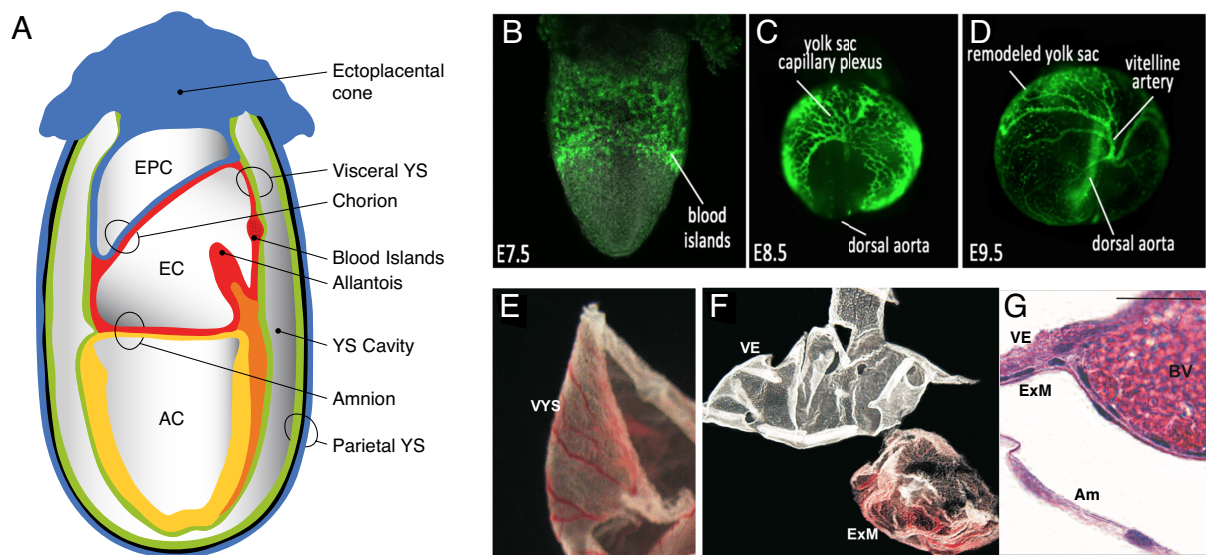


FIGURE 6 EXTRAEMBRYONIC TISSUES IN THE DEVELOPING MOUSE EMBRYO

(A) Extraembryonic tissues in mouse embryo at E7.5 (B) VEGFR2/Flk1 expression labeling nascent vessels in the blood islands at E7.5; (C) ϵ -globin-GFP labeled erythroblasts filling the primitive capillary plexus at E8.5, and (D) after vascular remodeling at E9.5. (E) Microdissected intact visceral yolk sac. (F) The visceral yolk sac of an E9.5 mouse embryo after enzymatic separation and microdissection of the avascular visceral endoderm and the reddish extraembryonic mesoderm. (G) Haematoxylin-eosin staining of a paraffin section through the visceral yolk sac of an E17.5 mouse fetus, which is approximately 2 days before birth. Scale bar: 25 μ m. Copyright permissions: (B-C) reproduced according to CC BY 3.0 from (**Garcia et al., 2014**); (E-G) reproduced with author's permission from (**Dobrev et al., 2010**). AC: amniotic cavity; Am: amnion; BV: blood vessel; EC: exocoelom; EPC: ectoplacental cavity; ExM: extraembryonic mesoderm; VE: visceral endoderm; VYS: visceral yolk sac; YS: yolk sac.

Between E7 and E7.5, extraembryonic mesodermal cells in the visceral YS proliferate and form mesodermal cell masses or angioblastic cords, which will originate the blood islands (**Figure 6A-B**). These structures consist of central erythroblasts that accumulate hemoglobin, and outer cells that flatten and form the endothelium. After the blood islands have formed, endothelial cell precursors (angioblasts) divide and differentiate into endothelial cells that

migrate distally into the YS and form the primitive capillary plexus (**Drake et al., 2000**). Blood island primitive erythroblasts enter the newly generated vasculature where they continue to divide for several days (**Palis et al., 2001b**). Macrophage progenitors can be found starting at E7.0 and after an initial expansion in the YS, these progenitors can be found in the bloodstream and embryo proper (**Palis et al., 1999**). The first differentiated macrophages can be identified in the YS between E9.0-9.5 (**Takahashi et al., 1989**).

This recently formed network of vessels then undergoes rapid morphological changes as the established vessels expand and increase in diameter, and smaller vessels fuse to form the larger vessels of the YS and embryo (**Figure 6D**) (**Udan et al., 2013**). At E8.5, the second wave of hematopoietic progenitors (discussed above) is generated in the remodeled YS vascular plexus by EHT, showing a direct relationship between endothelial and hematopoietic cells. Analysis of EMP-derived BFU-Es show increased numbers in the YS at E8.5-9.0 which decrease between E10.5 and E11.5, consistent with their migration to the liver rudiment (**Palis et al., 1999**). The first definitive erythrocytes can only be found at E12 as they egress from the FL, suggesting that EMP-derived erythroid progenitors cannot fully differentiate in the YS environment.

Little is known about the niche properties of the YS and the stromal cells that provide the required signals for cell proliferation and differentiation. E9.5 YS-derived endodermal and mesodermal cell lines have been shown to support *in vitro* growth of hematopoietic cells and influence differentiation of the hematopoietic precursors in a similar manner to that found *in vivo* (**Yoder et al., 1994**). More recently, the role of the YS endothelial compartment has been evaluated. Endothelial-specific deletion of *Kitl* using Tie2^{Cre}Kitl^{fllox} mice results in a reduction of EMPs in the YS at E11.5 at the same proportion as that found using Kitl mutant mice, suggesting a specific role for YS endothelial cells as niche components (**Azzoni et al., 2018**).

PLACENTA

The placenta is a vital organ during embryonic development and acts as the interface between the fetal and maternal environments. It is needed for the exchange of gases, nutrients and product waste between the mother and the embryo (**Fowden et al., 2012**). The most important cell types in the placenta are the cells of the trophoblast lineage, which derive from the trophectoderm, making up the majority of the fetal part of the placenta and providing the main structural and functional components of the two blood systems (reviewed in (**Rossant et al., 2001**)). The complex vascular network named the placental labyrinth is composed of the fetal vascular compartment that arises from the embryo allantois, and the maternal components that originate from the maternal vasculature and decidual cells (reviewed in (**Downs et al., 2020**)).

The placenta had not been recognized as a hematopoietic organ until recently, although the presence of hematopoietic progenitors had been reported since the 60s (**Till et al., 1961; Dancis et al., 1968; Melchers, 1979**). Recent studies re-addressed the role of the placenta in embryonic hematopoiesis and showed that this organ is a source of multipotential hematopoietic progenitors before FL colonization (**Alvarez-Silva et al., 2003**). Hematopoietic stem cells can be found in the placenta starting at E11 and reaching a peak at E12 (**Gekas et al., 2005; Ottersbach et al., 2005**). The numbers of HSCs found in placenta are however low (12-50 cells at E12), and decrease drastically after E13 (**Gekas et al., 2005; Ottersbach et al., 2005**). The local production of HSCs has been investigated. The placenta of a mouse model lacking a functional circulatory system was shown to still harbor hematopoietic activity, suggesting that the placenta could generate *de novo* hematopoietic cells with multilineage potential (**Rhodes et al., 2008a**), however, direct evidence for HSC emergence from the placenta is yet to attain.

The role of the hematopoietic microenvironment in the placenta has been poorly characterized. Human placenta-derived stromal cell lines with pericyte characteristics were shown to support *in vitro* maintenance of cord blood hematopoietic progenitors and hematopoietic cells were found in close contact with pericytes/perivascular cells in the placenta by immunostaining (**Robin et al., 2009**). In the mouse, laser-capture microdissection allowed the identification of endothelial cells as *Kitl* producers and important for HSPC regulation in the placenta (**Sasaki et al., 2010**). Platelet-derived growth factor β also seems to be an important component of the placental hematopoietic niche as in the absence of this ligand, trophoblast cells upregulate *Epo* expression, causing ectopic erythropoiesis (**Chhabra et al., 2012**).

AGM

The AGM is an embryonic region that contains the dorsal aorta, the genital ridge (that will originate the gonads), and the mesonephros (that will originate the kidneys). It develops from the para-aortic splanchnopleura (PSp) that derives from lateral plate mesoderm (**Figure 2**). The dorsal aorta is composed of endothelial cells that develop from two distinct mesodermal sources, the dorsolateral cells derive from paraxial mesoderm, which also gives rise to somites, while the ventral floor originates from splanchnic mesoderm (**Pardanaud et al., 1996**). The AGM and major embryonic vessels autonomously generate the first HSCs through an EHT process (**Cumano et al., 1996; Medvinsky et al., 1996; de Bruijn et al., 2000, 2002; Zovein et al., 2008; Bertrand et al., 2010; Kissa et al., 2010**). Hematopoietic stem cell clusters in the aorta can be found both dorsally and ventrally, however, HSC activity has been localized exclusively to the ventral aspect of the aorta, suggesting a ventral polarity in the development of HSCs (**Figure 7**) (**Taoudi et al., 2007; Mascarenhas et al., 2009**).

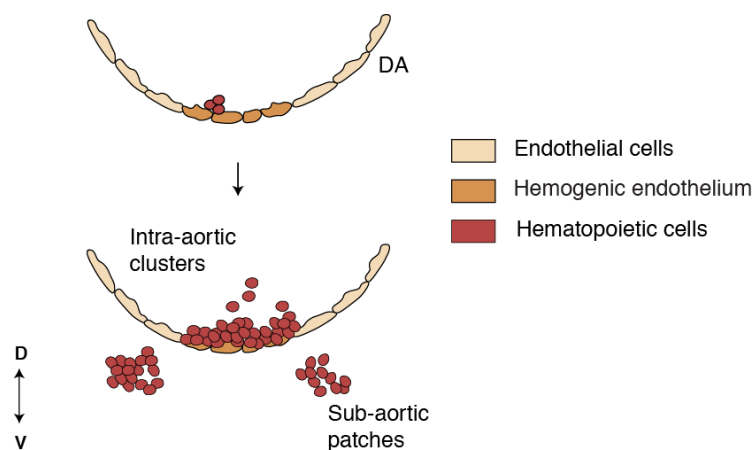


FIGURE 7 ENDOTHELIAL-TO-HEMATOPOIETIC TRANSITION IN THE DORSAL AORTA

Intra-embryonic hematopoietic clusters (IAHCs) containing newly formed HSCs in mammals. IAHC originate sub-aortic patches in mice. D – dorsal; DA – dorsal aorta; V – ventral. Adapted from (Elsaid et al., 2020b).

Distinct AGM-derived stromal cell lines have been generated and shown to have the capacity to support HSC repopulating activity after co-culture (Ohneda et al., 1998; Xu et al., 1998; Durand et al., 2006). More than a hundred stromal clones from sub-dissected AGM regions have been generated to compare the capacity of distinct anatomical sub-locations to support HSCs (Oostendorp et al., 2002) (reviewed in (Dzierzak et al., 2008; Gao et al., 2018). More recently, studies in human AGM identified secreted proteins that can promote hematopoiesis (Crosse et al., 2020).

FETAL LIVER

The FL is formed early in embryonic development, with the development of the hepatic diverticulum at around E8.5 (Wilson et al., 1963). It is colonized by hematopoietic cells at E10 and does not generate HSCs de novo (Figure 8) (Johnson et al., 1975; Houssaint, 1981; Palis et al., 1999). Hematopoietic stem and progenitor cells that are generated in YS and AGM, migrate to the FL where they undergo proliferation and differentiation before homing to the BM where they reside in the adult (Palis et al., 1999; Ema et al., 2000). Indeed, the FL accommodates the hematopoietic system from E10 until E18, being the major hematopoietic organ during embryonic development. The FL represents the only organ where HSCs expand more than 30-fold, generating the pool that will be maintained throughout adulthood (Ema et al., 2000; Gekas et al., 2005). The FL and its role in hematopoiesis will be extensively discussed further in this chapter.

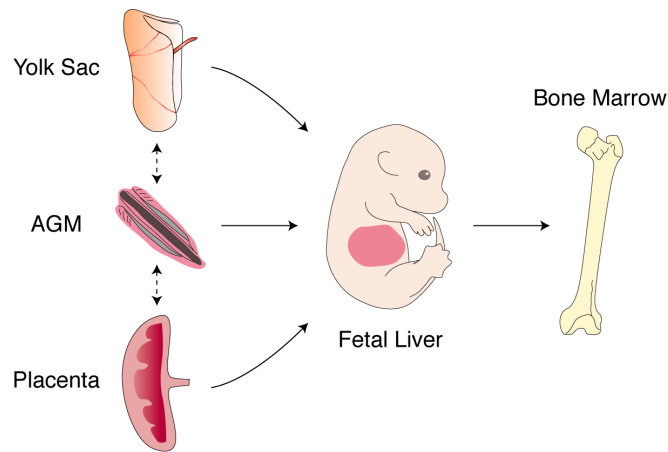


FIGURE 8 CIRCULATION OF HSPCs IN THE EMBRYO

Hematopoietic progenitors generated in the YS or AGM travel through circulation and can be found in the placenta. These progenitors are then retained in the FL where they expand and differentiate and colonize the BM in late gestation. YS – yolk sac; AGM – aorta-gonads-mesonephros; FL – fetal liver; BM – bone marrow.

FETAL AND ADULT BONE MARROW

The BM is the ultimate site of hematopoiesis in the embryo, being the production hub for blood cells throughout life. LSK cell homing and colonization of mouse BM was reported at E15.5-E16.5, coinciding with the fetal bone marrow (FBM) vascularization (**Gekas et al., 2005; Coşxkun et al., 2014; Cao et al., 2019**), although LT-HSC activity cannot be detected before E17.5 (**Christensen et al., 2004**). In mice, HSCs migrating out of the FL also seed the spleen, starting at E15.5, with HSC activity still being detected a few weeks after birth (**Wolber et al., 2002; Christensen et al., 2004; Bertrand et al., 2006**). In humans, although early colonization of fetal long bones was reported at ~10 weeks post-conception (wpc) (**Charbord et al., 1996**), the hematopoietic shift from the FL to the FBM occurs later, around 20 wpc (**Figure 2**).

Coşkun and colleagues showed that hematopoietic progenitor cells reside in the vascularized regions of fetal long bones, stage at which they are still proliferative (**Coşxkun et al., 2014**). Previous work showed that in the BM, HSCs are cycling during the first 3 weeks after birth and become quiescent thereafter (**Bowie et al., 2006**). The shift to a quiescence state seems to be dependent on the cellular composition of the microenvironment (**Coşxkun et al., 2014**). LSK cells isolated from *Osx*^{-/-} FBM (that lack osteolineage cells (**Nakashima et al., 2002**) and some stromal populations (**Mizoguchi et al., 2014**)) form multi-lineage colonies *in vitro*, but fail to repopulate transplanted recipients (**Mizoguchi et al., 2014**). LSK cells exhibited dysregulated cell cycle progression and defective homing ability, suggesting that osteolineage and/or mesenchymal cells are necessary to establish and sustain BM LT-HSCs (**Coşxkun et al., 2014**). Additionally, E15.5 CD105⁺Thy1⁻ mesenchymal progenitors

transplanted under the kidney capsule give rise to donor-derived chondrocytes and can create an ectopic niche with the recruitment of host-derived marrow and HSCs, evidencing the importance of endochondral ossification for HSC niche formation (**Chan et al., 2009**).

Osteopontin (OPN), also known as secreted phosphoprotein 1 (SPP1) has been suggested to be an important niche factor in BM. Its concentration in stromal cells is inversely correlated with HSC proliferation in the adult BM (**Stier et al., 2005**). The OPN dominant form thrombin-cleaved osteopontin (trOPN) is highly expressed in FBM (at the trabecular bone surface), but neglectable-to-none in the early-mid gestation FL (**Cao et al., 2019**). trOPN receptor $\alpha 4\beta 1$ integrin is upregulated in fetal compared to adult HSCs. The differential concentration of the divalent metal cations, Ca^{2+} , Mg^{2+} , and Mn^{2+} between FL and FBM, being highly prevalent in the latter, was assumed to activate $\alpha 4\beta 1$ in HSCs, possibly hindering their expansion in the BM (**Cao et al., 2019**).

CXCL12 expression in the vicinity of vascular endothelial cells in FBM supports the hematopoietic colonization of the organ (**Nagasawa et al., 1996; Ara et al., 2003**). HSCs isolated from CXCL12^{-/-} embryos failed to colonize the BM in long-term repopulation assays whereas their migration ability could be rescued by enforced expression of CXCL12 under the control of vascular-specific Tie-2 regulatory sequences (**Ara et al., 2003**). Moreover, the CXCL12-mediated migration of HSPCs *in vitro* seems to be enhanced by the presence of KITL, indicating an additive effect, only found in fetal HSCs (**Christensen et al., 2004**). This suggests an important role of KITL for HSC seeding and homing during embryonic development.

CXCL12-GFP knock-in mice allowed the identification of CXCL12-abundant reticular (CAR) cells, a population with high expression of CXCL12, distributed near sinusoidal endothelial cells and at a lower extent near the endosteum (**Sugiyama et al., 2006**). These cells show *in vitro* potential to differentiate into adipocytic and osteoblastic lineages (**Omatsu et al., 2010**). Specific markers have been identified for mesenchymal cells in BM. Leptin receptor (Lepr) is highly enriched in *Kitl*-GFP-expressing perivascular stromal cells (**Ding et al., 2012**) and Lepr⁺ cells were shown to largely overlap with CAR cells (**Zhou et al., 2014**). A rare population of Nestin⁺ cells that contains all of the fibroblastic CFU (CFU-F) activity within the mouse BM, capable of generating mesospheres (mesenchymal spheres) *in vitro*, multipotent and with self-renewal potential was identified as a mesenchymal stem cell (MSC) population (**Méndez-Ferrer et al., 2010**). Later, two different Nestin-GFP populations were discriminated based on the fluorescence intensity by microscopy. Rare quiescent *Nestin-GFP^{bright}* cells, positive for the pericyte marker NG2 and α -smooth muscle actin, are enriched for CFU-F activity and express *Cxcl12* and *Kitl*. These cells are located alongside arterioles, close to dormant HSCs (**Kunisaki et al., 2013**), and associated with sympathetic nerves, that

regulate their CXCL12 expression through the β_3 -adrenergic receptor (**Méndez-Ferrer et al., 2010**). *Nestin-GFP^{dim}* cells have a reticular shape, are mitotically active and line sinusoids, largely overlapping with Lepr⁺-cells (~80%) (**Kunisaki et al., 2013**). Selective ablation of mouse Nestin⁺ cells (**Méndez-Ferrer et al., 2010**) or CAR cells (**Omatsu et al., 2010**) significantly impacts the maintenance of HSCs. The structural differences of the blood vessels and perivascular populations seem to be associated with heterogeneity in HSC function. Besides, the selective deletion of *Cxcl12* from arteriolar NG2⁺ cells, but not sinusoidal Lepr⁺ cells, significantly reduced the HSCs compartment in the BM and a similar effect was observed by deletion of *Kitl* in LepR⁺; but not NG2⁺ cells, evidencing the differential contribution of perivascular populations in the cytokine production (**Asada et al., 2017**). BM microenvironment is illustrated in **Figure 9**.

Nestin⁺ cells can be prospectively isolated using PDGFR α and CD51 markers in the mouse and human fetal and adult BM (**Pinho et al., 2013**) and show MSC's properties and enrichment of *Cxcl12*, *Vcam1*, *Angpt1*, *Opn* and *Kitl* genes. Of note, in humans, these cells represent a small subset of CD146⁺ cells (**Pinho et al., 2013**), the latter harboring all the CFU-F activity in BM (**Sacchetti et al., 2007**). Co-culture of human PDGFR α ⁺CD51⁺ mesenspheres with human FBM CD34⁺ cells in a serum-free, but cytokine supplemented (TPO, KITL, FLT3L) culture media can expand MPPs that engraft immunodeficient mice (**Pinho et al., 2013**).

In the FL, Nestin-GFP⁺NG2⁺ cells, associated with portal vessels, form a niche promoting HSC expansion during the FL development that is no longer found after birth. Concomitantly, the phenotype of the portal vessel transits from Neuropilin-1⁺Ephrin-B2⁺ arterial to EphB4⁺ venular vessels (**Khan et al., 2016**). This role of Nestin⁺NG2⁺ cells in FL is opposite to that in BM, where Nestin⁺NG2⁺ arteriolar pericytes were proposed to maintain HSC quiescent (**Kunisaki et al., 2013**).

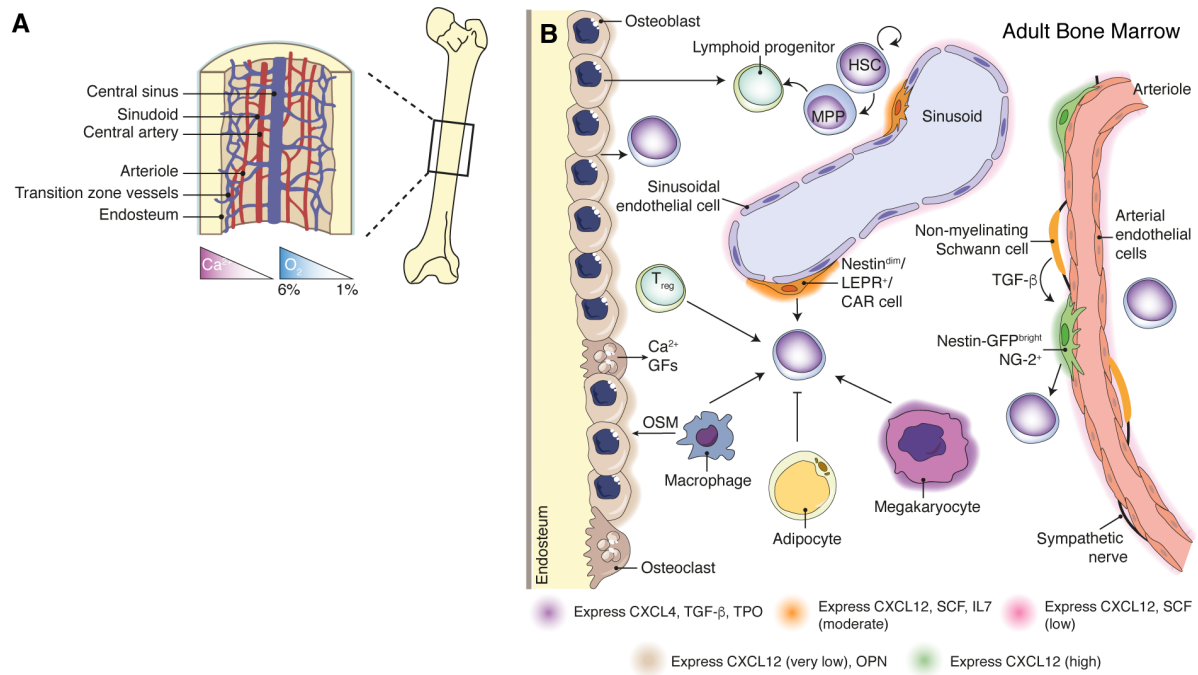


FIGURE 9 ADULT BONE MARROW NICHE

Distinct BM stromal cell populations have been described as modulators of HSC functions by expression of soluble factors. BM Nestin-GFP^{high}, NG2⁺ perisinusoidal cells and Nestin-GFP^{dim}, LepR⁺, CAR sinusoidal cells are key regulators of HSC maintenance, contributing differentially for cytokine production. Macrophages, T_{reg}, megakaryocytes were also reported to contribute to HSC maintenance and mobilization. Sympathetic nerves regulate HSC mobilization through a tight control of CXCL12 expression by stromal cells. Non-myelinating Schwann cells activate latent TGF-β into its active form, which may contribute for HSC maintenance. The role of osteoblasts in HSC and progenitor regulation is still unclear. Adipocytes may negatively affect HSC maintenance. Adapted from (Soares-da-Silva et al., 2020).

THE FETAL LIVER STROMA AS A NICHE FOR HEMATOPOIESIS

LAW OF ATTRACTION: WHAT BRINGS HEMATOPOIETIC PROGENITORS TO THE DEVELOPING LIVER?

Hematopoietic stem cells emerge from the dorsal aorta directly into circulation and can, therefore, be found in different locations (**Cumano et al., 1996; Medvinsky et al., 1996**). These cells can travel through the umbilical arteries to the placenta and return to the embryo through the umbilical veins. The umbilical veins drain directly into the liver by fusing with the intrahepatic vascular plexus of the vitelline veins, forming the hepatic sinus. Cells traveling in the right umbilical vein can bypass the liver directly to the vena cava through the *ductus venosus*, a structure only present during fetal development, and can be directed into other embryonic regions through the heart. The liver is also irrigated by the vitelline veins, which transport blood from the yolk sac, and eventually mature to become the portal vein. Another route for newly formed HSCs is to travel from the dorsal aorta through the subcardinal vein to the liver or inferior cardinal vein to the heart. From the heart, circulating cells can reach the developing lungs or upper half of the body (**Kiserud, 2005**). However, it is in the developing FL that HSCs establish and remain until they migrate to the BM.

Hematopoietic stem or progenitor cells but also EMPs, colonize the FL after liver bud formation at E10.5 (**Johnson et al., 1975; Palis et al., 2001a**). Distinct mechanisms of FL colonization have been proposed, mostly relying on cell adhesion-mediated processes and/or chemoattraction (cytokines, chemokine signaling and growth factors) (**Figure 10**) (**Hayashi et al., 2019**).

Several cell adhesion molecules have been identified in embryonic HSPCs, including integrins, selectins, cadherins and others. Hematopoietic progenitors express vascular-endothelial cadherin (VE-Cadherin/CD144) as they emerge from the YS (in the case of EMPs) or AGM (in the case of HSCs) that is downregulated thereafter and undetectable in BM HSCs (**Fraser et al., 2002; Taoudi et al., 2005**). FL CD34⁺ progenitors express higher levels of the integrins $\beta 1$ (CD29), $\alpha 2$ (CD49b) and $\alpha 5$ (CD49e), similar levels of integrins $\alpha 4$ (CD49d) and $\alpha 6$ (CD49f), E- and P-selectins (CD62E and CD62P respectively) and CD11b and CD11c molecules, but lower levels of integrin $\beta 2$ (CD18), CD11a and CD44 than their adult BM equivalent (**Roy et al., 1999**). Seminal studies showed that HSPCs lacking $\beta 1$ integrin were unable to colonize the FL but were still present in the circulation and capable of generating all blood lineages, suggesting a role for integrin-mediated cell adhesion in FL colonization (**Hirsch et al., 1996**). Integrin receptors result from the dimerization of α and β subunits and analysis of the expression of the α chain partner of $\beta 1$ integrin in LSK progenitors revealed integrins $\alpha 4$ and $\alpha 6$ as the most predominant (**Sugiyama et al., 2013**). FL CD34⁺ progenitors

bind the ECM component fibronectin through the integrin receptors $\alpha 4\beta 1$ and $\alpha 5\beta 1$ (also known as VLA-4 and VLA-5) and this binding has been proposed to be modulated by the integrin $\alpha 2\beta 1$ (GPIIb or CD41), also expressed in these cells (**Figure 10**) (**Roy et al., 1999**; **Emambokus et al., 2003**). Other ECM components have been tested for adhesion of FL CD34⁺ progenitors such as type I and type IV collagen and laminin, however, only type IV collagen promotes adherence at levels equivalent to that of fibronectin, through $\alpha 2\beta 1$ integrin (VLA-2) (**Roy et al., 1999**). In the FL, hepatoblasts (defined as DLK1⁺ cells) are the major producers of ECM components, namely vitronectin and fibronectin (**Sugiyama et al., 2013**). Embryos lacking hepatoblasts can still form the liver bud but die between E10.5-E12.5 (**Nishina et al., 1999**). These FL show decreased expression of vitronectin and fibronectin that may play an important role in FL colonization by HSCs and YS EMPs, although this role has not been specifically assessed (**Sugiyama et al., 2013**). Integrins can also mediate cell-cell interactions. Cellular bound counterparts of VLA-4 include the vascular cell adhesion molecule-1 (VCAM-1/CD106), expressed by FL hepatoblasts (**Sugiyama et al., 2010a**).

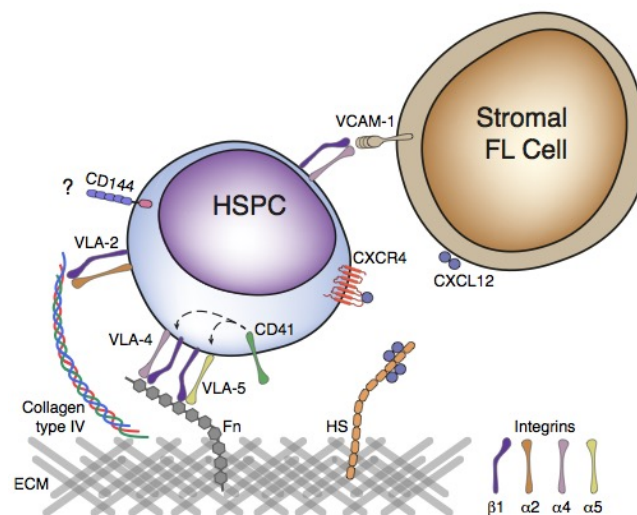


FIGURE 10 MECHANISMS OF FETAL LIVER COLONIZATION

HSPC express a variety of cell adhesion molecules on their surface that can favor attachment in a specific location. $\beta 1$ integrin is essential for colonization of FL by HSPC and can bind to the ECM components fibronectin and type IV collagen through dimerization with $\alpha 4$ and $\alpha 5$ or $\alpha 2$ integrins, respectively. VLA-4 and VLA-5 binding to fibronectin has been proposed to be modulated by CD41. VLA-4 ($\alpha 4\beta 1$ integrin) can also mediate cell-cell interactions by binding with VCAM-1, expressed in the surface of FL stromal cells. Expression of the cadherin CD144 in HSCs as they emerge is thought to play a role in FL colonization, although no binding partner has been described in FL. CXCL12, a potent chemoattractant is expressed by FL stromal cells and binds to CXCR4, expressed in the surface of HSPC. CXCL12 gradients can be generated as this chemokine binds to proteoglycans in the ECM, namely to heparan sulfate (HS). HSPC – hematopoietic stem and progenitor cell; FL – fetal liver; ECM – extracellular matrix; VLA – very late antigen; Fn – fibronectin; HS – heparan sulfate. Reproduced from (**Soares-da-Silva et al., 2020**).

Cytokine and chemokine signaling can also stand at the basis of FL colonization. The stromal cell-derived factor-1 (SDF-1), commonly known as CXC chemokine ligand 12 (CXCL12), acts through binding to its receptor CXCR4 present in HSPCs and has been extensively studied in the adult BM – reviewed in (Yu et al., 2016; Wei et al., 2018). CXCL12 expression is stabilized at the cell surface or in the surrounding ECM through proteoglycans binding, allowing the creation of chemokine gradients essential for cell migration (Schumann et al., 2010). In the FL, CXCL12 is expressed by DLK1⁺ hepatoblasts (Chou et al., 2010) and Nestin⁺NG2⁺ pericytes (Khan et al., 2016). The role of CXCL12 in FL colonization was analyzed using CXCL12^{-/-} embryos. At early stages (E12.5-E14.5), the number of HSCs was similar in the FL of mutant animals and controls. By E16.5 FL HSCs were reduced by more than 2-fold and an abnormally high number was found in circulation. These observations indicate that CXCL12 is an important factor for retaining HSCs in FL, but not for its initial colonization (Ara et al., 2003).

Owing to the particular architecture of fetal circulation, FL is in an anatomically privileged location. Even if HSCs are traveling directly within the embryo through the subcardinal vein, or the umbilical veins after passing in the placenta, the FL is the first intraembryonic organ they encounter. Whether a passive retainment of circulating cells (e.g. through β 1-integrin) occurs, or specific signals directly promote chemoattraction of HSPCs to FL is still unclear.

POPULATIONS PRESENT IN THE FETAL LIVER DURING EMBRYOGENESIS

Endoderm germ layer forms during gastrulation and gives rise to the primitive gut tube that is patterned into foregut, midgut and hindgut regions. The foregut, located in the anterior endoderm, adjacent to the developing heart, generates the liver, alongside with the lung, thyroid, and pancreas (Tremblay et al., 2005). Embryonic liver development starts at around E8.5-E9 (~4 wpc in humans) with the formation of the hepatic diverticulum, an extension of the ventral foregut epithelium that invades the septum transversum mesenchyme (STM) and forms a liver bud, by E9.5 (Wilson et al., 1963; Severn, 1971). The liver bud originates from a single-sheet of columnar endodermal epithelia with a gut morphology (Figure 11A), that then transitions to pseudostratified epithelial hepatoblasts (Figure 11B) (Bort et al., 2006). At this stage, these cells are separated from the STM by a basement membrane rich in laminin and composed of other ECM molecules, such as nidogen, type IV collagen, fibronectin, and heparan sulfate proteoglycan (Shiojiri et al., 2004). A process of extensive hepatoblast proliferation follows, during which the cells outgrow the liver bud, disrupting the basement membrane, into the STM (Figure 11C) (Douarin, 1975). The other constituents of the organ, sinusoidal endothelial cells, mesothelial, sub-mesothelial and hepatic stellate cells have a mesoderm origin (reviewed by (Yang et al., 2019)), as described below. Angioblasts or endothelial progenitor cells are found delimiting the basement membrane, like a loose

necklace of cells, and were shown to promote liver organogenesis. In Flk-1^{-/-} mutant embryos, which lack endothelial cells, hepatic specification occurs, but proliferation and migration into the STM are impaired (**Matsumoto et al., 2001**). At E10.5-E11.0 (~5-6 wpc in humans), hematopoietic cells colonize the FL that rapidly becomes the major fetal hematopoietic organ (**Johnson et al., 1975; Migliaccio et al., 1986; Palis et al., 2001a**).

Hepatoblasts

Hepatoblasts are bipotent immature FL cells that differentiate into hepatocytes – the major parenchymal liver's cell type – or cholangiocytes – the biliary epithelial cells. At the onset of liver development, bipotent hepatoblasts express the liver-specific transcription factors (TFs) Hematopoietically-expressed homeobox protein (HHEX) (**Bogue et al., 2000**), Prospero homeobox protein 1 (PROX1) (**Dudas et al., 2004**), and co-express the hepatocytes' markers Alpha-fetoprotein (AFP), Albumin (ALB) (**Cascio et al., 1991**), Cytokeratin 18 (CK18) (**Tanimizu et al., 2003**), Hepatocyte nuclear factor 4 α (HNF4 α) (**Li et al., 2000**) and cholangiocytes' markers such as Cytokeratin 19 (CK19) (**Tanimizu et al., 2004**). Other markers expressed by hepatoblasts are listed in **Table 3**.

Different markers have been used to isolate hepatoblasts, namely delta like non-canonical Notch ligand (DLK1 or preadipocyte factor 1 (Pref-1)) (**Tanimizu et al., 2003**), CD324/E-cadherin (**Nitou et al., 2002**), CD326/EpCAM (**Tanaka et al., 2009**), and leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) (**Prior et al., 2019**). DLK1 is strongly expressed by hepatoblasts in the E10.5 liver bud and continues to be highly expressed until around E16.5, being significantly downregulated thereafter and absent in mature hepatocytes and cholangiocytes (**Tanimizu et al., 2003; Tanaka et al., 2009**). E-Cadherin, present at the onset of liver outgrowth, is downregulated by the time hepatoblasts migrate to the STM, disrupting the epithelial sheet, although it can be used as a hepatoblast-specific marker after E12.5 (**Nitou et al., 2000; Margagliotti et al., 2007**). Transient EpCAM expression labels newly formed hepatoblasts but is significantly reduced after E12, while expression after E16 specifically labels bile ducts (**Tanaka et al., 2009**). Recently, combining multicolor clonal genetic lineage tracing, organoid cultures and analysis of single-cell RNA sequencing, Lgr5 was shown to mark a subpopulation of *bonafide* bipotential hepatoblasts at E9.5-E10, being at the origin of the hepatoblast pool (**Prior et al., 2019**).

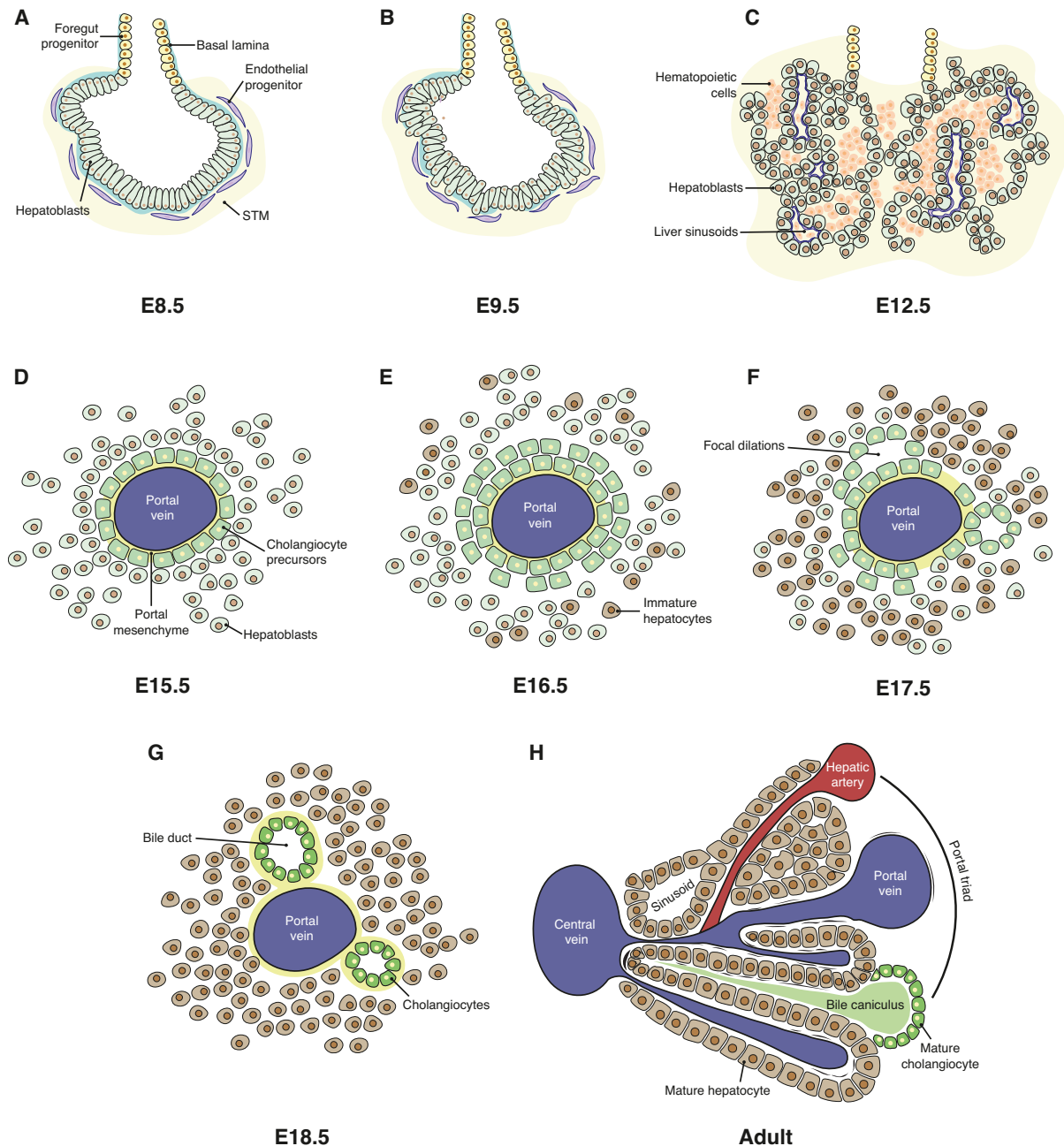


FIGURE 11 ONTOGENY OF THE FETAL LIVER

Liver development is initiated with the formation of the hepatic diverticulum at E8.5 (**A**). The single layer of columnar endodermal epithelial cells (**A**) thickens and transitions to a pseudostratified epithelium, resulting in the delamination and migration of hepatoblasts into the STM, forming the liver bud by E9.5 (**B**). Extensive hepatoblasts proliferation follows, originating hepatic chords intermingled with the hepatic mesenchyme and surrounding the sinusoids. From E10.5, hematopoietic cells colonize the FL that rapidly becomes the major fetal hematopoietic organ up until E15.5 (**C**). From E12.5-E15.5 the FL continues to enlarge, expanding both hematopoietic and hepatic compartments. Structural changes are only evident after hepatoblast-to-cholangiocyte specification occurs around the portal vessels, forming a monolayered ductal plate (**D**), that evolves into a bi-layer at E16.5 (**E**). Focal dilations (**F**) evolve into bile duct structures in late gestation (**G**). Adult liver architecture is characterized by portal triads where a hepatic artery, a portal vein and a bile duct form a complex structure, and a central vein, to which plates of hepatocytes lined by sinusoids converge (**H**). This architecture is only recognizable a few weeks after birth. Reproduced from (Soares-da-Silva et al., 2020).

Endothelial cells

The main blood vessels in the adult liver are the portal and central veins and the hepatic artery. Up until birth, the hepatic artery is absent and embryonic circulation is sustained by a transient afferent vascular system, the extraembryonic umbilical and vitelline veins (**Figure 12A**) (**Collardeau-Frachon et al., 2008**).

The portal vein arises early in the liver development, between E10.5-E12.5 in mouse (4-6 wpc in human) (**Collardeau-Frachon et al., 2008; Swartley et al., 2016**). The hepatic sinusoids are the first vessels to appear, by E10-E10.5, originating from the pre-existing vitelline vessels. The latter branches throughout the STM, by angiogenesis, receiving signals from the surrounding mesenchyme (**Figure 11C**) (**Swartley et al., 2016**). Hepatoblasts were also identified as a positive stimulator of sinusoid morphogenesis and maturation (**Takabe et al., 2012**). Stabilin 2 (STAB-2) and lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) (commonly used as a marker of lymphatics) – hyaluronan receptors – start to be expressed in sinusoidal endothelial cells (SECs) at E9.5 and E10.5, respectively, and continues to be expressed thereafter (**Nonaka et al., 2007; Takabe et al., 2012**). Of note, lymphatic vessels were only reported after birth (**Swartley et al., 2016**). At E9.5, endothelial cells, located around the liver diverticulum (**Figure 11A**) express both CD31/PECAM-1 and Flk-1 (**Sugiyama et al., 2010b**). CD31 and Flk1 expression in SECs is strong in early stages of liver development, but it is downregulated with time. In adult livers, endothelial cells of portal and hepatic veins strongly express CD31, but in SECs is absent or weakly detected (**Sugiyama et al., 2010b; Takabe et al., 2012**). Primitive SECs also strongly express Flk-1, contrarily to endothelial cells of portal and hepatic veins (**Sugiyama et al., 2010b**). During embryonic liver development, portal vessels express the arterial markers Ephrin-B2 and Neuropilin-1, but not the venular marker EphB4. This expression profile is inverted at the end of gestation, with the transition into a venular phenotype (**Wang et al., 1998; Khan et al., 2016**). Liver endothelial cells are heterogeneous and different markers should be used for their identification according to their vascular location.

Mesothelial and sub-mesothelial cells

Mesothelial cells (MCs) compose a single epithelial layer (mesothelium) lining the liver parenchyma on the surface of lobes. From E12.5, MCs are characterized by the expression of cytokeratin, CD200, glycoprotein M6A (GPM6A), podoplanin (PDPN/Gp38), podocalyxin-like protein 1 (PODXL) and mesothelin (MSLN) (**Lua et al., 2016**). PODXL is highly expressed in immature MCs, being downregulated during development, while MSLN is upregulated. MCs proliferate during liver development and remain quiescent after birth. Wilm's tumor-1 (WT1) is mainly expressed by MCs (**Onitsuka et al., 2010**). WT1^{-/-} embryos show incomplete lobulation compared with control littermates at E13.5, reduced numbers of Flk1-PODXL^{high} MCs, DLK1+

hepatoblasts, and total FL cells, suggesting that hepatic development was impaired due to defective MCs. This is supported by the observation that fetal MCs express growth factors (PTN, MDK, and HGF) involved in hepatic development (Ijpenberg et al., 2007; Onitsuka et al., 2010).

Underneath these sheets of MCs, there is a population of cells expressing Desmin, Nerve growth factor receptor (NGFR/p75NTR) and Platelet-derived growth factor receptor α (PDGFR α /CD140a), associated with type IV collagen of the basal lamina, commonly referred as “sub-mesothelial cells” (sub-MCs) or capsular fibroblasts. The expression of activated leukocyte cell adhesion molecule (ALCAM/CD166) and WT1 was also observed in MC and sub-MC around E11-14 and before that, in the STM by E9-10 (Asahina et al., 2011; Lua et al., 2016).

Hepatic stellate cells and/or pericytes

Although the terms hepatic stellate cells and pericytes have been used by many authors as synonyms, it is not consensual that they represent the same population. In adult liver, there is a population of perisinusoidal cells residing in the space of Disse between hepatocytes and sinusoidal endothelial cells that store vitamin D lipids (Wake, 1971), and that are major players in liver fibrinogenesis (Guyot et al., 2006). MesP1-expressing mesoderm has been considered their earliest ancestry, which gives rise to the STM that will contribute to the liver mesothelium and mesenchymal cells. Migration inward of MC and sub-MC from the liver surface is believed to give rise to hepatic stellate cells and perivascular mesenchymal cells (Asahina, 2012). Hepatic stellate cells express Desmin, p75NTR, but not the MC markers ALCAM, WT1, and Gp38 (Asahina et al., 2010).

Gerlach et al. isolated CD146⁺CD45⁻CD56⁻CD34⁻ cells from fetal and adult human livers and identified them as pericytes, a distinct population from hepatic stellate cells (Gerlach et al., 2012). They showed that these cells express NG2 and vimentin but not GFAP *in situ*, being found around periportal blood vessels, but not pericentral or within the space of Disse. Considering mesenchymal properties, these cells exhibit high osteogenic and myogenic, but low adipogenic or chondrogenic differentiation potential, in *in vitro* differentiation assays. In mouse, a population characterized by the expression of Nestin and NG2 was identified as periportal pericytes, that expresses mesenchymal markers and shows trilineage mesenchymal capacity *in vitro* (Khan et al., 2016).

TABLE 3 FETAL LIVER CELL TYPES, GENE EXPRESSION AND MARKERS USED FOR ISOLATION¹ – study performed in human.

Cell type	Gene expression	Markers used for isolation
Hepatoblasts	<i>Hhex, Prox1, Alb, Afp, Ck8, Ck18, Ck19, Met, Hnf6, Oc2, Hnf4a, Ttr, Foxm1, Tbx3, Dlk1, Lgr5, Epcam, Cdh1, Cd29, CD49f, Liv2, Prom1, Anpep</i>	DLK1(Tanimizu et al., 2003) E-Cadherin (Nitou et al., 2002) EpCAM (Tanaka et al., 2009) LGR5 (Prior et al., 2019) CD29, CD49f (Suzuki et al., 2000) LIV2 (Watanabe et al., 2002) AFP (Nierhoff et al., 2005)
Hepatocytes	<i>Alb, Afp, Ttr, G6p</i>	-
Cholangiocytes	<i>Sox9, Hnf6, Oc2, Spp1, Ck19</i>	EpCAM (Tanaka et al., 2009; Yang et al., 2017)
Endothelial cells	<i>Flk1, Flt1, Ve-cadh, Pecam1, Mcam, Tek, Tie, Lyve1, Kdr</i>	CD31 (Khan et al., 2016)
Mesothelial cells	<i>cytokeratin, Cd200, Gpm6a, Alcam, Gp38, Wt1, Podxl, Mslh</i>	PODXL (Onitsuka et al., 2010)
Sub-mesothelial cells	<i>Alcam, Desmin, Nestin, p75NTR, Pdgfra, Wt1</i>	-
Hepatic stellate cells	<i>Desmin, p75NTR, Foxf1, Lhx2, Hlx</i>	p75NTR (Tan et al., 2017b)
Pericytes	<i>NG2, Nestin, Vimentin, aSMA, Pdgfra, Pdgfrb, Dlk1, Itgav, Endoglin, Vcam, Mcam, Nr2f2</i>	Nestin, NG2 (Khan et al., 2016) CD146 ¹ (Gerlach et al., 2012)

SIGNALS PROVIDED BY THE FETAL LIVER STROMA

Signals needed for HSC and downstream progenitors

Emerging imHSCs lack long-term reconstitution activity in conventional or Rag2^{-/-} immunocompromised mice but can reconstitute NK-deficient Rag2^{γc}^{-/-} animals (**Cumano et al., 2001; Bertrand et al., 2005a**). After co-culture with the OP9 BM stromal cell line in the presence of thrombopoietin (TPO) or with E10.5 FL rudiments, CD31⁺Kit⁺CD45⁻ imHSCs acquire an adult HSC phenotype (LSK CD150⁺CD48⁻) and develop LTR activity in Rag2^{-/-} or conventional mice as they upregulate MHC class I molecules (**Kieusseian et al., 2012**). These experiments suggest that the FL provides the signals necessary for the maturation of newly formed HSC. TPO is the main regulator of megakaryocyte differentiation and platelet production (**Kaushansky, 1995; Eaton et al., 1997**) but has also been shown to promote survival and proliferation of BM hematopoietic progenitors *in vitro* (**Ku et al., 1996**), proliferation of fetal hematopoietic progenitors *in vivo* (**Alexander et al., 1996**) or expansion of BM HSC following transplantation (**Fox et al., 2002**). The survival and proliferation effects of TPO are enhanced when used in combination with other early cytokines, namely FMS-like tyrosine kinase 3 ligand (FLT3L) and KITL both in murine and human adult BM cells (**Ramsfjell et al., 1996; Borge et al., 1997**). The highest levels of TPO in the adult are found in the liver (**Lok et al., 1994**). It can be detected in FL as early as E10.5, having a strong impact on HSC expansion and survival in this organ (**Petit-Cocault et al., 2007**). Indeed, several cytokines/chemokines/growth factors are important for HSC proliferation, maintenance and survival, namely KITL, FLT3L, IGF, angiopoietin-3, angiopoietin-like 2, Wnts, Ephrin2a, CSF1, EPO, Cxcl12 and IL-6 – reviewed in (**Sauvageau et al., 2004**). In FL, these cytokines are expressed by hepatoblasts or other stromal cells, such as stellate cells or pericytes (see **Table 4** and **Figure 12**) (**Charbord et al., 2005; Chou et al., 2010; Khan et al., 2016; Tan et al., 2017b**).

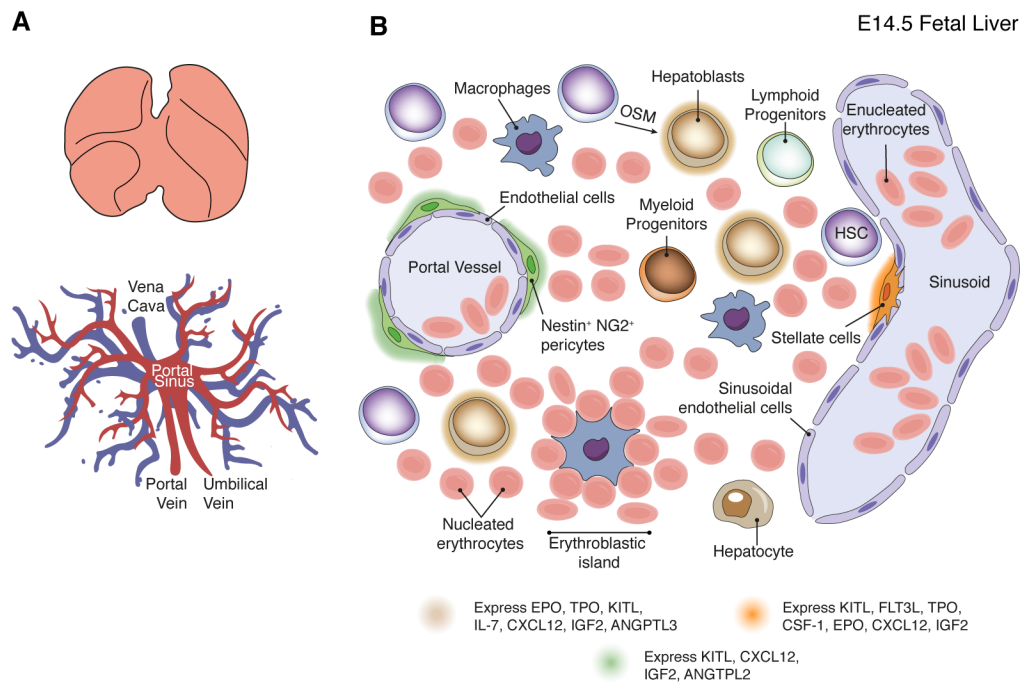


FIGURE 12 FETAL LIVER NICHE

Data available for FL stromal populations is scarce and, consequently, evidence for a direct role of stromal populations in HSC expansion or differentiation is not reported. The FL microenvironment includes cytokine-producing hepatoblasts, pericytes and stellate cells, together with a high number of hematopoietic progenitors and erythroid cells. Adapted from (Soares-da-Silva et al., 2020).

The hypothesis that stem cells are regulated by their environment has been proposed by Schofield (Schofield, 1978) and states that stem cell properties are maintained by the surrounding cells designated “niche”. Although this theory has been validated in different tissues, it has not been directly demonstrated in hematopoiesis (Lander et al., 2012). Stromal regulation of hematopoiesis has been proposed by many and early studies of co-culture of hematopoietic progenitors with either FL fibroblast or epithelial-stromal cell lines showed erythroid and myeloid support (Tsai et al., 1986; Hata et al., 1993). More than 200 FL stromal cell lines have been developed and tested for maintenance or expansion of HSC, however, only a few were able to maintain their repopulating activity over a 3-week co-culture period (Wineman et al., 1996). The functional heterogeneity observed was not due to distinct cytokine production as all cell lines expressed similar cytokine profiles, including Flt3l, Kitl, TPO, leukemia inhibitory factor (LIF), IL-6, IL-11, granulocyte-colony stimulating factor (G-CSF), granulocyte/macrophage-colony stimulating factor (GM-CSF), IGF-1 and transforming growth factor- β (TGF- β). Importantly, the successful stromal cell lines have in common the expression of *Dlk1*, and overexpression of this factor is sufficient to enable hematopoietic support (Moore et al., 1997). Although these cell lines only maintain HSC potential and do not promote HSC expansion, some FL cells have been described to do so. Recent studies have addressed the role of specific FL populations *in vivo* or *in vitro*. It is the case of *DLK1*⁺

hepatoblasts that are able to expand LT-HSC around 20-fold after a 3 week co-culture period (Chou et al., 2013). This expansion seems to be contact-dependent as DLK1⁺ conditioned medium only allows expansion of ST-HSC and cells cultured in transwell inserts did not show the same expansion levels. Although these results are promising, it is important to mention that these cultures were supplemented with KITL, TPO and FLT3L and it is still unclear which mechanisms underlie HSC expansion in DLK1⁺ cell co-cultures. Moreover, DLK1 knockdown in human hepatoblasts results in decreased hematopoietic support *in vitro* (Gerlach et al., 2019). The role of hepatoblasts *in vivo* has been difficult to assess as transgenic mice with hepatoblast deficiencies die between E10.5 and E12.5 and studies of liver development do not usually investigate the hematopoietic compartment (Nishina et al., 1999). DLK1 has been used to identify hepatoblasts but it is also expressed by the majority of Nestin⁺ cells surrounding the portal vessels, a cell type that has been implicated as part of the FL niche (Tanimizu et al., 2003; Chou et al., 2010, 2013; Khan et al., 2016). HSC numbers are modestly reduced when Nestin⁺NG2⁺ pericytes are selectively eliminated and the remaining HSC are less proliferative (Khan et al., 2016). So far, this is the only study that addresses the role of a specific cell type in HSC expansion and maintenance *in vivo*. Nestin⁺ cells are the major producers of CXCL12 at E14.5 when compared to Nestin⁻ cells, therefore, the reduction in FL HSC could result from a defect in HSC expansion together with a displacement of cells into circulation. Moreover, Nestin⁺ cells seem to be a transient population, important for HSC localization around the portal vessels during FL hematopoiesis but are no longer present postnatally, a stage at which HSC migrate and reside in the BM. These cells have also been described in adult BM and ablation of Nestin⁺NG2⁺ pericytes alters HSC localization away from arterioles (Kunisaki et al., 2013). In the rat, fetal hepatic stellate cells were shown to express VCAM-1 and to secrete CXCL12 and HGF, revealing a potential role for the hematopoietic and hepatic development (Kubota et al., 2007). Accordingly, mouse FL hepatic stellate cells isolated as p75NTR⁺ cells express a range of hematopoietic cytokines, *Csf1*, *Igf2*, *Tpo*, *Kitl*, *Epo*, *Igf1*, *Il11*, *Flt3l* and Oncostatin M (*Osm*, involved in hepatic maturation) and were therefore proposed as likely niche components (Tan et al., 2017b).

TABLE 4 CYTOKINE SIGNALING IN FETAL LIVER

FL Supportive Stroma		Hematopoietic Progenitors	
Pathway Involved	Expressing Cell	Receptor	Effect
KITL	DLK1 ⁺ Hepatoblasts (Chou et al., 2010; Sugiyama et al., 2011) Nestin ⁺ cells (Khan et al., 2016) Stellate cells (Tan et al., 2017b)	Kit (CD117)	BM HSC survival and self-renewal (Barker, 1994; Miller et al., 1997)
ANGPTL2	Nestin ⁺ cells (Khan et al., 2016)	PirB	BM HSC proliferation (Zhang et al., 2006)
ANGPTL3	DLK1 ⁺ Hepatoblasts (Chou et al., 2010)	PirB	BM HSC maintenance (Zheng et al., 2011) BM HSC proliferation (Zhang et al., 2006)
FLT3L	Stellate cells (Tan et al., 2017b)	FLT3 (CD135)	FL HSPC proliferation (Lyman et al., 1993)
TPO	DLK1 ⁺ Hepatoblasts (Chou et al., 2010; Sugiyama et al., 2011; Brouard et al., 2017) Stellate cells (Tan et al., 2017b)	MPL (TPO-R, CD110)	FL HSC survival and proliferation (Petit Cocault et al., 2007)
CSF1	Stellate cells (Tan et al., 2017b)	CSF1 Receptor	Commitment to macrophage lineage (Rieger et al., 2009)
EPO	DLK1 ⁺ Hepatoblasts (Sugiyama et al., 2011) Stellate cells (Tan et al., 2017b)	EPO Receptor	Proliferation and differentiation of FL erythroid progenitors (Lin et al., 1996)
CXCL12	DLK1 ⁺ Hepatoblasts (Chou et al., 2010) Nestin ⁺ cells (Khan et al., 2016) Stellate cells (Kubota et al., 2007)	CXCR4	BM HSC engraftment post-transplantation (Lai et al., 2014; McDermott et al., 2015) FL HSC retainment (Ara et al., 2003)
IL-7	VCAM1 ⁺ ALCAM ⁺ DLK1 ⁺ Hepatoblasts (Tsuneto et al., 2013)	IL-7R α	Lymphocyte expansion (Peschon et al., 1994)
IGF2	DLK1 ⁺ Hepatoblasts (Chou et al., 2010) Nestin ⁺ cells (Khan et al., 2016) Stellate cells (Tan et al., 2017b)	IGF2-R	FL and BM HSC proliferation (Zhang et al., 2004)

Signals needed for other hematopoietic lineages

FL stroma also supports the differentiation of committed hematopoietic progenitors towards distinct lineages. IL-7 promotes survival and proliferation of lymphoid progenitors and controls the determination of the B cell lineage (**Sudo et al., 1989; Peschon et al., 1994**). In FL, IL-7 is produced by VCAM1⁺ALCAM⁺DLK1⁺ hepatoblasts (**Tsuneto et al., 2013**) and controls the number of lymphoid progenitors that develop into the B-cell lineage by stabilizing the B-cell transcriptional signature (**Berthault et al., 2017**). For instance, EPO is produced by DLK1⁺ hepatoblasts and is required for terminal proliferation and differentiation of erythroid progenitors (**Sugiyama et al., 2011**). Also, TPO expressing Ter119⁻CD45⁻CD51⁺VCAM1⁺PDGFR α ⁻ FL hepatoblasts support the production of megakaryocytes from adult BM HSC in a contact-dependent manner (**Brouard et al., 2017**).

OPEN QUESTIONS IN THE FIELD

EX VIVO EXPANSION OF HSCs

Hematopoietic stem cells are the only cells of the hematopoietic compartment with the potential to replenish all mature blood cells and to divide without triggering differentiation programs, a process known as self-renewal. The mechanisms conveying these properties have been under investigation over the last 60 years, however, to date, they remain poorly understood. The concept that specific BM niche/microenvironment components regulate the fate of HSCs has been proposed by many authors. (**Gomes et al., 2016**) analyzed not only HSCs but also different hematopoietic progenitors and found both HSCs and MPPs located near or in contact with the same mesenchymal progenitor CAR-cells, expressing CXCL12 and KITL, fundamental to maintain the HSC pool, and IL-7 that acts as a short-range signal for lymphoid differentiation. These observations suggest that both maintenance and multilineage differentiation are locally regulated by the same niche (**Gomes et al., 2016**). In the FL, HSCs expand considerably and differentiate, producing different mature lineages. If a given stromal population is also involved in the regulation of both processes, by the expression of different cytokines, or different cell populations contribute with different cytokines is still unresolved.

Hematopoietic stem cell transplantation is a widely used cell therapy intervention in the treatment of hematologic, autoimmune and genetic disorders. However, this therapy is still associated with high mortality rates, mainly due to infection, graft-versus-host disease (GvHD) and organ dysfunction, urging the need for improvement (**Tanaka et al., 2016**). The most common source of HSCs for transplantation is the BM or mobilized circulating HSPCs. However, matching of major histocompatibility complex antigens is needed to avoid GvHD (**Schuster et al., 2012; Walasek et al., 2012**). Cord blood (CB) can be successfully used as a source of partially mismatched HSCs, as it is readily available through CB banks and elicits low levels of GvHD. Low numbers of HSCs in CB and consequent absent or delayed reconstitution lead to post-transplantation infections, limiting the use of CB in adult patients (**Ruggeri et al., 2014**).

In vitro generation of HSCs would overcome some of the current clinical difficulties that transplantation faces, however, despite countless efforts to derive HSC from pluripotent stem cell sources, generation of HSCs *in vitro* has not yet been achieved – reviewed in (**Freire et al., 2020**). Another possibility to obtain higher numbers of HSCs would be to expand them *ex vivo* prior to transplantation. Distinct cytokine/growth factor cocktails have shown promising for expansion of HSCs, yet, limited success was reported in clinical studies due to a lack of LT-HSC expansion and rather proliferation of downstream progenitors together with

undesirable stem cell differentiation – reviewed in (Kumar et al., 2017; Tajer et al., 2019). Cytokines currently used for *ex vivo* HSC expansion include KITL, TPO, IL-3 and FLT3L. *Kitl* and *Tpo* knockout mice show normal fetal development but reduced HSC numbers in the adult, suggesting that these cytokines are important for stem cell survival and proliferation in adulthood but might not be the drivers of HSC expansion during the embryonic period (Fujita et al., 1989; de Sauvage et al., 1996). There is growing evidence that the physical and mechanical properties of the microenvironment could impact HSC decisions – reviewed in (Kumar et al., 2017). A combination of cytokines with stroma-derived ECM components – fibronectin and collagen – has shown encouraging results (Wohrer et al., 2014; Wilkinson et al., 2019). Moreover, culture of BM HSPCs in tropoelastin, the most elastic biomaterial known, induces a sixfold increase of LSK cells without cytokine supplementation, suggesting that tropoelastin mediates a similar effect in survival and proliferation of LSK cells and could replace exogenous cytokines (Holst et al., 2010). Most cytokines studied have a role in HSC function in the BM, a site where HSC expansion does not occur in physiological conditions. The same cytokines have been found in FL stroma, but whether these are responsible for fetal HSC expansion is not known. Most likely, the FL expansion of newly generated HSCs results from intrinsic cellular properties together with a suitable microenvironment, physical cues included. Efforts have been made to replicate the embryonic microenvironment where HSCs expand, particularly by co-culture with FL hepatic or mesenchymal cells ((Wineman et al., 1996; Chou et al., 2010, 2013; Khan et al., 2016)). Most strategies used so far focused on specific cell populations, either hepatoblasts or mesenchymal cells, but overlooked the possibility that distinct populations might need to interact. Only the co-culture of HSCs in a system where all the cytokine-expressing populations of the FL are present would reproduce the FL microenvironment. Such a system where hepatoblasts, endothelial and mesenchymal cells are cultured together in a 3D aggregate has already been devised, but no co-culture with hematopoietic cells has been attempted (Takebe et al., 2015). Liver cell culture models have been extensively developed for pharmacological and toxicological research or as a source for transplantation, to obtain an *in vitro* system that resembles a mature liver – reviewed in (Godoy et al., 2013), and for that reason are not suitable for recreating the FL niche environment.

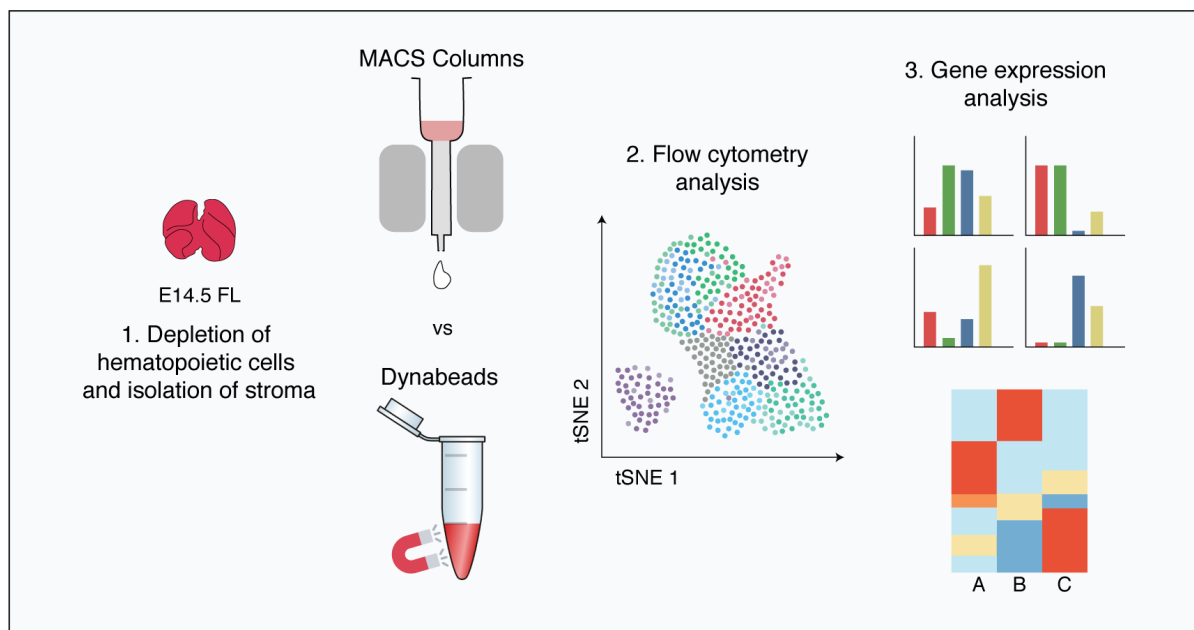
Whereas the FL interactions between different cell types have been correlated with their contribution to the massive transient expansion of the hematopoietic system, other niche factors need to be addressed. Sigurdsson et al. reported the role of FL bile acids (BAs) as chemical chaperones, critical to sustain high protein production by expanding LT-HSCs without triggering endoplasmic reticulum stress (Sigurdsson et al., 2016). Inhibiting the biosynthesis of BAs *in vivo* resulted in reduced numbers of Lin⁻, LSKs and LSK CD48⁻CD150⁺

in the FL, with no apparent effect in the number of HSCs in the mother's BM (**Sigurdsson et al., 2016**). Comparison of FL and BM HSCs transcriptomes demonstrated that FL HSCs metabolism relies on oxygen-dependent pathways, which may be a requirement for extensive energy production during expansion. Contrary to BM HSCs, FL HSCs use oxidative phosphorylation (aside from glycolysis), have higher number of mitochondria and up-regulate genes associated with antioxidant and DNA repair pathways, that are speculated to confer protection from reactive oxygen species-mediated (geno)toxicity (**Manesia et al., 2015**).

Exploring the complexity of HSC niches (cellular composition, cytokine and growth factors milieu, physical properties, oxygen availability, etc.) will improve our understanding of HSC self-renewal capacity (recently reviewed by (**Wilkinson et al., 2020**)), that is currently insufficient to devise efficient strategies for HSC expansion *ex vivo*.

CHAPTER III – NON-HEMATOPOIETIC POPULATIONS IN THE FETAL LIVER AT THE PEAK OF HSC EXPANSION

The adult hematopoietic system originates in the embryo, with hematopoietic stem cells emerging in the major arteries and migrating to the developing liver. It is in the fetal liver environment that these cells expand and differentiate. The mechanisms and/or cell populations that enable hematopoietic stem cell proliferation are currently unknown. Here, we characterize the distinct cell types present in the fetal liver at E14.5, the age at which hematopoietic stem cells are at the peak of expansion. We outline a protocol we optimized for isolating non-hematopoietic cells and devised a multicolor flow cytometry scheme that distinguishes 6 major populations, three of unknown lineage affiliation. RNA-sequencing analysis of the 3 major populations, CD324⁺, CD31⁺ and Kit⁺CD24⁺ cells revealed the latter as associated to the hematopoietic lineage. We have therefore identified a previously unidentified population in the fetal liver that represents around 10% of total fetal liver cells at E14.5.



Keywords: fetal liver; stroma; hematopoietic stem cells; flow cytometry; unsupervised analysis

FOREWORD

The FL is colonized by hematopoietic progenitors from E10.5-E11 and supports the hematopoietic system until HSPCs migrate to the fetal BM at around E17.5, being considered the major hematopoietic organ during embryogenesis (**Palis et al., 1999; Christensen et al., 2004**). The role of the FL stroma in the support of the hematopoietic system has been assessed and distinct populations described to secrete hematopoietic cytokines – hepatic progenitors (**Chou et al., 2010; Sugiyama et al., 2011**), stellate cells (**Tan et al., 2017b**) and Nestin⁺NG2⁺ pericytes (**Khan et al., 2016**) – however none has been shown critical for HSC expansion.

While the adult liver is mostly composed of hepatocytes, endothelial cells, cholangiocytes and mesenchymal subsets including stellate cells (**Yang et al., 2019**), the FL composition during embryogenesis is distinct. Histological analysis of the FL shows that in early time-points, E11.5-E15.5, it is mostly composed of hematopoietic cells, and only after E16.5 the architecture starts resembling the one found in the adult, however a fully mature liver is only observed after birth (**Crawford et al., 2010**).

The protocols used for the isolation of adult liver cells are based on perfusion of enzymatic digestion cocktails (**Liu et al., 2017**). Conversely, perfusion of the mouse FL is challenging. Isolation of FL cells for the study of the stromal compartment has been mostly performed by enzymatic digestion, followed by enrichment of subsets of interest or direct cell surface labeling with markers for hepatoblasts, endothelial, stellate and hematopoietic cells (**Sugiyama et al., 2011; Chou et al., 2013; Khan et al., 2016; Tan et al., 2017b**).

A standardized protocol for the recover and identification of the FL populations at distinct time-points is lacking. As mentioned above, the FL is mostly composed of hematopoietic cells (representing more than 90% of total FL cells), and the identification of rare cell subsets can be hindered when this is not taken into account. Here, we devised a strategy for the enrichment of the stromal compartment – encompassing all non-hematopoietic cells – by depletion of hematopoietic cells using the pan-hematopoietic marker CD45 and the erythroid marker Ter119. We compared distinct protocols for FL cell isolation and selected the one with the best results regarding cell survival for the characterization of the FL using a panel of 13 cell surface markers. This approach allowed the identification of epithelial, endothelial and mesenchymal cells, and 3 clusters of cells of unidentified lineage affiliation. Gene expression analysis confirmed the identity of the epithelial cells as hepatoblasts and RNA-Seq analysis of the most frequent population without lineage affiliation, as belonging to the hematopoietic lineage.

CHARACTERIZATION BY FLOW CYTOMETRY

OPTIMIZATION PROTOCOL FOR ANALYSIS OF STROMAL CELL POPULATIONS: ELIMINATION OF HEMATOPOIETIC CELLS

We analyzed, by immunohistochemistry and flow cytometry, the size of the hematopoietic compartment in E14.5 FL, and detected a large percentage (>80%) of Ter119⁺ erythroid cells and around 5% of CD45⁺ hematopoietic cells (**Figure 13A-B**). To analyze the stromal compartment, we performed depletion of Ter119⁺CD45⁺ hematopoietic cells by two methods – Dynabeads and MACS columns. Both methods allowed a recovery of Ter119⁻CD45⁻ cells of more than 70%, with the highest depletion efficiency being obtained using MACS Columns ($94.2 \pm 2.5\%$ vs $77.9 \pm 13.8\%$ with Dynabeads) (**Figure 13C**).

To characterize the Ter119⁻CD45⁻ compartment by flow cytometry, we designed various antibody panels including epithelial, endothelial, mesenchymal, adhesion molecules and progenitor cell markers. We identified a minimal antibody panel that allows discrimination of major populations: CD31 expressed by endothelial cells, CD324 and CD54 expressed by epithelial cells, CD24 and Kit that distinguishes 3 populations: P1 (Kit⁺CD24⁻), P2 (Kit⁺CD24⁺) and P3 (Kit⁻CD24⁺), and a population negative for CD31, CD324, CD24 and Kit (DN) that expresses mesenchymal markers. A summary of the expression of the markers analyzed in these populations can be found in **Table 5**.

Because the use of MACS columns could lead to loss of stromal cells by unspecific binding to the columns, we compared by flow cytometry the cells obtained after Dynabeads or MACS columns depletion. For that, we used the minimal antibody panel (CD31, CD54, CD24, Kit) together with CD166 and VCAM1 for analysis of E15.5 FL cells. We performed an unsupervised analysis of the data obtained and represented it as t-SNE and heatmap of the clusters obtained, allowing an unbiased analysis of the existing differences (**Figure 13D-G**). The results from Dynabeads and MACS columns were merged for analysis, preserving the initial annotation and were represented as tSNE plots. Cells depleted by Dynabeads or MACS columns are represented in black in **Figure 13D** and **13E**, respectively. Clustering according to the expression of the surface markers analyzed is overlaid in the tSNE plot (**Figure 13F**) and presented as heatmap (**Figure 13G**). Ter119⁻CD45⁻ cells obtained by Dynabeads depletion are distributed evenly in the tSNE plot, suggesting that all clusters are represented in this condition, with nevertheless, reduction of the cells from cluster A (pink) and clusters I-L (blue). On the other hand, Ter119⁻CD45⁻ cells obtained by MACS columns are evenly distributed in the tSNE, with exception of clusters E-H (dark blue), that are less represented. Overall, these results indicate that depletion with MACS columns is more efficient and that the cells obtained by this method are more representative of the stromal compartment of the FL.

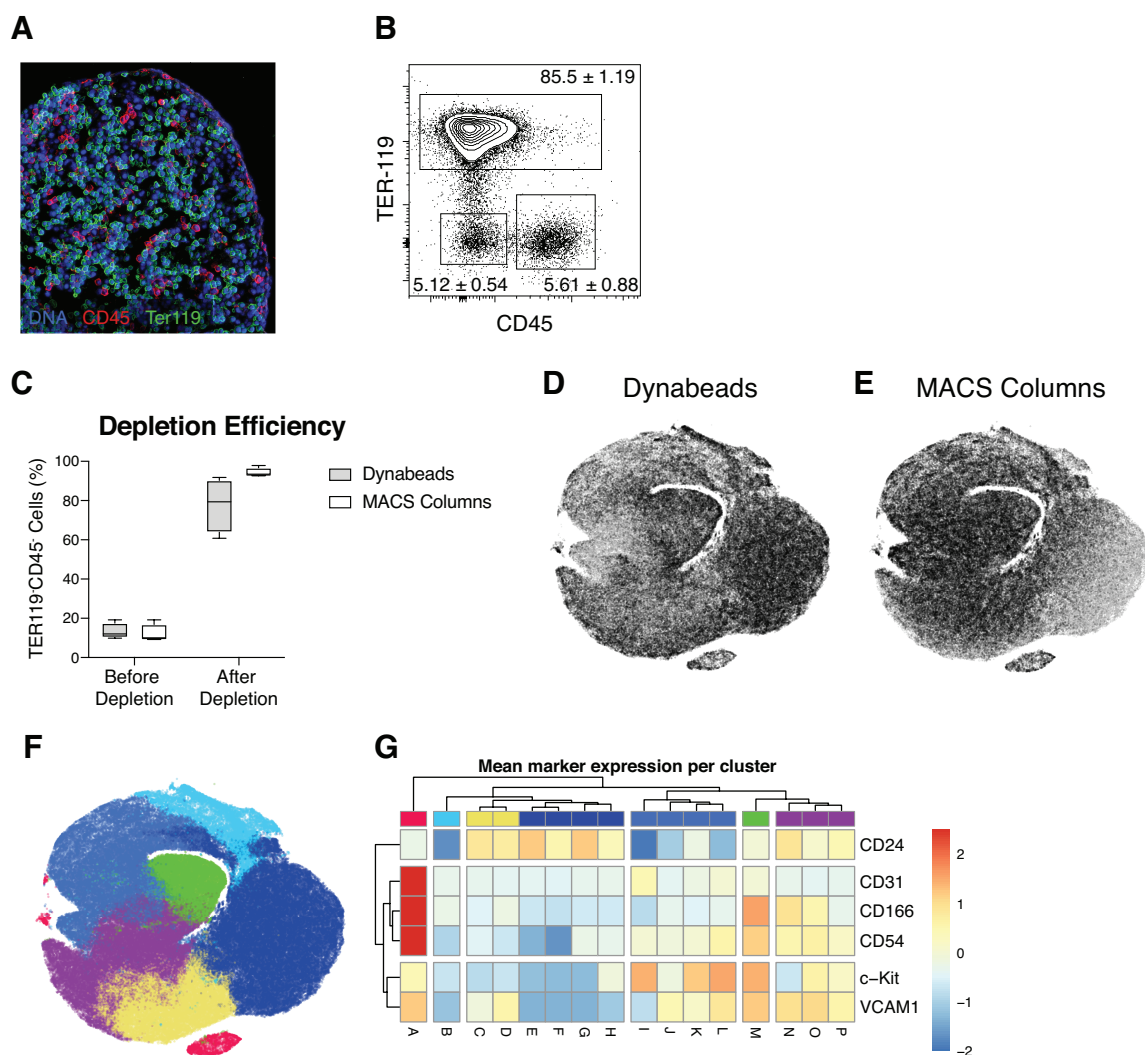


FIGURE 13 DEPLETION OF HEMATOPOIETIC CELLS

(A) Immunohistochemistry staining of E14.5 fetal liver tissue section with anti-CD45 (red) and anti-Ter119 (green) antibodies and DAPI (blue). (B) Flow cytometry profile of E14.5 fetal liver cells according to the expression of Ter119 and CD45. (C) Depletion efficiency of hematopoietic cells (Ter119 and CD45) using Dynabeads (grey) and MACS columns (white). (D and E) tSNE analysis of E15.5 Ter119⁺CD45⁺ fetal liver cells obtained by Dynabeads or MACS columns depletion, respectively. (F) tSNE representation of clusters obtained by unsupervised hierarchical clustering based on the expression of the surface markers CD31, CD54, CD24, Kit, CD166 and VCAM1 of E15.5 Ter119⁺CD45⁺ cells and respective heatmap (G). See also **Supplementary Figure 1**.

TABLE 5 EXPRESSION OF SURFACE MARKERS IN FETAL LIVER CELLS

Surface Marker	Populations					
	CD31 ⁺	CD54 ⁺	DN	P1	P2	P3
CD54	++	++	-	-	-	-
CD31	++	-	-	-	-	-
CD24	-	-	-	-	++	++
CD117	-	-	-	++	++	-
CD90	+	-	-	-	-	-
Sca1	+	-	-	-	-	-
Nrp1	-	-	-	-	-	-
AA4.1	+	-	-	-	-	-
EpCAM	-	-	-	-	-	-
LPAM1	-	-	+	+	-	-
CD146	++	+	+	+	-	-
Gp38	-	+	+	+	-	-
PDGFR α	-	+	+	-	-	-
CD51	-	++	++	+	-	-
CD324	+	++	-	-	-	-
NG2	+	-	+	-	-	-
CD166	-	++	+	-	-	-
VCAM1	+	++	+	-	-	-

*OPTIMIZATION PROTOCOL FOR ANALYSIS OF STROMAL CELL POPULATIONS:
OBTAINING SINGLE CELL SUSPENSIONS*

We obtained FL cell suspensions as described previously (**Ramond et al., 2014; Berthault et al., 2017**), a protocol devised for isolation of FL hematopoietic cells based on mechanical digestion of the tissue by aspiration through a 26-gauge needle. This method, however, may not be suitable for analysis of stromal populations that are more sensitive, resulting in increased cell death when used later than E16.5. To investigate the best protocol for the isolation of stromal FL cells, we compared mechanical with enzymatic digestion, namely Collagenase IV + DNase I and Liberase TH, at distinct time-points. Mechanical

digestion results in 60-70% cell viability up until E16.5, values that decrease to less than 50% after this time-point. We then compared cell viability after treatment with enzymatic digestion methods: a combination of 0,5 mg/ml Collagenase IV with 50 mg/ml DNase I or 0,05 mg/ml Liberase TH (**Figure 14A**). Enzymatic digestion with Liberase TH provided the best results in overall cell survival. We then performed flow cytometry analysis of the cells after mechanical or Liberase digestion and depletion with MACS columns to compare if there was an enrichment of specific populations between the two digestion methods. We performed the same analysis as described above with cell surface staining with CD31, CD54, CD24, Kit, CD166 and VCAM-1 and unsupervised tSNE analysis and hierarchical clustering (**Figure 14B-E**). The results show that clusters A and B (green) are less represented when FL is digested with Liberase, but there is an enrichment of clusters H and I (light blue), cluster Q (orange) and clusters R and S (pink) (**Figure 14B-E**), suggesting that enzymatic digestion is a better method for analysis of non-hematopoietic cells.

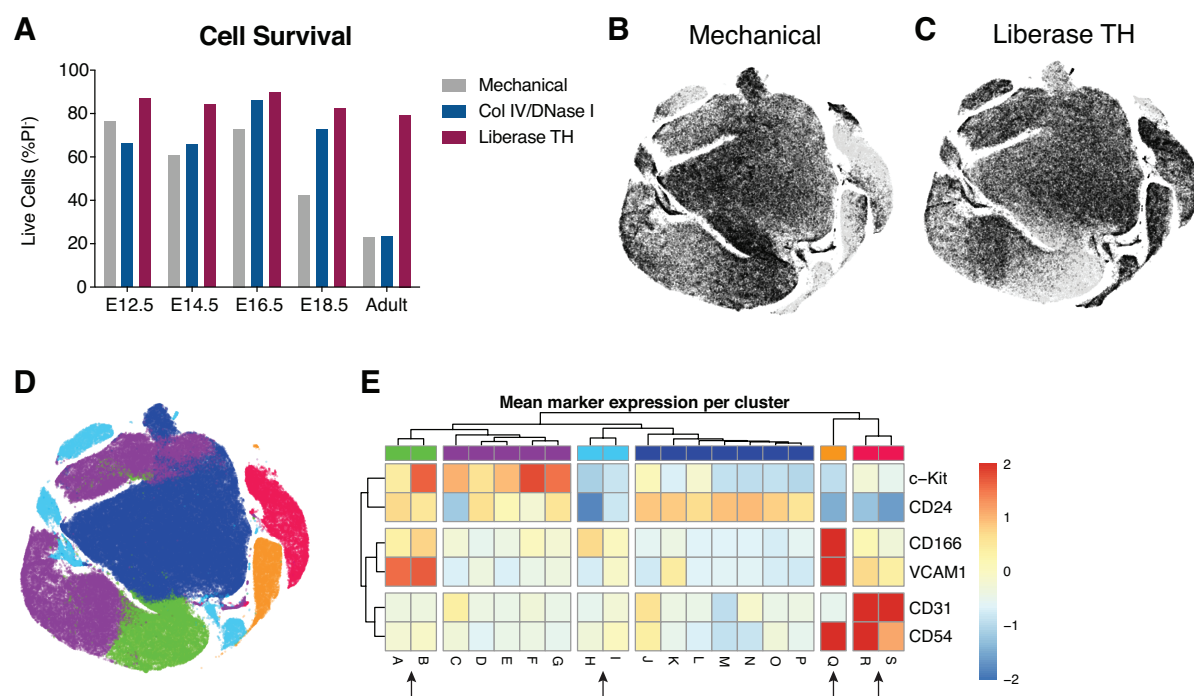


FIGURE 14 DIGESTION OF FETAL LIVER

(A) Cell survival analysis of E12.5, E14.5, E16.5, E18.5 and adult liver cells after mechanical or enzymatic digestion, obtained by Annexin V flow cytometric analysis. (B and C) tSNE analysis of E15.5 Ter119⁺CD45⁺ fetal liver cells obtained by mechanical or Liberase TH digestion, respectively. (D) tSNE representation of clusters obtained by unsupervised hierarchical clustering based on the expression of the surface markers CD31, CD54, CD24, Kit, CD166 and VCAM1 of E15.5 Ter119⁺CD45⁺ cells and respective heatmap (E). See also **Supplementary Figure 2**.

PANEL DESIGN TO IDENTIFY STROMAL POPULATIONS

After optimization of the protocols for FL cell isolation and depletion of hematopoietic cells, we aimed at characterizing the FL stromal compartment at the time-point where HSC expansion peak occurs, E14.5. For that, we stained Ter119⁺CD45⁺ E14.5 FL cells with a panel of 13 surface antibodies that included epithelial, endothelial, mesenchymal, adhesion molecules and progenitor cell markers. We performed unsupervised tSNE analysis and hierarchical clustering and obtained 4 distinct clusters (**Figure 15A**). The largest cluster represents a mix of 3 smaller clusters (purple, blue and dark blue, representative of P1, P2 and P3 cells identified in **Table 5**) that include cells characterized by expression of CD24 and Kit (**Figure 15B**). Orange cluster (cluster J) represents epithelial cells as cells express high levels of epithelial cadherin (CD324). These cells also express high levels of the adhesion molecules CD166 and CD54 and the integrin alpha V (CD51). Light blue cluster (cluster K) does not express the epithelial marker CD324 nor the endothelial marker CD31 and is characterized by expression of CD166, CD51 and Gp38 (or Podoplanin). Clusters L and M (pink) are characterized by high levels of the endothelial marker CD31 and express CD146 and CD54. The two clusters segregate due to distinct expression levels of CD90 and Sca1 (**Figure 15B**). We were able to obtain the same populations through manual gating of the flow cytometry data (**Supplementary Figure 3**). Of note, the majority of Ter119⁺CD45⁺ cells (Clusters A through I) have an undefined lineage origin as they do not express epithelial, endothelial or mesenchymal markers.

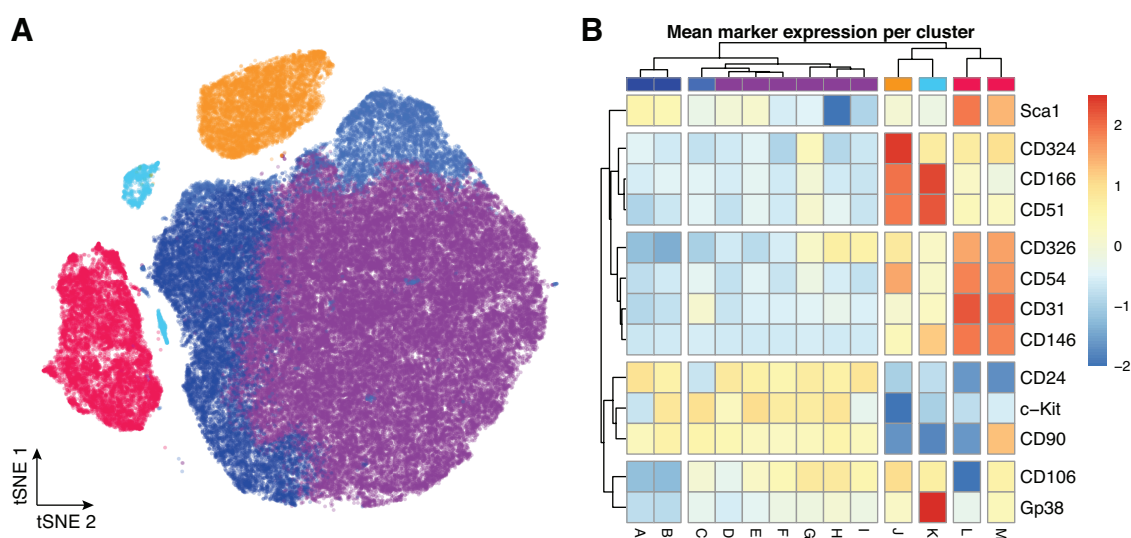


FIGURE 15 T-SNE ANALYSIS OF E14.5 TER119⁺CD45⁺ FETAL LIVER CELLS

(A) tSNE analysis of Ter119⁺CD45⁺ E14.5 FL cells overlaid with clusters obtained by unsupervised hierarchical clustering based on expression of the surface markers Sca1, CD324, CD166, CD51, CD326, CD54, CD31, CD146, CD24, Kit, CD90, CD106 and Gp38 and respective heatmap (B). See also **Supplementary Figure 3**.

CHARACTERIZATION BY GENE EXPRESSION

To characterize the FL stromal compartment, we performed gene expression analysis in the 6 major populations identified above. For that, we sorted E14.5 Ter119⁻CD45⁻ FL cells based on cell surface expression of CD54, CD31, CD24 and Kit (gating strategy in **Figure 16A**). We evaluated the expression of hepatocyte (*Afp*, *Krt18*, *Hnf4a*), cholangiocyte (*Hnf6*, *Krt19*), endothelial (*Flk1*) and mesenchymal (*Wt1*, *Acta2*) transcripts (**Figure 16B**). Epithelial cells sorted based on the expression of CD54 had transcripts for the hepatic genes *Afp*, *Krt18* and *Hnf4a*, and the cholangiocyte gene *Hnf6*, suggesting that they are hepatic bipotent progenitors, or hepatoblasts. CD31⁺ cells expressed the endothelial transcript *Flk1* and the mesenchymal transcript *Acta2*. Cells negative for all markers (DN) did not express hepatic nor endothelial transcripts, however expressed the cholangiocyte marker *Krt19* and the mesenchymal genes *Wt1* and *Acta2*, suggesting that these cells represent a heterogeneous population of cholangiocytes and mesenchymal subsets. P1, P2 and P3 cells did not express any of the analyzed transcripts.

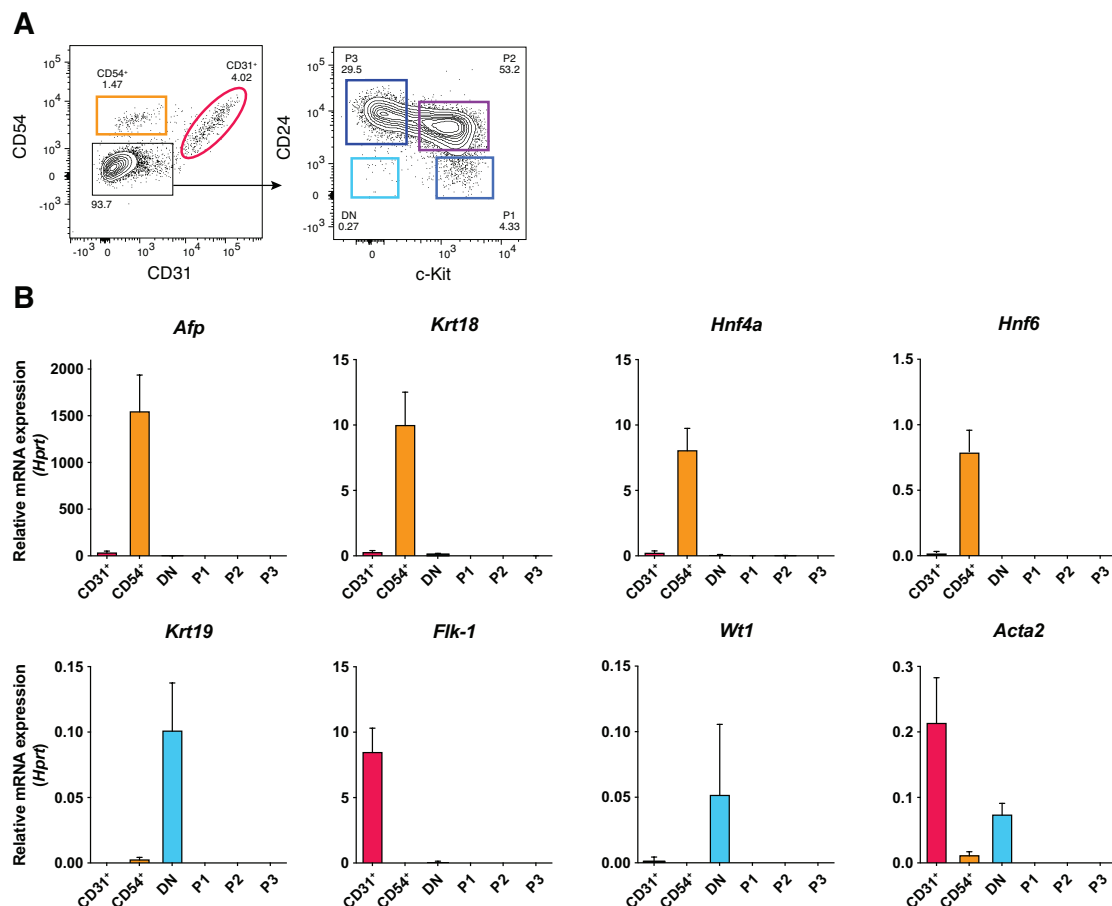


FIGURE 16 GENE EXPRESSION ANALYSIS OF E14.5 FETAL LIVER CELLS

(A) Flow cytometry plot indicating the gating strategy used for sorting E14.5 Ter119⁻CD45⁻ cells based on CD54, CD31, CD24 and Kit. (B) Gene expression analysis of the hepatocyte (*Afp*, *Krt18*, *Hnf4a*), cholangiocyte (*Hnf6*, *Krt19*), endothelial (*Flk1*) and mesenchymal (*Wt1*, *Acta2*) transcripts of the sorted cells. Relative mRNA expression was compared to that of *Hprt*.

RNA-SEQ ANALYSIS IDENTIFIES P2 CELLS AS BEING AFFILIATED TO THE HEMATOPOIETIC LINEAGE

Since gene expression analysis did not provide information on the lineage affiliation of the most frequent population in the Ter119⁻CD45⁻ compartment, CD24⁺Kit⁺ or P2 cells, we performed RNA sequencing analysis of these cells and compared with CD54⁺CD324⁺ and CD31⁺ cells (**Figure 17A**). We compared gene expression between the three populations by differential expression analysis and the genes upregulated >2-fold are listed in **Supplementary Table 1, 2 and 3**. Within the highest expressed transcripts in CD31⁺ cells are the endothelial-specific genes *Kdr*, *Lyve1*, *Flt1*, *Tek*, *Pecam1* and *Cdh5* (**Figure 17A**). Within the highest expressed transcripts in CD324⁺ cells are *Afp*, *Alb*, *Dlk1*, *Gulo* and *Serpina6*, hepatic and metabolism specific genes. The highest expressed transcripts in P2 cells include *Myb*, *Runx3*, *Klf1*, *Epor*, *Gata1* and *Bcl11a*, hematopoietic specific transcripts (**Figure 17A**). The overexpressed genes were subjected to gene ontology analysis using Enrichr (**Chen et al., 2013**) (**Figure 17B** and **Supplementary Figure 4**). The top biological processes identified in CD31⁺ cells include endothelial cell migration processes, angiogenesis and vasculature, in P2 cells include hematopoietic processes such as leukocyte adhesion and erythroid or myeloid differentiation, and the ones identified in CD324⁺ cells include metabolic processes (**Figure 17B**). These results further correlate to the tissues and cell types that overexpress this set of genes, with CD31⁺ cells being comparable to HUVEC cell line, P2 cells analogous with erythroblasts and CD324⁺ cells similar to hepatocytes and HEPG2 cell line (**Supplementary Figure 4**).

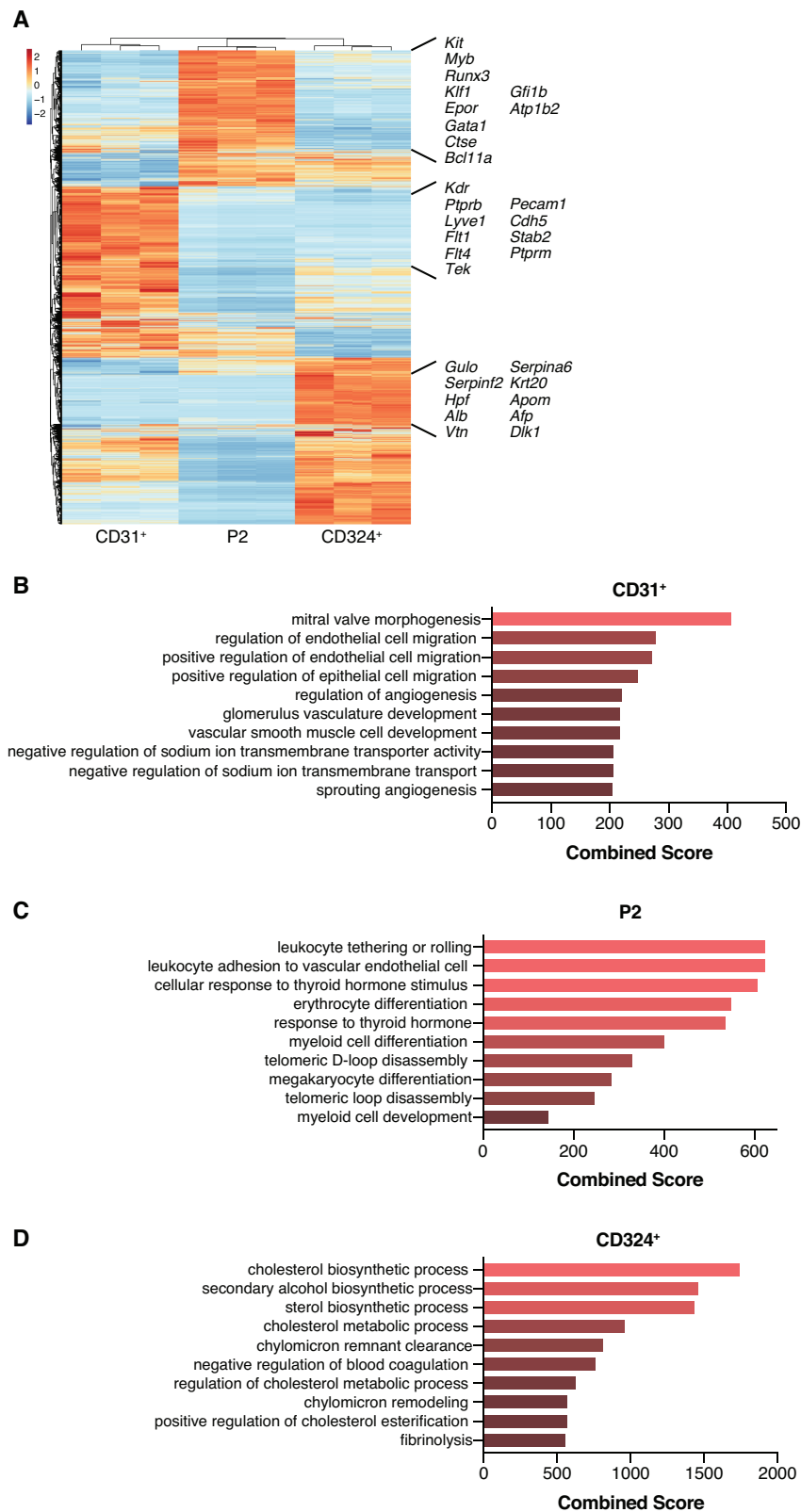


FIGURE 17 RNA-SEQ ANALYSIS OF E14.5 FETAL LIVER CELLS

(A) CD31⁺, P2 and CD324⁺ cells from E14.5 FL were sorted and subjected to RNA sequencing. Differentially expressed genes are represented as heatmap and 10 most differentially expressed genes are listed (n=3 independent litters). **(B-D)** Gene set enrichment analysis on genes with more than two-

fold difference in expression level using *Enrichr* web-application; top 10 significantly associated GO Biological Processes. See also **Supplementary Figure 4**.

DISCUSSION

Here we described an optimized protocol for the analysis of the FL stromal compartment, by enzymatic digestion of FL using Liberase TH and depletion of Ter119⁺CD45⁺ hematopoietic cells using MACS columns. The combination of these methods allows an increase in live cells and a better characterization of the non-hematopoietic compartment with identification of less frequent cell subsets. Previous studies addressed specific populations either by isolation of hepatoblasts by Dlk1⁺ cell enrichment (**Chou et al., 2013**) or using reporter mouse models for Nestin⁺ cells (**Khan et al., 2016**). Few studies aimed at characterizing the FL stroma using small antibody panels that allow identification of hepatic, endothelial and stellate cells (**Sugiyama et al., 2011; Tan et al., 2017b**). We analyzed Ter119⁻CD45⁻ cells with various combinations of 18 cell surface markers that have been used for the characterization of stromal cells in other tissues (**Crane et al., 2017**), with few additions, and that recognize epithelial, endothelial and mesenchymal lineages. From that panel, we selected the most promising markers for analysis in a single 13-color flow cytometry panel.

Using a panel of 13 cell surface markers we were able to identify the cell types already described in the literature: epithelial, endothelial and mesenchymal cells, but also 3 undescribed populations that represent the majority (>70%) of Ter119⁻CD45⁻ cells, characterized by expression of CD24 and Kit (P1, P2 and P3).

Gene expression analysis showed that CD54⁺ cells expressed hepatic and cholangiocyte genes, and could, therefore belong to the hepatoblast lineage. Cells negative for all markers expressed *Krt19*, a cholangiocyte marker and mesenchymal genes such as *Wt1* and *Acta2*, suggesting they are a heterogeneous population, and could be further segregated based on the expression of CD146, CD51, CD166 and Gp38. P1, P2 and P3 cells did not express any of the transcripts and remained of undefined lineage affiliation. RNA sequencing analysis of P2 cells revealed a hematopoietic affiliation, with the highest expressed genes being *Kit*, *Myb*, *Runx3*, *Epor* and *Gata1*, although these cells do not express the erythroid marker Ter119 nor the pan-hematopoietic marker CD45. These cells represent, together with P1 and P3, >70% of Ter119⁻CD45⁻ cells and 10-15% of total FL cells and are therefore, the subject of the next chapter of this thesis.

MATERIALS AND METHODS

Mice

C57BL/6J mice were purchased from Envigo. 6-8-week-old mice or timed pregnant females were used. Timed-pregnancies were generated after overnight mating, the following morning females with vaginal plug were considered to be at E0.5. All animal manipulations were performed according to the ethic charter approved by French Agriculture ministry and to the European Parliament Directive 2010/63/EU.

Cell suspension

E14.5 FLs were dissected under a binocular magnifying lens. FLs were recovered in Hanks' balanced-salt solution (HBSS) supplemented with 1% fetal calf serum (FCS) (Gibco) and three methods were tested: mechanical digestion – FLs were passed through a 26-gauge needle of a 1-ml syringe to obtain single-cell suspensions; Collagenase IV/DNAse I treatment: FLs were cut in 1 mm² pieces and incubated with 0,5 mg/ml Collagenase IV +50 mg/ml DNAse I solution for 15-20 min at 37°C; Liberase TH treatment: FLs were cut in 1 mm² pieces and incubated with 0,05 mg/ml Liberase TH (Roche) for 6-8 minutes at 37°C. Enzymatic treatment was stopped by addition of cold HBSS + 1% FCS followed by centrifugation. Before staining, cell suspensions were filtered with a 100 µm cell strainer (BD).

Hematopoietic cell depletion

FLs were depleted of hematopoietic cells (Ter119⁺CD45⁺) using MACS columns (Miltenyi Biotec) or Dynabeads (Invitrogen) according to manufacturer instructions. Briefly, for MACS columns depletion, cells were incubated with biotinylated Ter119 and CD45 antibodies for 20-30 min at 4°C, washed, incubated with anti-biotin microbeads (Miltenyi Biotec) for 20 min at 4°C and passed through a LS column (Miltenyi Biotec). The flow-through was collected and used for cell surface staining. For Dynabeads depletion, cells were incubated with biotinylated Ter119 and CD45 antibodies for 20-30 min at 4°C, followed by incubation with pre-washed Dynabeads for 30 min with rotation at 4°C. After incubation, the tube containing the cells was placed in a magnetic holder and supernatant containing unbound cells was collected for cell surface staining. Hematopoietic cell depletion was evaluated at all times by incubation with a fluorochrome-labeled streptavidin.

Flow cytometry and cell sorting

Cell suspensions were stained for 20-30 min at 4°C in the dark with antibodies listed in **Supplementary Table 4**. Dead cells were excluded by incorporation of Propidium Iodide. Stained cells were analyzed on a SONY SP6800 (SONY Biotechnology) or a BD SYMPHONY (BD Biosciences) and were sorted with a BD FACSAria III (BD Biosciences) according to the guidelines for the use of flow cytometry and cell sorting (**Cossarizza et al., 2019**). Data were

analyzed with FlowJo software (v.10.5.3, BD Biosciences) or R packages as described in “Bioinformatic Analysis”.

Cell viability analysis

For viability analysis using Annexin V, cells were stained with surface markers, incubated with Annexin V APC in binding buffer (10 mM HEPES pH 7.4, 0.14M NaCl, 2.5mM CaCl₂) for 15 min at room temperature, followed by incubation with Propidium Iodide before analysis on a BD LSR Fortessa.

Gene expression by RT-PCR

Cells were sorted directly into lysis buffer and mRNA was extracted (RNeasy Micro Kit (Qiagen)), reverse-transcribed (PrimeScript RT Reagent Kit (Takara Bio)), and quantitative PCR was performed with Taqman Master Mix (Applied Biosystems) (see **Supplementary Table 5** for probes). qPCR reactions were performed on a Quantstudio3 thermocycler (Applied Biosystems), gene expression was normalized to that of Hprt and relative expression was calculated using the $2^{-\Delta Ct}$ method.

RNA-sequencing and analysis

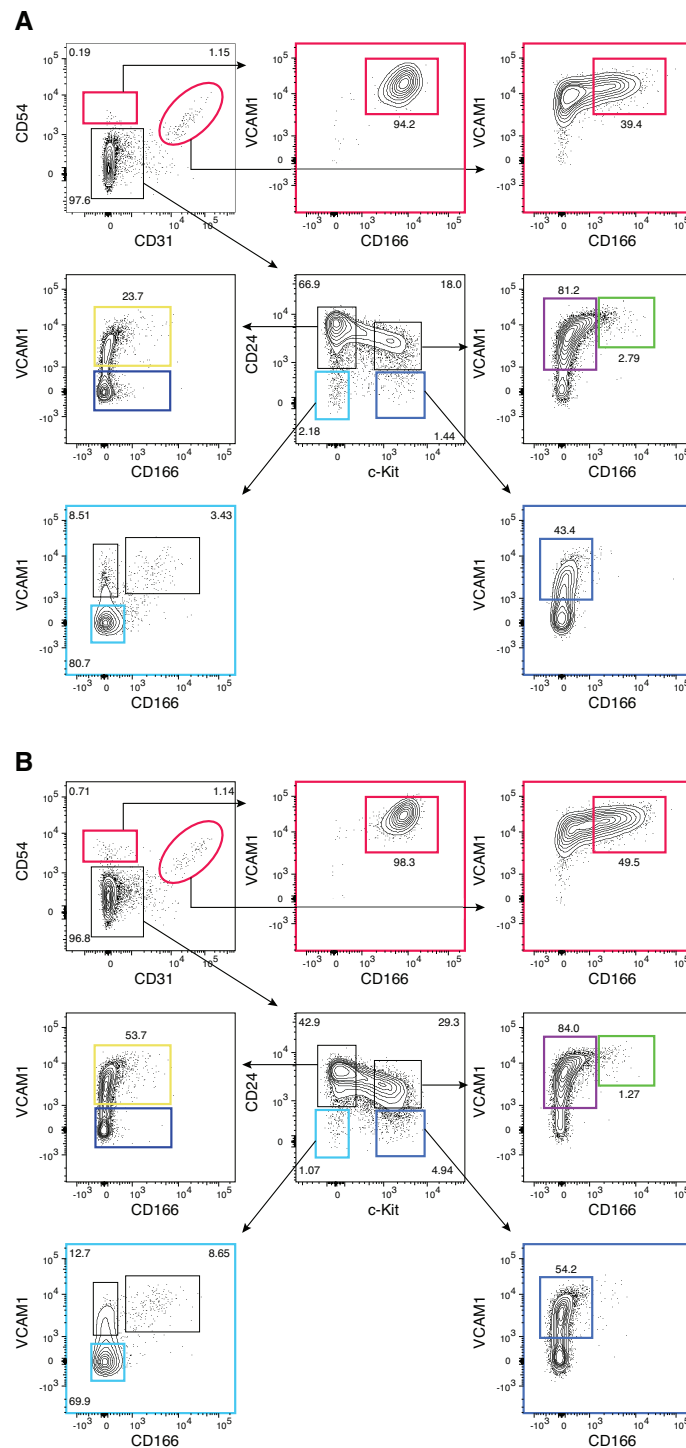
Total RNA from sorted E14.5 FL cells was extracted using the RNeasy Micro kit (Qiagen) following manufacturer instructions and rRNA sequences were eliminated by enzymatic treatment (Zap R, Clontech). cDNA libraries were generated using the SMARTer Stranded Total RNA-Seq Kit – Pico Input Mammalian (Clontech). The single read RNA-seq reads were aligned to the mouse reference genome GRCm38 using STAR. The number of reads aligned to genes was counted using FeatureCounts (**Liao et al., 2014**). The R package DESeq2 (**Love et al., 2014**) was used to normalize reads and identify differentially expressed genes with statistically significance using the negative binomial test ($p < 0.05$, Benjamini-Hochberg correction). More than 2-fold differentially expressed genes in 2 by 2 comparisons were used for Enrichr gene-set enrichment analysis (**Chen et al., 2013**). Top 10 terms from the Gene Ontology Biological Process 2018, ARCHS4 Tissues and ARCHS4 Cell-lines were retrieved. Expression datasets are available in NCBI Gene Expression Omnibus under Accession Number GSE138960.

Bioinformatic Analysis

Flow cytometry data analysis was performed in FCS files of live CD45⁺Ter119⁻ cell fraction using R packages “Rtsne”, “Rphenograph” and “pheatmap” using Rv3.5.0. Heatmaps and hierarchical clustering were generated using R packages “pheatmap” and “Rphenograph” (**Levine et al., 2015**).

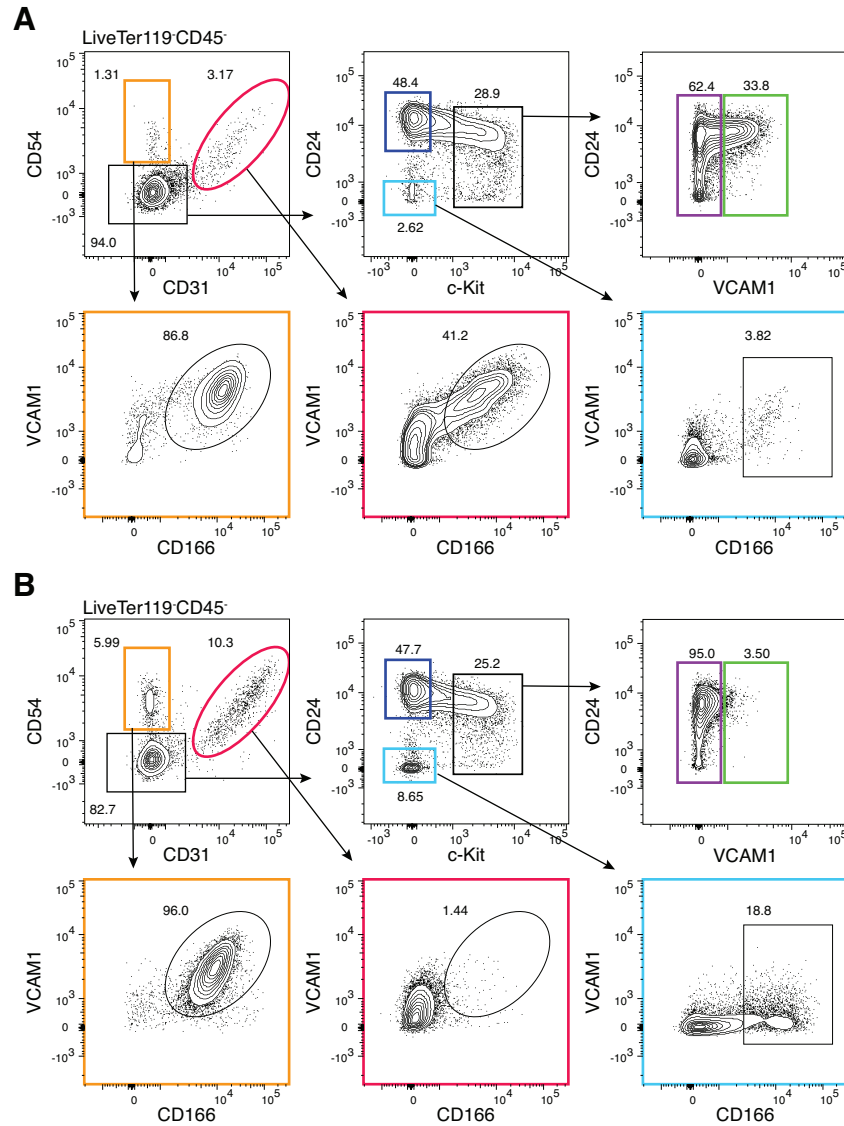
SUPPLEMENTARY INFORMATION

SUPPLEMENTARY FIGURES



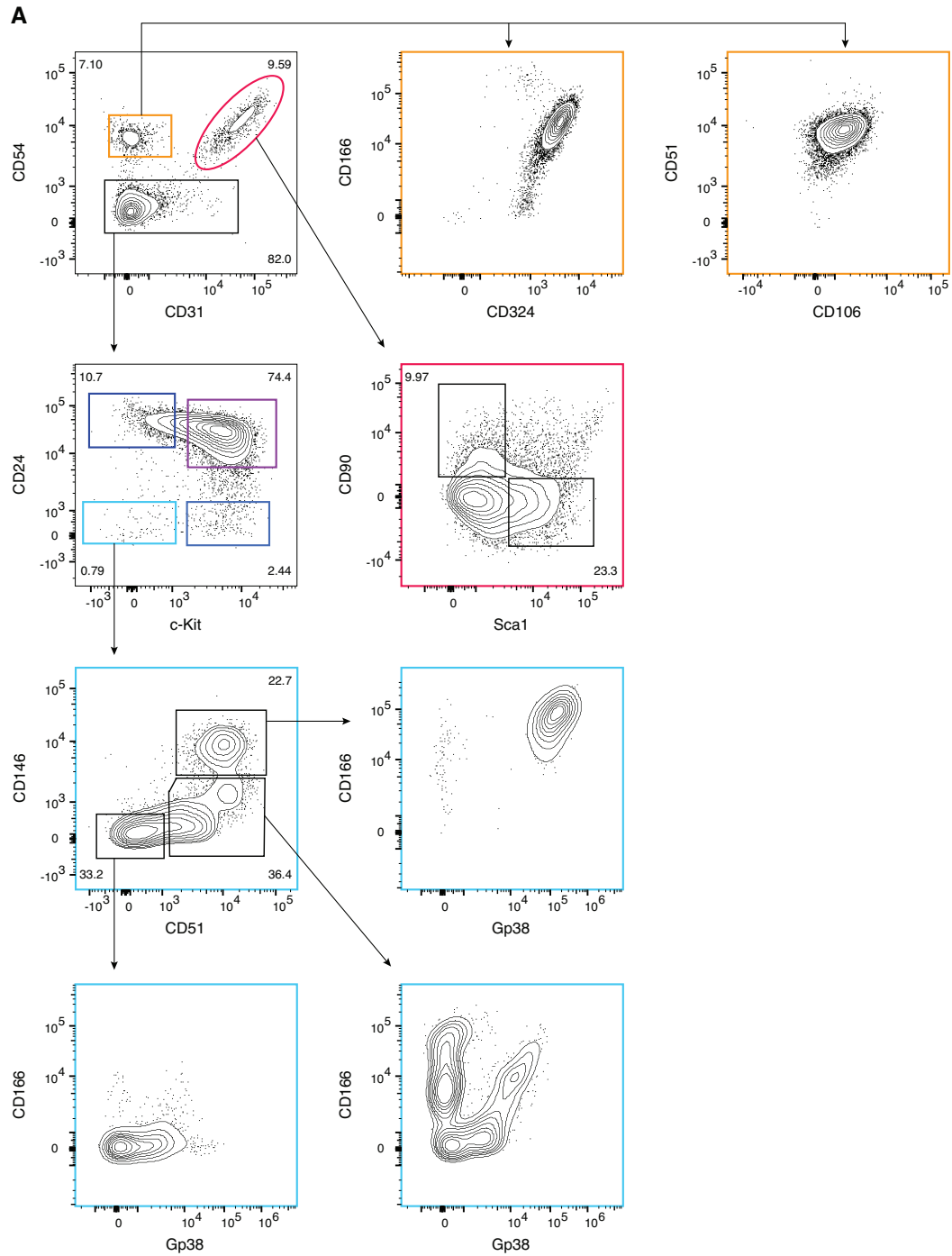
SUPPLEMENTARY FIGURE 1 FLOW CYTOMETRY PROFILES OF E14.5 TER119-CD45- CELLS OBTAINED BY DEPLETION WITH DYNABEADS OR MACS COLUMNS

(A) Representative FACS plot showing the cell types obtained by depletion of Ter119 and CD45 cells E15.5 FL cells using Dynabeads. **(B)** Representative FACS plot showing the cell types obtained by depletion of Ter119 and CD45 cells E15.5 FL cells using MACS Columns.



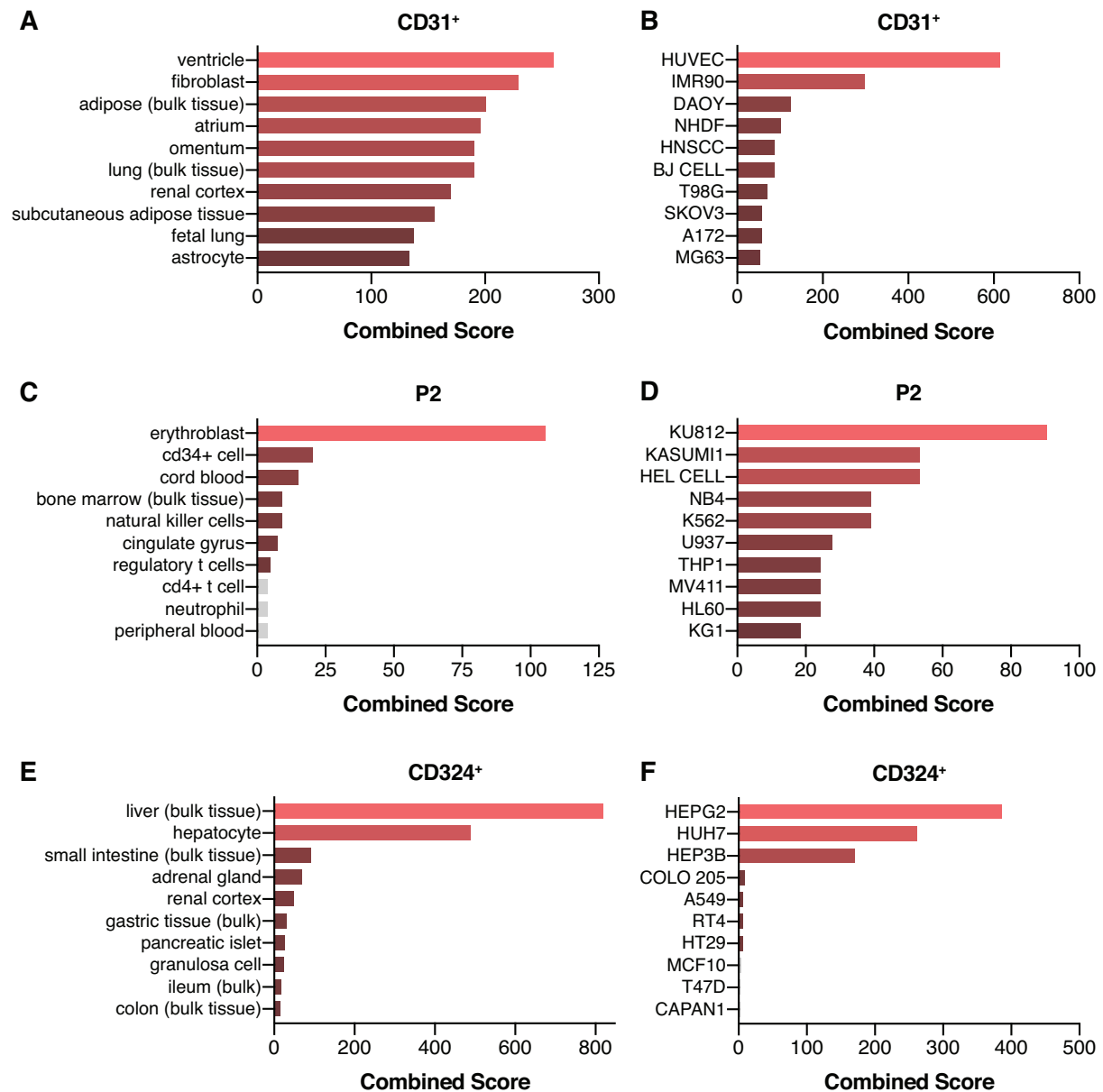
SUPPLEMENTARY FIGURE 2 FLOW CYTOMETRY PROFILES OF E15.5 FETAL LIVER CELLS OBTAINED BY MECHANICAL OR ENZYMATIC DIGESTION

(**A**) Representative FACS plot showing the cell types obtained by mechanical isolation of E15.5 FL cells. (**B**) Representative FACS plot showing the cell types obtained by enzymatic digestion of E15.5 FL cells.



SUPPLEMENTARY FIGURE 3 MANUAL GATING IDENTIFYING POPULATIONS OBTAINED BY UNSUPERVISED ANALYSIS OF E14.5 FETAL LIVER CELLS

(A) Representative FACS plot illustrating the gating strategy for the clusters obtained by unsupervised analysis of E14.5 Ter119⁺CD45⁺ FL cells.



SUPPLEMENTARY FIGURE 4 ARCH4 TISSUE AND CELL LINES

(A-F) Gene set enrichment analysis on genes with more than two-fold difference in expression level using *Enrichr* web-application; top 10 significantly associated ARCHS4 Tissues (A, C, E) and ARCHS4 Cell-lines (B, D, F).

SUPPLEMENTARY TABLES

SUPPLEMENTARY TABLE 1 LIST OF SUBMITTED GENES FOR ENRICHR ANALYSIS – CD31⁺

<i>Mpo</i>	<i>Tinagl1</i>	<i>Mtss1l</i>	<i>Fam174b</i>	<i>Bend4</i>	<i>Tmtc2</i>
<i>Hivep3</i>	<i>Amotl1</i>	<i>Fmn12</i>	<i>Eng</i>	<i>Mef2c</i>	<i>Smarca2</i>
<i>Ldb2</i>	<i>Igfbp7</i>	<i>Vim</i>	<i>Mkl1</i>	<i>Tbc1d4</i>	<i>Evl</i>
<i>Ptprb</i>	<i>Sparc</i>	<i>Stard9</i>	<i>Col5a2</i>	<i>Ccdc126</i>	<i>Plcg2</i>
<i>Kdr</i>	<i>Pxdn</i>	<i>Id3</i>	<i>Camk2d</i>	<i>Slc38a6</i>	<i>Parp14</i>
<i>Stab2</i>	<i>Hip1</i>	<i>Ehd3</i>	<i>Plpp1</i>	<i>Zfp69</i>	<i>Myom1</i>
<i>Nrp2</i>	<i>Srgap1</i>	<i>Pea15a</i>	<i>Crybg3</i>	<i>Ddah2</i>	<i>Rab27b</i>
<i>Rasgrp3</i>	<i>Epas1</i>	<i>Adam12</i>	<i>Unc13b</i>	<i>Fyn</i>	<i>Zmiz1</i>
<i>Flt1</i>	<i>Hecw2</i>	<i>Ophn1</i>	<i>Sall2</i>	<i>Plek</i>	<i>Peak1</i>
<i>S100a16</i>	<i>Dock4</i>	<i>Fbn1</i>	<i>Cnn2</i>	<i>Prickle1</i>	<i>Gnb4</i>
<i>Fat4</i>	<i>Ccdc80</i>	<i>Plxna4</i>	<i>Inpp4b</i>	<i>Rap2a</i>	<i>Myo1c</i>
<i>Mrc1</i>	<i>Nrp1</i>	<i>Zfp462</i>	<i>Zfp608</i>	<i>Nfatc1</i>	<i>Khdrbs3</i>
<i>Plpp3</i>	<i>Cd38</i>	<i>Ston1</i>	<i>Prkce</i>	<i>Zyx</i>	<i>Tubb1</i>
<i>Cdh5</i>	<i>Pecam1</i>	<i>Cmtm3</i>	<i>Adamts10</i>	<i>Thbs1</i>	<i>F2r</i>
<i>Fgd5</i>	<i>Map1b</i>	<i>Fam171a1</i>	<i>Snx33</i>	<i>Cerk</i>	<i>Dip2c</i>
<i>Oit3</i>	<i>Nes</i>	<i>Efnb1</i>	<i>Adgrl2</i>	<i>Spry4</i>	<i>Zeb1</i>
<i>Lyve1</i>	<i>Sema6d</i>	<i>Npl</i>	<i>Pbx1</i>	<i>Mrv1</i>	<i>Gja1</i>
<i>Ptprm</i>	<i>Fzd4</i>	<i>Hpgd</i>	<i>Epb41l2</i>	<i>Sh3tc2</i>	<i>Dlgap4</i>
<i>Cd93</i>	<i>Tshz2</i>	<i>Fli1</i>	<i>Ets2</i>	<i>Homer3</i>	<i>Shisa5</i>
<i>Gas7</i>	<i>Mest</i>	<i>Plvap</i>	<i>Efna5</i>	<i>Itgb3</i>	<i>1700112E06Rik</i>
<i>Plk2</i>	<i>Adamts1</i>	<i>Ldlrad4</i>	<i>Slc31a2</i>	<i>Dock11</i>	<i>Sema6b</i>
<i>Tek</i>	<i>Ckap4</i>	<i>Vav3</i>	<i>Klhl6</i>	<i>Map4k2</i>	<i>Klhl5</i>
<i>Mb21d2</i>	<i>Ccnj1</i>	<i>Gdpd5</i>	<i>Fos</i>	<i>Prkg1</i>	<i>Tmsb10</i>
<i>Tm4sf1</i>	<i>Dse</i>	<i>Ica1</i>	<i>Pdzd2</i>	<i>Pls3</i>	<i>Klhl8</i>
<i>Adgrf5</i>	<i>Gata4</i>	<i>Dock6</i>	<i>Kank3</i>	<i>Fosb</i>	<i>Lats2</i>
<i>Col4a1</i>	<i>Mmp15</i>	<i>Rcsd1</i>	<i>Sh3rf1</i>	<i>Pf4</i>	<i>Csnk1e</i>
<i>Fbxl7</i>	<i>Sema4c</i>	<i>Zfpm2</i>	<i>Ttc7b</i>	<i>Plcb3</i>	<i>Crim1</i>
<i>Ets1</i>	<i>Wwtr1</i>	<i>Clec1b</i>	<i>Itpkb</i>	<i>Rcn3</i>	<i>Oaz2</i>
<i>Stox2</i>	<i>Adam19</i>	<i>Mpdz</i>	<i>Snrk</i>	<i>Ubash3b</i>	<i>Mast4</i>
<i>Stab1</i>	<i>Slc43a2</i>	<i>Itga8</i>	<i>Plcg1</i>	<i>Fam107b</i>	<i>Gramd1a</i>
<i>Flt4</i>	<i>Serpinh1</i>	<i>Clca3a1</i>	<i>Ahnak</i>	<i>Sgcb</i>	<i>Rgl2</i>
<i>Dab2</i>	<i>Sorbs3</i>	<i>Adamts9</i>	<i>Igfbp5</i>	<i>Vwf</i>	<i>Plcb4</i>
<i>Palld</i>	<i>Ppp1r18</i>	<i>Tanc1</i>	<i>Ptpn18</i>	<i>Klhl36</i>	<i>Plxnc1</i>

<i>Msr3</i>	<i>Prr5l</i>	<i>Palm</i>	<i>Gatm</i>	<i>Ankrd44</i>	<i>Ago3</i>
<i>Npr1</i>	<i>Slc9a3r2</i>	<i>Itprl2</i>	<i>Itga6</i>	<i>Prkd2</i>	<i>Atp11a</i>
<i>Hmcn1</i>	<i>Dpp4</i>	<i>Plekho1</i>	<i>Mid1</i>	<i>Lhfp12</i>	<i>Ggta1</i>
<i>Akap12</i>	<i>Adarb1</i>	<i>Trps1</i>	<i>Phactr2</i>	<i>Tspan6</i>	<i>Fam188b</i>
<i>Sema6a</i>	<i>Vstm4</i>	<i>Ptgs1</i>	<i>Lcp1</i>	<i>Slc25a24</i>	<i>Akt3</i>
<i>Gpr182</i>	<i>Amotl2</i>	<i>Itga9</i>	<i>Wdfy2</i>	<i>Rab27a</i>	<i>Mark4</i>
<i>Maf</i>	<i>Dock1</i>	<i>Phldb1</i>	<i>App</i>	<i>Cebpd</i>	<i>Atrnl1</i>
<i>Armxc4</i>	<i>Dbn1</i>	<i>Nucb2</i>	<i>Nectin2</i>	<i>Bmpr2</i>	<i>Crip2</i>
<i>Prex2</i>	<i>Col4a2</i>	<i>Slc9a9</i>	<i>Vangl2</i>	<i>Pde7b</i>	<i>Cd55</i>
<i>Notch1</i>	<i>Nhs12</i>	<i>Rilp1</i>	<i>Slc36a4</i>	<i>Cachd1</i>	<i>Arap3</i>
<i>Gimap4</i>	<i>Sgk1</i>	<i>Dgkh</i>	<i>Gpsm1</i>	<i>Chst14</i>	<i>Gp1ba</i>
<i>Cttnbp2nl</i>	<i>Pear1</i>	<i>Tmem2</i>	<i>Ttc28</i>	<i>S100a13</i>	<i>Il20rb</i>
<i>Arhgap31</i>	<i>Sash1</i>	<i>Dusp3</i>	<i>Rasal2</i>	<i>Shank3</i>	<i>Znrf1</i>
<i>Tspan18</i>	<i>Obsl1</i>	<i>Cd84</i>	<i>Csrp2</i>	<i>Serpina6a</i>	<i>Sh3bgr13</i>
<i>Rgs3</i>	<i>Fam102a</i>	<i>Stk39</i>	<i>Gfod1</i>	<i>Copg2</i>	<i>Sun2</i>
<i>Ednrb</i>	<i>Calcl1</i>	<i>Tpm4</i>	<i>Wls</i>	<i>S100a10</i>	<i>Dlg2</i>
<i>Ramp2</i>	<i>Rps6ka2</i>	<i>Lbh</i>	<i>Ppic</i>	<i>Arrb2</i>	<i>Zfp354c</i>
<i>Tspan7</i>	<i>Rgl1</i>	<i>Hdac9</i>	<i>Ptrf</i>	<i>Runx1t1</i>	<i>4931406P16Rik</i>
<i>Plekha1</i>	<i>Il6st</i>	<i>Rarb</i>	<i>Dusp1</i>	<i>Mylip</i>	<i>Sox4</i>
<i>Eml1</i>	<i>Stard8</i>	<i>Fstl1</i>	<i>Asap2</i>	<i>Jak3</i>	<i>Pced1b</i>
<i>Shroom2</i>	<i>Nav1</i>	<i>Arhgap28</i>	<i>Mmrn1</i>	<i>Megf11</i>	<i>Skap2</i>
<i>Dchs1</i>	<i>F8</i>	<i>Pik3c2b</i>	<i>Pam</i>	<i>Ksr1</i>	<i>Tnik</i>
<i>Klhl3</i>	<i>Rapgef5</i>	<i>Bgn</i>	<i>Ecm1</i>	<i>Slc44a2</i>	<i>Tmem181a</i>
<i>Smad6</i>	<i>Adgra2</i>	<i>Zswim6</i>	<i>Nbea</i>	<i>Tcf4</i>	<i>Klhl24</i>
<i>Meis2</i>	<i>Lrrc32</i>	<i>Mxra7</i>	<i>Aff3</i>	<i>Trem1</i>	<i>Capg</i>
<i>Prkch</i>	<i>Dpysl3</i>	<i>Abcc9</i>	<i>Tecpr1</i>	<i>2810025M15Rik</i>	<i>Fam102b</i>
<i>Colec12</i>	<i>Rin2</i>	<i>Reep1</i>	<i>Inpp5a</i>	<i>Pde3a</i>	<i>Lrmp</i>
<i>Nr2f2</i>	<i>Tns2</i>	<i>Arhgap29</i>	<i>Ankrd50</i>	<i>Angpt1</i>	<i>Hcls1</i>
<i>Cd36</i>	<i>Itga2</i>	<i>Acer2</i>	<i>Alox12</i>	<i>Eogt</i>	<i>Limk1</i>
<i>Hspg2</i>	<i>Il1a</i>	<i>Arhgap25</i>	<i>Prune2</i>	<i>Pbbp</i>	<i>Fnbp1</i>
<i>Plxnd1</i>	<i>Parp8</i>	<i>Plagl1</i>	<i>Rapgef2</i>	<i>Tcaf1</i>	<i>Cblb</i>
<i>Man1c1</i>	<i>Maged2</i>	<i>Abcg1</i>	<i>Itga2b</i>	<i>Armxc1</i>	
<i>Fam43a</i>	<i>Nedd9</i>	<i>Pros1</i>	<i>Prkcq</i>	<i>Ccnyl1</i>	
<i>Trp53i11</i>	<i>Asah2</i>	<i>Fam219a</i>	<i>Arhgef28</i>	<i>Rnf157</i>	
<i>Dysf</i>	<i>Hivep2</i>	<i>Maml2</i>	<i>Marcks1</i>	<i>Mctp1</i>	

SUPPLEMENTARY TABLE 2 LIST OF SUBMITTED GENES FOR ENRICHR ANALYSIS – P2

<i>Kit</i>	<i>Kcnn4</i>	<i>Klf1</i>	<i>Slc38a5</i>	<i>Rgcc</i>	<i>Wrm</i>
<i>Myb</i>	<i>Ikzf1</i>	<i>Dyrk3</i>	<i>Rfx2</i>	<i>Myh7b</i>	<i>Emilin2</i>
<i>Runx3</i>	<i>Gm11427</i>	<i>Add2</i>	<i>Tarsl2</i>	<i>4930523C07Rik</i>	<i>Frat2</i>
<i>Spn</i>	<i>Fcho1</i>	<i>Epor</i>	<i>Bbs12</i>	<i>Ank1</i>	<i>Bsn</i>
<i>Gna15</i>	<i>Spire1</i>	<i>Rpia</i>	<i>Ampd3</i>	<i>Fam234b</i>	<i>Nfix</i>
<i>Def6</i>	<i>Sema4d</i>	<i>Gata1</i>	<i>Mgat4a</i>	<i>Hsph1</i>	<i>Taf1c</i>
<i>Sphk1</i>	<i>Unc13d</i>	<i>Il1rl1</i>	<i>Adamts3</i>	<i>Pcyt1b</i>	<i>Dut</i>
<i>Kcnab2</i>	<i>A730089K16Rik</i>	<i>Cited4</i>	<i>Ptpu</i>	<i>Ssx2ip</i>	<i>Car2</i>
<i>Epdr1</i>	<i>Adcy7</i>	<i>Casp3</i>	<i>Samd14</i>	<i>Ppm1l</i>	<i>Noa1</i>
<i>Ptpn7</i>	<i>Mfsd2b</i>	<i>Fam132a</i>	<i>Lmo2</i>	<i>Ammecr1</i>	<i>Slc19a1</i>
<i>Ubash3a</i>	<i>Ermap</i>	<i>Pmm1</i>	<i>Mylk3</i>	<i>Sapcd2</i>	<i>Art4</i>
<i>Ydjc</i>	<i>Itga4</i>	<i>Ctse</i>	<i>Arhgap9</i>	<i>Tmem56</i>	<i>Pfas</i>
<i>Samsn1</i>	<i>Gfi1b</i>	<i>Gpc4</i>	<i>Recql4</i>	<i>Fuca2</i>	<i>5730508B09Rik</i>
<i>Ces2g</i>	<i>Klf9</i>	<i>Ptk2b</i>	<i>Helb</i>	<i>Reps2</i>	<i>Noc4l</i>
<i>Mbp</i>	<i>Them6</i>	<i>Cox6b2</i>	<i>Cldn13</i>	<i>Mylpf</i>	<i>Ubxn2b</i>
<i>Apbb1ip</i>	<i>Mns1</i>	<i>Gcnt1</i>	<i>Sppl2b</i>	<i>6030468B19Rik</i>	<i>Pfkm</i>
<i>Bcl11a</i>	<i>Prps1</i>	<i>Syngap1</i>	<i>Orc2</i>	<i>Ttll12</i>	<i>Abcg4</i>
<i>Atp1b2</i>	<i>Tspo2</i>	<i>Rhag</i>	<i>Endod1</i>	<i>Ddx21</i>	

SUPPLEMENTARY TABLE 3 LIST OF SUBMITTED GENES FOR ENRICHR ANALYSIS – CD324⁺

<i>Fgb</i>	<i>Car3</i>	<i>Cxcl12</i>	<i>Slc12a7</i>	<i>Scp2-ps2</i>	<i>Plekha6</i>
<i>Agt</i>	<i>Rgn</i>	<i>Rbp1</i>	<i>Slc39a14</i>	<i>C77080</i>	<i>Habp4</i>
<i>Igfbp1</i>	<i>Krt18</i>	<i>Errfi1</i>	<i>Ggcx</i>	<i>Dbi</i>	<i>Pgm3</i>
<i>Serpina6</i>	<i>Gys2</i>	<i>Qprt</i>	<i>Scpep1</i>	<i>P2rx4</i>	<i>Scp2</i>
<i>Apoa2</i>	<i>Cyp2c68</i>	<i>Tenm3</i>	<i>Tln2</i>	<i>Bphl</i>	<i>Slc25a15</i>
<i>Apom</i>	<i>Cdh1</i>	<i>Anxa4</i>	<i>Vwce</i>	<i>Sdc1</i>	<i>Tceal8</i>
<i>Plg</i>	<i>Gldc</i>	<i>Hmgcs1</i>	<i>Mvk</i>	<i>Pter</i>	<i>Galk1</i>
<i>Serpina1a</i>	<i>Pcsk9</i>	<i>Cxadr</i>	<i>Sigirr</i>	<i>Ldlr</i>	<i>Acadl</i>
<i>Vtn</i>	<i>Dgat2</i>	<i>Cth</i>	<i>Bdh1</i>	<i>Tkfc</i>	<i>Plekha7</i>
<i>Ambp</i>	<i>Pah</i>	<i>Atp8a2</i>	<i>Gstz1</i>	<i>Nsdhl</i>	<i>Dap</i>
<i>Alb</i>	<i>Bex2</i>	<i>Phyh</i>	<i>Aldh6a1</i>	<i>Slc19a2</i>	<i>Dopey2</i>
<i>Fga</i>	<i>Dsp</i>	<i>Pard3</i>	<i>Gpc6</i>	<i>Tspan12</i>	<i>Rdh11</i>
<i>C3</i>	<i>Dlk1</i>	<i>Dpyd</i>	<i>Abtb2</i>	<i>Insig1</i>	<i>Ebp</i>
<i>Serpina1b</i>	<i>Tmem176b</i>	<i>Gpt2</i>	<i>Slc36a1</i>	<i>Stard10</i>	<i>Insr</i>
<i>Serpinf2</i>	<i>Proz</i>	<i>Tst</i>	<i>Hadh</i>	<i>Dnajc12</i>	<i>Lmbrd2</i>
<i>Ahsg</i>	<i>Gsn</i>	<i>Glud1</i>	<i>Hsd17b7</i>	<i>Pcyox1</i>	<i>Selenos</i>
<i>Habp2</i>	<i>Dusp9</i>	<i>Paqr9</i>	<i>Grk3</i>	<i>Pxmp4</i>	<i>Acat2</i>
<i>Itih2</i>	<i>Abcc2</i>	<i>Galm</i>	<i>Dhcr7</i>	<i>Pecr</i>	<i>Pgrmc1</i>
<i>A2m</i>	<i>Prodh2</i>	<i>Pid1</i>	<i>Amacr</i>	<i>Cdc14b</i>	<i>Herpud1</i>
<i>F2</i>	<i>Fn1</i>	<i>Smoc1</i>	<i>Gstp3</i>	<i>Abhd2</i>	<i>Trim2</i>
<i>Anpep</i>	<i>Aass</i>	<i>Ablim1</i>	<i>Hmgcr</i>	<i>Mob3b</i>	<i>Mocs2</i>
<i>Apoa1</i>	<i>Prox1</i>	<i>Peg3</i>	<i>Lss</i>	<i>Hook1</i>	<i>Scd1</i>
<i>Ugt2b34</i>	<i>Krt8</i>	<i>Atf5</i>	<i>Ctps2</i>	<i>Shmt1</i>	<i>Tgm2</i>
<i>Hpx</i>	<i>Slc38a4</i>	<i>Cped1</i>	<i>0610040J01Rik</i>	<i>Deptor</i>	<i>Bnip3</i>
<i>Gulo</i>	<i>F10</i>	<i>Sec14l2</i>	<i>Crot</i>	<i>Acs11</i>	<i>Fdxr</i>
<i>Afp</i>	<i>Hsd17b2</i>	<i>Timd2</i>	<i>Sox5</i>	<i>Tpmt</i>	<i>Pir</i>
<i>Ttr</i>	<i>Bex1</i>	<i>Myo5b</i>	<i>Gm6781</i>	<i>Pafah2</i>	<i>Ube2l6</i>
<i>Serpinc1</i>	<i>Abca8b</i>	<i>Fam46a</i>	<i>Ddah1</i>	<i>Mpc2</i>	<i>Acadm</i>

<i>Aldob</i>	<i>Igf2</i>	<i>Fras1</i>	<i>Rnf43</i>	<i>Ebpl</i>	<i>Coq8a</i>
<i>Hc</i>	<i>Hnf4a</i>	<i>Alcam</i>	<i>Dqx1</i>	<i>1300017J02Rik</i>	<i>Nadsyn1</i>
<i>Itih4</i>	<i>Cgn</i>	<i>Idh1</i>	<i>Asl</i>	<i>Mt1</i>	<i>Tfeb</i>
<i>Apoh</i>	<i>Rtl1</i>	<i>Synj2</i>	<i>Trim14</i>	<i>P4hb</i>	<i>Plxna3</i>
<i>Itih1</i>	<i>A1cf</i>	<i>Epb41l1</i>	<i>Ppm1k</i>	<i>Acot2</i>	<i>Arhgef40</i>
<i>Fgg</i>	<i>Gstm1</i>	<i>Fggy</i>	<i>Ptpn21</i>	<i>H2-K1</i>	<i>Iah1</i>
<i>Hmgcs2</i>	<i>Lipc</i>	<i>Msmo1</i>	<i>Reep6</i>	<i>Llgl2</i>	<i>Cpq</i>
<i>Knq1</i>	<i>Ndrq1</i>	<i>Gcat</i>	<i>Fdft1</i>	<i>Tob1</i>	<i>Mettl7a1</i>
<i>Proc</i>	<i>Apoe</i>	<i>Fkbp11</i>	<i>Gm5424</i>	<i>Asb13</i>	<i>Sfxn2</i>
<i>H2-Q10</i>	<i>Masp1</i>	<i>Zim1</i>	<i>Hip1r</i>	<i>Acaa2</i>	<i>Pcyt2</i>
<i>Pzp</i>	<i>Itgb5</i>	<i>Ocln</i>	<i>Morc4</i>	<i>Gpam</i>	<i>Sord</i>
<i>Slc27a2</i>	<i>Lrp1</i>	<i>Pygl</i>	<i>Gstt2</i>	<i>Pdzk1</i>	<i>Gnpnat1</i>
<i>Itih3</i>	<i>Abat</i>	<i>Sc5d</i>	<i>Slc25a23</i>	<i>Foxp4</i>	<i>Wnk4</i>
<i>Serpinf1</i>	<i>Reln</i>	<i>Gstm4</i>	<i>Zdhhc9</i>	<i>Dbt</i>	<i>Slc25a10</i>
<i>Cpn1</i>	<i>Dennd5b</i>	<i>Grip1</i>	<i>Prdm16</i>	<i>Fbxo21</i>	<i>Map3k5</i>
<i>Cpb2</i>	<i>Mttp</i>	<i>Nectin1</i>	<i>Ttc38</i>	<i>Grhpr</i>	<i>Kif21a</i>
<i>Spp2</i>	<i>F5</i>	<i>Nipsnap1</i>	<i>Aldh3a2</i>	<i>Gm6652</i>	<i>Bex4</i>
<i>Gc</i>	<i>Pcbd1</i>	<i>Ank3</i>	<i>Atp8b1</i>	<i>Fads1</i>	<i>Abcd3</i>
<i>Serpina1e</i>	<i>Lpl</i>	<i>Cyp51</i>	<i>Ass1</i>	<i>Bex3</i>	<i>Plpp5</i>
<i>Rbp4</i>	<i>Myrf</i>	<i>Soat2</i>	<i>Lrig1</i>	<i>Sqle</i>	<i>Mmab</i>
<i>Krt20</i>	<i>Abca1</i>	<i>Pgm2</i>	<i>Acad11</i>	<i>Ak4</i>	<i>Glrx</i>
<i>Cfi</i>	<i>Gpc3</i>	<i>Ank2</i>	<i>Rnf128</i>	<i>Echs1</i>	
<i>Apoc1</i>	<i>Met</i>	<i>Csad</i>	<i>Aldh7a1</i>	<i>Entpd5</i>	
<i>Aldh1b1</i>	<i>Cdh2</i>	<i>Dst</i>	<i>Hdac6</i>	<i>Hsf2</i>	
<i>Apob</i>	<i>Pde4dip</i>	<i>Tm7sf2</i>	<i>Dhcr24</i>	<i>Cryz</i>	

SUPPLEMENTARY TABLE 4 LIST OF ANTIBODIES USED FOR FLOW CYTOMETRY

Protein	Clone	Fluorochrome	Manufacturer	Catalog #
AA4.1	AA4.1	APC	eBioscience	17-5892-82
Annexin V	-	APC	SONY	3804600
PDGFRα	APA5	PE-Cy7	Invitrogen	25-1401-82
CD146	ME-9F1	FITC	Miltenyi	130-102-230
CD166	eBioALC48	APC	eBioscience	17-1661-82
CD24	M1/69	BV510	SONY	1109155
CD24	M1/69	PE-Cy7	BD Biosciences	560536
CD24	M1/69	FITC	BD Biosciences	553261
CD31	MEC13.3	BUV737	BD Biosciences	565097
CD31	MEC13.3	PE	BD Biosciences	553373
CD324	DECMA-1	PE-Cy7	SONY	1336545
CD324	DECMA-1	AF647	SONY	1336535
CD45	30-F11	Biotin	SONY	1115520
CD51	RMV-7	BV421	BD Biosciences	740062
CD54	3E2	BUV395	BD Biosciences	740222
CD54	YN1/1.7.4	Pacific Blue	Biolegend	116116
CD90.2	30-H12	AF700	SONY	1126600
Kit	2B8	APC-Cy7	SONY	1129130
EpCAM	G8.8	BV605	SONY	1191135
Gp38	8.1.1	PE	BD Biosciences	566390
Gp38	8.1.1	APC	eBioscience	50-5381-82
LPAM1	DATK 32	BV421	BD Biosciences	562943
NG2	1E6.4	PE	Miltenyi	130-100-468
Nrp1	3E12	BV421	SONY	1326045
Sca1	D7	BV711	SONY	1140655
Ter119	TER-119	Biotin	SONY	1181020
VCAM1	429	PerCP-Cy5.5	SONY	1128575

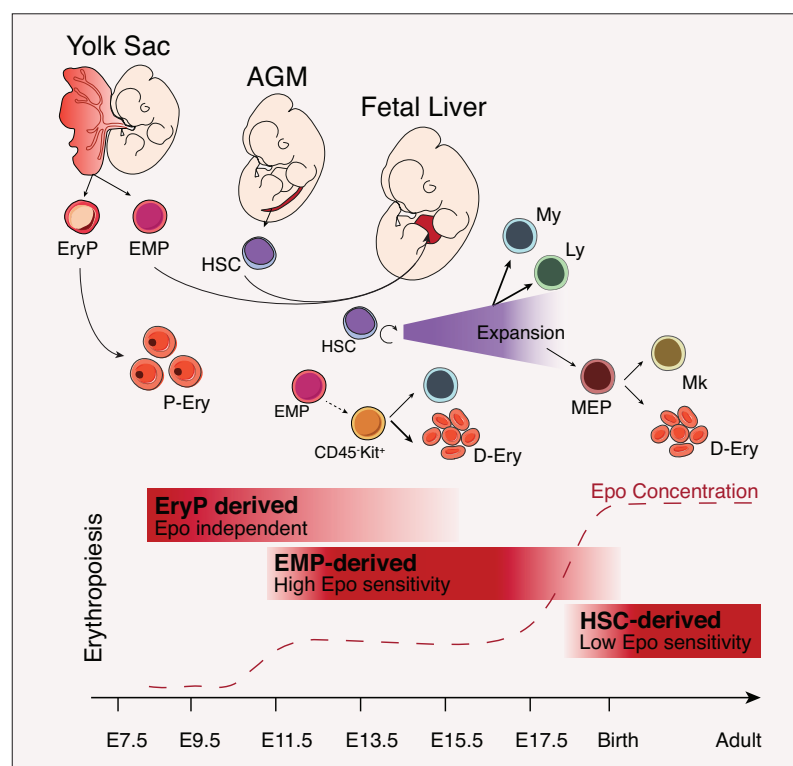
VCAM1	429	PE-Cy7	Biolegend	105720
Streptavidin	-	BV786	SONY	2626245
Streptavidin	-	BV650	BD Biosciences	566390

SUPPLEMENTARY TABLE 5 LIST OF TAQMAN PROBES USED

Gene	Gene ID	Taqman Assay ID
Acta2	11475	Mm01546133_m1
Afp	11576	Mm00431715_m1
Kdr (Flk-1)	16542	Mm01222421_m1
Hnf4a	15378	Mm01247712_m1
Onecut1 (Hnf6)	15379	Mm00839394_m1
Krt18	16668	Mm01601704_g1
Krt19	16669	Mm00492980_m1
Wt1	22431	Mm01337048_m1
Hprt	15452	Mm03024075_m1

CHAPTER IV – THE FETAL LIVER AS A HUB FOR YOLK SAC ERYTHROPOIESIS THROUGHOUT EMBRYO DEVELOPMENT

In the embryo, the first hematopoietic cells derive from the yolk sac and are thought to be rapidly replaced by the progeny of hematopoietic stem cells. We used three lineage tracing mouse models to show that, contrary to what was previously thought, hematopoietic stem cells do not contribute significantly to erythrocyte production up until birth. Lineage tracing of yolk sac erythromyeloid progenitors, that generate tissue-resident macrophages, identified highly proliferative erythroid progenitors that rapidly differentiate after intra-embryonic injection, persisting as the major contributors to the embryonic erythroid compartment. We show that erythrocyte progenitors of yolk sac origin require ten-fold lower concentrations of erythropoietin than their hematopoietic stem cell-derived counterparts for efficient erythrocyte production. We propose that, in a low erythropoietin environment in the fetal liver, yolk sac-derived erythrocyte progenitors efficiently outcompete hematopoietic stem cell progeny that fails to generate megakaryocyte and erythrocyte progenitors.



Keywords: erythropoiesis; embryo; yolk sac; fetal liver; hematopoietic stem cells

This chapter is based on the following publication:

Soares-da-Silva F, Freyer L, Elsaid R, Burlen-Defranoux O, Iturri L, Sismeiro O, Pinto-do-Ó P, Gomez-Perdiguero E, Cumano A (2020) **Yolk sac, but not HSC-derived progenitors, sustain erythropoiesis throughout murine embryonic life** bioRxiv doi: 10.1101/2020.02.27.968230, Under review

FOREWORD

Erythrocytes are the most abundant cells in circulation, they transport oxygen and have a half-life of around 22 days in the mouse, therefore, constant production in the BM is required to maintain the numbers of circulating RBCs.

Erythropoiesis is the process whereby HSCs progressively differentiate into erythromegakaryocyte and later into lineage-committed erythroid progenitors, immature BFU-E and the more mature CFU-E. CFU-E successively progress in differentiation through nucleated proerythroblast, basophilic, polychromatophilic and orthochromatic stages, enucleation and formation of RBCs. The distinct stages of erythroid differentiation are characterized by changes in surface expression of the progenitor marker Kit, of the transferrin receptor CD71, of the adhesion molecule CD44 and the mature erythroid marker Ter119 (**Kina et al., 2000; Aisen, 2004; Chen et al., 2009**).

During mouse embryogenesis, three overlapping hematopoietic waves emerge in distinct anatomic sites. The first blood cells arise in the YS blood islands at E7.5 and belong to the macrophage, erythroid and megakaryocytic lineages (**Palis et al., 1999**). Primitive erythrocytes are large nucleated cells that express a specific pattern of embryonic ($\epsilon\gamma$ - and $\beta\text{H1-}$) globins (**Kingsley et al., 2006**). Erythromyeloid progenitors arise in the YS around E8.5 (**Bertrand et al., 2005b**), differentiate into erythrocytes, megakaryocytes, macrophages and other myeloid lineages such as neutrophils, granulocytes and mast cells, but lack HSC activity (**Palis et al., 1999; McGrath et al., 2015**). EMP-derived erythrocytes resemble definitive erythrocytes and express embryonic $\beta\text{H1-}$ and adult $\beta\text{1-}$ but no $\epsilon\gamma$ -globins (**McGrath et al., 2011**). HSCs emerge after E8.5 (E8.5-E11.5) (**Cumano et al., 1996; de Bruijn et al., 2000; Taoudi et al., 2008; Bertrand et al., 2010; Kissa et al., 2010; Kieusseian et al., 2012**) in the major arteries, through an endothelial to hematopoietic transition process, rapidly enter circulation and colonize the FL where they expand and differentiate, generating the blood lineages. EMP that arise through a similar process in the YS (**Frame et al., 2016; Kasaai et al., 2017**) also converge to the FL where they are identified as $\text{Kit}^+\text{CD16/32}^+$ in contrast to $\text{Kit}^+\text{CD16/32}^-$ HSC.

In *c-Myb* mutants, where primitive hematopoiesis is preserved but HSC-derived hematopoiesis is lacking, YS-derived primitive erythrocytes suffice to maintain living embryos up until E15.5 (**Mucenski et al., 1991; Tober et al., 2008; Schulz et al., 2012**). In the absence of HSC activity, EMP-derived hematopoietic cells maintain viable embryos throughout development up until birth (**Chen et al., 2011**).

YS hematopoiesis has long been considered a transient wave devoted to the production of erythrocytes, megakaryocytes and a few myeloid cells that ensure oxygenation and tissue

hemostasis. HSC-derived hematopoiesis was thought to replace YS-derived cells after HSC migrate to the FL at E10.5 (Palis, 2016). Recently, growing evidence endows the YS with the capacity to contribute to tissue-resident cells such as macrophages that persist throughout life (Gomez-Perdiguerro et al., 2015) and mast cells (Gentek et al., 2018) maintained up until birth. Primitive erythrocytes were also shown to persist throughout gestation (Fraser et al., 2007) and EMP-derived cells contribute to the erythrocyte compartment for more than 20 days upon transplantation (McGrath et al., 2015). Nonetheless, it has been difficult to establish the temporal relative contribution of EMP or HSC-derived progenitors to erythropoiesis because they share surface markers and transcriptional regulators and are therefore indistinguishable.

Here we report a large population of Kit⁺CD45⁻Ter119⁻ erythroid progenitors unique to FL, comprising >70% of E14.5 Ter119⁻CD45⁻ cells (>10% of FL cells). These are the most actively proliferating progenitors at early stages and rapidly progress in erythroid differentiation. These cells that require *c-Myb* expression originate from YS EMP as they are co-labeled with microglia in the Cdh5^{CreERT2} Rosa26^{YFP} and labeled in the Csf1r^{MerCreMer} Rosa26^{YFP} lineage-tracer models induced at E7.5 and E8.5, respectively. They persist through fetal life and are the major contributors to the RBC compartment.

We show that HSC do not contribute significantly to embryonic erythropoiesis by tracing Flt3⁺ progenitors or YFP expressing cells in Cdh5^{CreERT2} Rosa26^{YFP} induced at E10.5 embryos. HSC-derived erythroid progenitors require more than 10-fold higher concentrations of EPO than their YS-derived counterparts, for erythrocyte differentiation. The limiting amounts of EPO available in the embryo (Suzuki et al., 2011) result in a selective advantage of YS-derived over HSC-derived erythropoiesis.

A UNIQUE POPULATION OF KIT⁺ CELLS REPRESENTS THE MAJORITY OF FL TER119⁻CD45⁻ CELLS

We detected in FL, by flow cytometry, a large fraction of Kit⁺ cells (>50%) expressing neither Ter119 (specific for erythrocytes) nor CD45 (a pan-hematopoietic marker) (**Figure 18A, Supplementary Figure 5A**). Single-cell surface marker expression data from E14.5 FL cells was projected as tSNE1 vs tSNE2 (**Figure 18A**) and three major clusters were defined by the expression of CD324 on epithelial cells, CD31 on endothelial cells and Kit. Combined analysis of Kit expression together with CD24 further defined 3 populations in the Ter119⁻CD45⁻CD31⁻CD324⁻ compartment: Kit⁺CD24⁻ (hereafter called P1), Kit⁺CD24⁺ (P2) and CD24⁺Kit⁻ (P3) cells (**Figure 18B; Supplementary Figure 5A**). P1 cells decreased while P2, and subsequently P3 cells increased as gestation progressed (**Figure 18C**). Numbers of P2 cells reached a maximum (around 10⁶ cells per FL) at E14-15 and decreased thereafter, although they were still detected around birth (E18.5) (**Figure 18D**). Kit⁺CD45⁻Ter119⁻Lin⁻ (P1

and P2) cells were also negative for the expression of Sca1 that marks multipotent progenitors, for CD16/32 that marks GM progenitors and for CD34 marking CMPs and therefore they fall in a gate that typically defines MEPs in the FL and the BM (**Supplementary Figure 5B**). Unlike their BM counterparts however, where all Kit⁺ cells co-expressed CD45, most FL Kit⁺ (P1 and P2) cells within the LK compartment did not express CD45 (**Figure 18D**) raising the possibility that they did not belong to the hematopoietic lineage. We, therefore, identified a major population of Kit⁺CD45⁻Ter119⁻ cells unique to FL of undefined lineage affiliation and origin.

P1 AND P2 CELLS IN THE FL HAVE AN ERYTHROID PROGENITOR SIGNATURE

RNA sequencing of the three major populations P2, CD324⁺ and CD31⁺ cells indicated that the highest expressed transcripts in P2 cells were *Myb*, *Bcl11a*, *Klf1*, *Gata1* and *Epor*, which are associated with erythrocyte differentiation (**Figure 18E**). The 122 genes upregulated >2-fold in P2 vs CD324⁺ cells were subjected to gene ontology analysis using *Enrichr* (**Chen et al., 2013**) (List of submitted genes in **Supplementary Table 2**). The top biological processes and tissue-associated genes revealed an erythrocyte/erythroblast profile (**Figure 18F**). These results were validated by Q-RT-PCR indicating that *Gata1*, *Lmo2*, *Klf1* and *Epor* expressions gradually increased from P1 to P3 cells, with the latter showing comparable expression levels of these transcripts to Ter119⁺ erythroblasts (**Figure 18G**). Hemoglobin transcripts for *Hbb-y*, *Hbb-bh1* and *Hbb-b1* were detected in P3 cells but only significantly expressed in Ter119⁺ cells. Multipotent hematopoietic associated transcription factors such as *c-Myb*, *Runx3* and *Bmi1* decreased as the erythroid-specific transcripts increased. The results above indicated that the CD45⁻Kit⁺ subsets (P1 and P2) are erythroid progenitors and suggested a hierarchy where immature P1 cells further differentiate into P2 and later lose Kit expression (P3) before acquiring Ter119 expression, the definitive marker of erythroid identity.

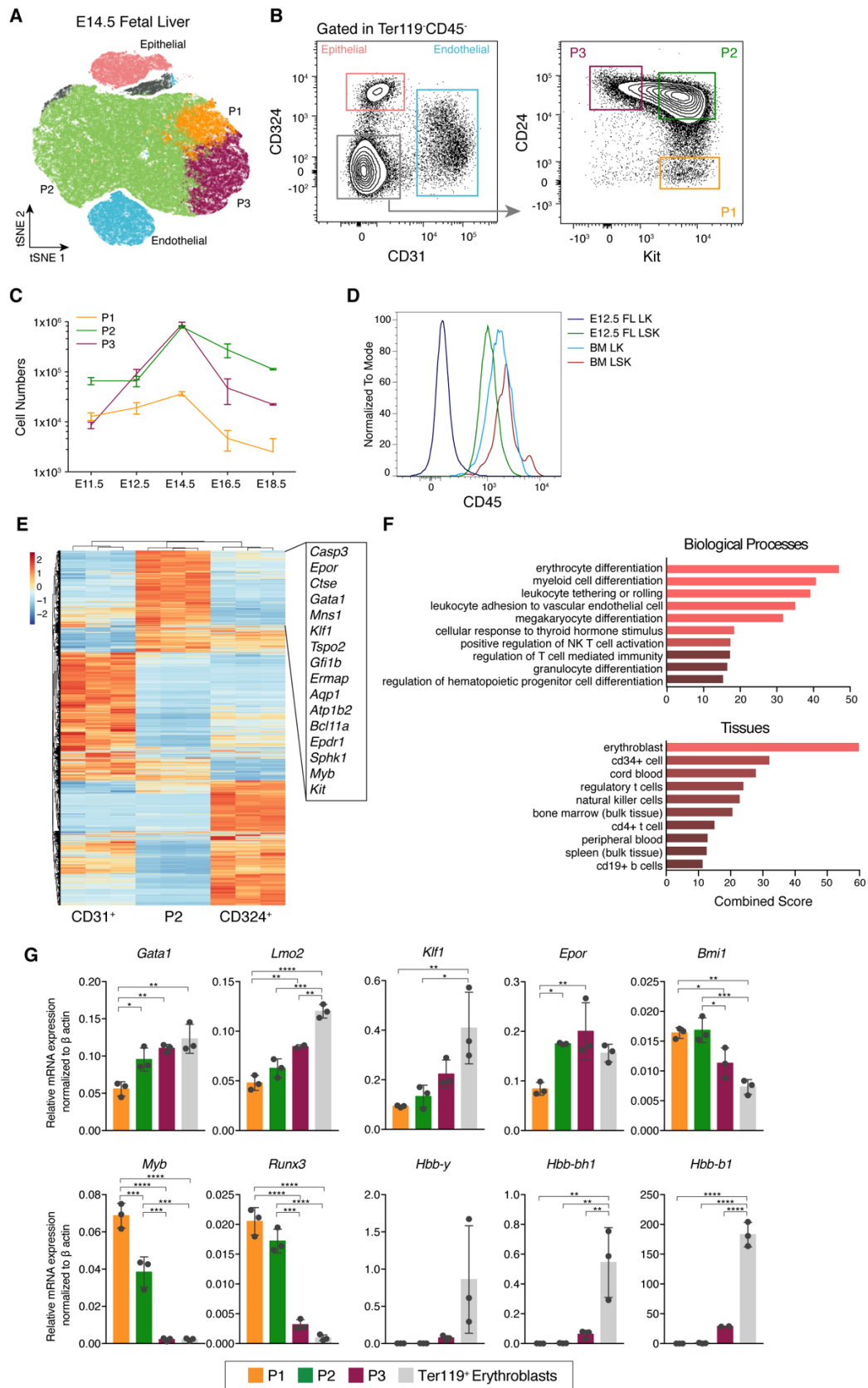


FIGURE 18 A POPULATION OF KIT⁺ CELLS UNIQUE TO FL REPRESENTS THE MAJORITY OF TER119⁺CD45⁻ CELLS AND HAS AN ERYTHROID PROGENITOR TRANSCRIPTIONAL SIGNATURE

(A) t-SNE analysis and hierarchical clustering of flow cytometry data of Ter119⁺CD45⁻ cells from E14.5 fetal livers stained with the surface markers CD31 (endothelial cells), CD324 (epithelial cells),

hepatoblasts), Kit and CD24. **(B)** Representative FACS plots of the clusters identified in **(A)** using the same color code. **(C)** TER119⁺CD45⁻ cell numbers from E12.5 and E18.5 **(D)** Histogram of CD45 expression in Lin⁻Kit⁺Sca1⁻ (LK) and Lin⁻Kit⁺Sca1⁺ (LSK) cells from E12.5 FL and adult BM. (E11.5 n=6, E12.5 n=17, E14.5 n=4, E16.5 n=8, E18.5 n=2). See also **Supplementary Figure 5**. **(E)** CD31⁺, P2 and CD324⁺ cells from E14.5 FL were sorted and subjected to RNA sequencing. Differentially expressed genes are represented as heatmap and most expressed genes are listed (n=3 independent litters). **(F)** Gene set enrichment analysis on genes with more than two-fold difference in expression level between P2 and CD324⁺ cells using *Enrichr* web-application; top 10 significantly associated GO Biological Processes and ARCHS4 Tissues are shown. Top biological process is erythrocyte differentiation (q-value <1.0e⁻⁶; Gene Ontology term GO:0030218) and top tissue associated is erythroblast (q-value <1.0e⁻¹⁴; ARCHS4 Tissues gene database). **(G)** E14.5 FL P1, P2, P3 and Ter119⁺ cells were sorted and gene expression of key erythroid genes (*Gata1*, *Lmo2*, *Klf1*, *Epor* and *Bmi1*), progenitor associated genes (*c-Myb* and *Runx3*) and hemoglobins (*Hbb-y*, *Hbb-bh1*, *Hbb-b1*) was analyzed. QPCR data were analyzed using the 2^{-ΔCt} method and normalized with *Actb*. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparison test **(G)** *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are represented as mean ± standard deviation from 3 independent experiments.

P1, P2 AND P3 CELLS REPRESENT INCREASINGLY MATURE STAGES WITHIN THE ERYTHROID LINEAGE

Erythroid differentiation has been characterized by the expression of CD71 (transferrin receptor) and CD44, in Ter119⁺ cells (**McGrath et al., 2017**). Imaging flow cytometry (**Figure 19A**) showed that the proerythroblast marker CD71 was low in P1, increased in P2 and highly expressed in all P3 cells (**Figure 19B**), indicating they correspond to consecutive stages of erythrocyte development.

In BM and FL, CFU-E are characterized as Kit⁺CD71⁺Ter119⁻ whereas low levels of Ter119 expression marks proerythroblasts that lost proliferative capacity (**McGrath et al., 2017**). P2 FL cells express CD71, but not Ter119, indicating that they correspond to CFU-E. P3 cells express low levels of Ter119 (**Figure 19B**) visible in 8% of them and express erythroid genes at levels similar to Ter119⁺ erythroblasts (**Figure 18G**) suggesting they correspond to proerythroblasts. CD71 expression is limited to P2 and P3 cells indicating that CD71 and CD24 are redundant markers in this context, further confirmed by conventional flow cytometry (**Supplementary Figure 5C**).

Administration of 3 doses of the nucleotide analog 5-ethynyl-2'-deoxyuridine (EdU) that labels newly synthesized DNA in E12.5 or E13.5 pregnant females (**Figure 19C**) indicated that P1 E13.5 FL cells are the highest proliferating cells (80% EdU⁺) compared to P2 and P3 cells (around 50% EdU⁺). E14.5 FL cells show the same level of EdU incorporation (40-50%) in all 3 subsets (**Figure 19D-E**). Cell proliferation was further assessed by analyzing the expression of the nuclear protein Ki-67 that in association with DAPI allows distinguishing cells in G0, G1 and G2M phases of the cell cycle. Consistent with the EdU labeling experiments, P1 showed the lowest frequency of cells in G0 (~10%) and the highest frequency of cells expressing Ki-67, from which around 20% were actively synthesizing DNA (DAPI⁺) (**Figure 19F**). By contrast, P3 cells show the lowest frequency of proliferating cells (**Figure 19F**). Taken together these results indicated that P1 are the most proliferating cells and, as they transit onto the P2 and further into the P3 subset, lose proliferative activity.

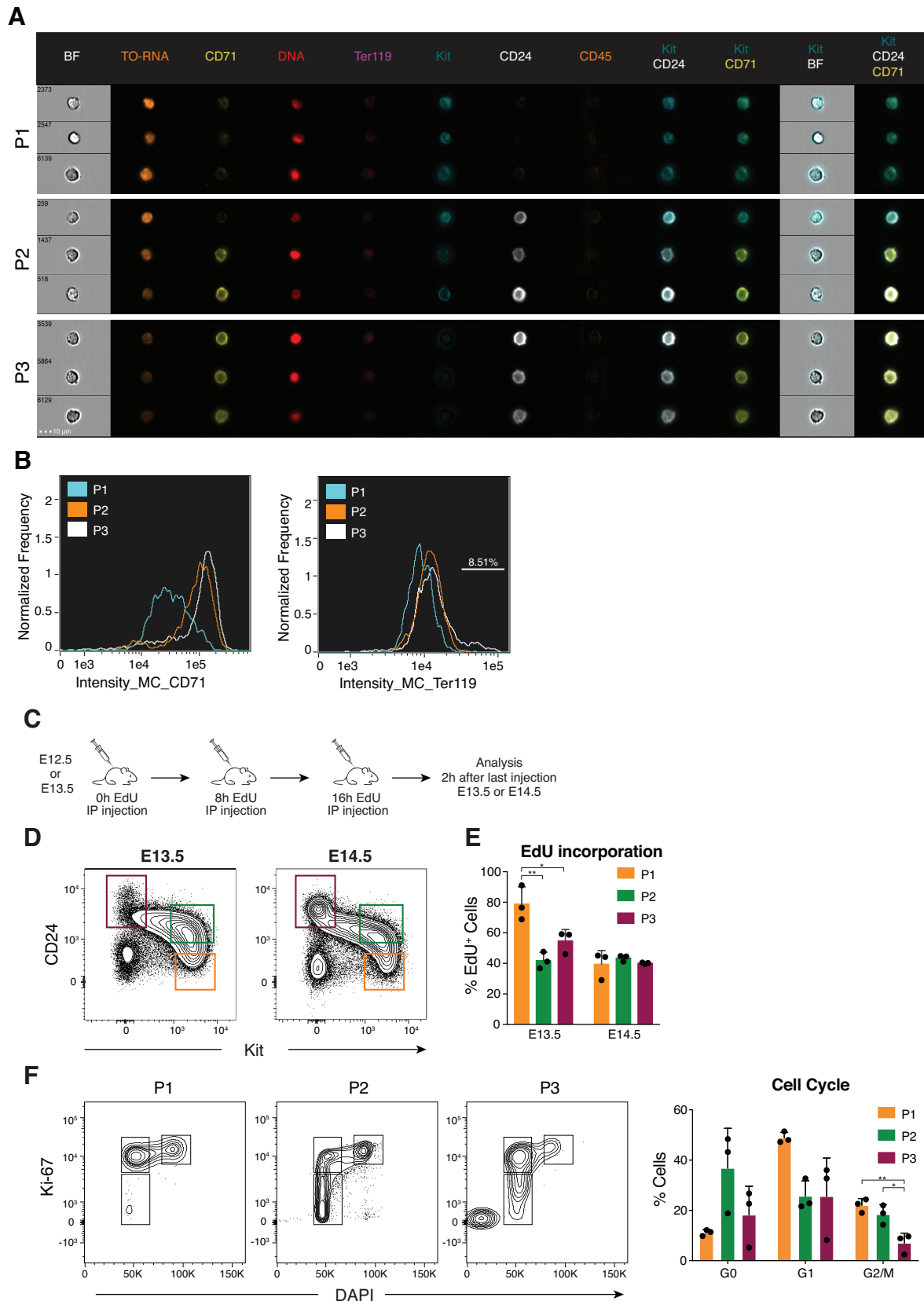


FIGURE 19 P1, P2 AND P3 CELLS REPRESENT INCREASINGLY MATURATION STAGES WITHIN THE ERYTHROID LINEAGE

(A) E13.5 Ter119⁺ CD45⁺ cells were analyzed by imaging flow cytometry using CD71, Ter119, Kit, CD24 and CD45 as surface markers, DRAQ5 to label nuclei and Thiazole Orange (TO) to label RNA. Representative images of P1, P2 and P3 cells. (B) Expression of CD71 and Ter119 was assessed in

P1, P2 and P3 cells and plotted as a histogram. **(C)** Experimental design of cell cycle analysis using EdU labeling. E12.5 or E13.5 pregnant mice were injected intraperitoneally with 100 μ g of EdU at 0h, 8h and 16h. Fetal livers were collected 2h after the last injection and EdU expression was analyzed on Ter119⁺CD45⁺CD54⁺CD31⁻ cells using the indicated gates **(D)**. **(E)** Percentages of EdU incorporation in P1, P2 and P3 cells at E13.5 and E14.5 (n=3). **(F)** Cell cycle analysis of E14.5 fetal liver cells using Ki-67 and DAPI. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparison test **(C)** *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are represented as mean $\pm$ standard deviation from 3 independent experiments.

P2 AND P3 CELLS ARE COMMITTED ERYTHROID PROGENITORS WHEREAS P1 RETAIN RESIDUAL MYELOID POTENTIAL

To assess the differentiation potential of CD45⁺Kit⁺ FL cells we performed differentiation assays in liquid cultures and semi-solid colony assays **(Figure 20A-B)**. Limiting-dilution analysis showed that P1 and P2 gave rise to hematopoietic colonies at frequencies similar to that of LK (Lin⁻CD45⁺Kit⁺Sca1⁻) cells (1 in 1 for LK and P1, 1 in 2 for P2 cells) while P3 cells did not divide significantly in culture (less than 1 colony in 2592 wells analyzed) **(Figure 20A)**. Both P1 and P2 cells generated a majority of CFU-E (more than 50% of plated cells) in semi-solid colony assays. P1 cells generated also BFU-E (<5%), CFU-M (5%) and CFU-Mk (5%) whereas control (LK) generated a majority of myeloid colonies (CFU-G, CFU-GM and CFU-GEMM) **(Figure 20B)**.

To address the lineage relationship between P1 and P2 cells we performed liquid culture differentiation assays. After 6 and 12 hours in culture, 15% and 40% of P1 cells had upregulated CD24 expression, respectively **(Figure 20C)**. After 24h in culture, all P1 cells had differentiated to P2 cells, analyzed by upregulation of CD24 and loss of Kit expression **(Figure 20C)**. While P1 cells started to upregulate the definitive erythroid marker Ter119 after 48 hours in culture, P2 cells had already started to express this marker after 18h of culture, demonstrating that they represent a more committed progenitor **(Figure 20D-F)**.

To probe their differentiation potential *in vivo* we injected P2 cells purified from E13.5 UBC-GFP embryos into E13.5 C57/BL6 recipient embryos, *in utero* **(Figure 20G)**. FL and blood collected three days later indicated that GFP⁺P2 originated exclusively GFP⁺Ter119⁺ cells whereas LK generated a majority of myeloid cells detected both in FL and in the blood **(Figure 20H-I)** while none gave rise to lymphocytes.

These results demonstrated that P2 FL cells are committed erythroid progenitors while P1 cells retain residual *in vitro* myeloid differentiation potential.

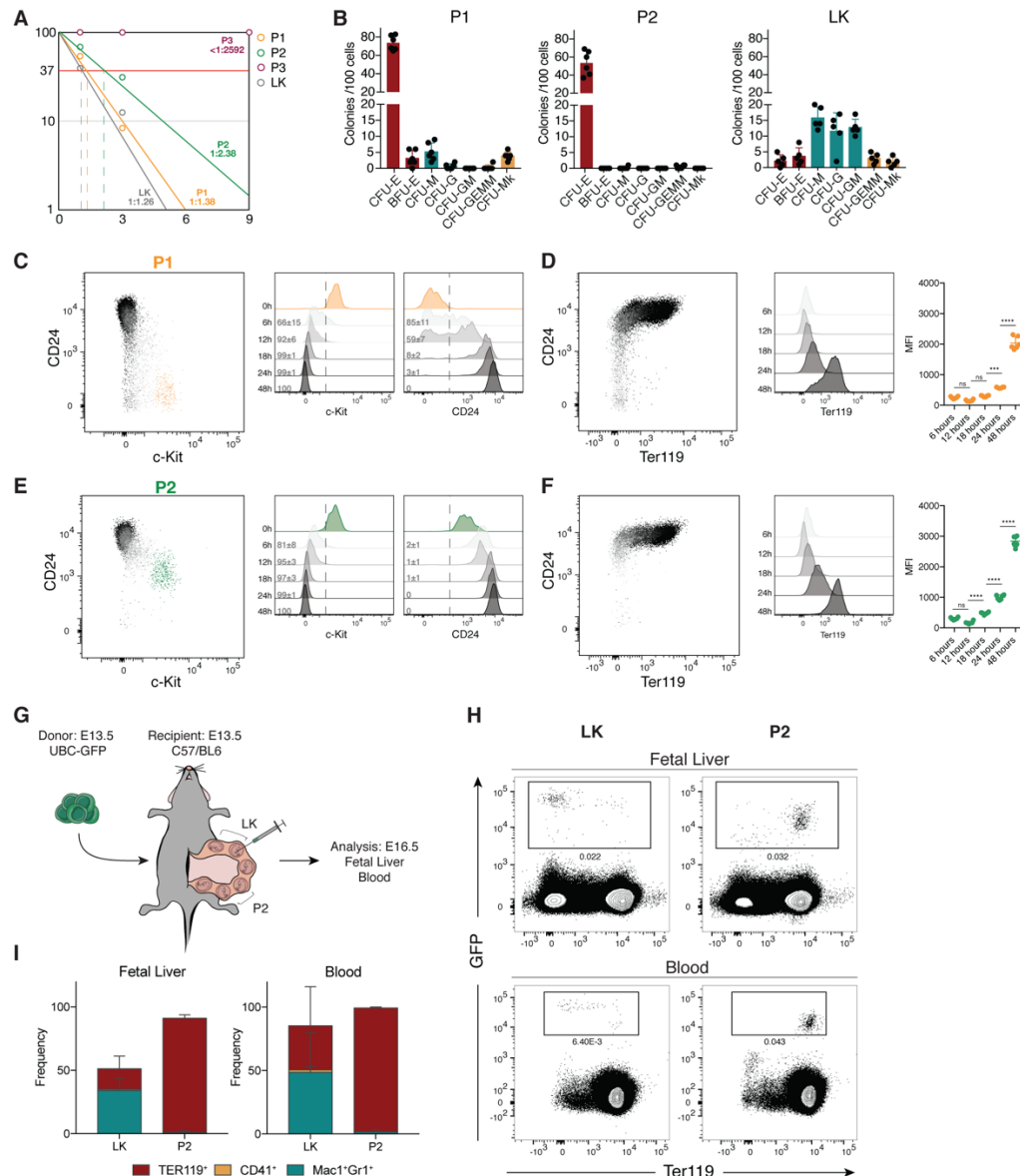


FIGURE 20 P2 AND P3 CELLS ARE COMMITTED ERYTHROID PROGENITORS WHEREAS P1 RETAIN RESIDUAL MYELOID POTENTIAL

(A) Frequencies of colony-forming cells in P1, P2, P3 and Lin⁻ CD45⁺ Kit⁺ (LK) cells from E13.5 FL (n= 288 wells from 3 independent experiments of each population). (B) *In vitro* lineage potential of E13.5 P1, P2, P3 and LK cells in semi-solid cultures. CFU-E colonies were quantified at 3 days and BFU-E, CFU-M, CFU-G, CFU-GM, CFU-GEMM and CFU-Mk at 7 days of culture (n= 6, 3 independent experiments). (C) Representative FACS plots and histograms showing upregulation of CD24 and loss of Kit after culture of P1 cells. (D) Representative FACS plots and histograms of upregulation of Ter119 and respective mean fluorescence intensity (MFI) analysis of P1 cells after culture. (E) Representative FACS plots and histograms showing maintenance of CD24 and loss of Kit after culture of P2 cells. (F) Representative FACS plots and histograms of upregulation of Ter119 and respective mean fluorescence intensity (MFI) analysis of P2 cells after culture. (G) Schematic representation of transplantation experiment. E13.5 C57/BL6 pregnant females were anesthetized, the peritoneal cavity was opened, and the uterus exposed. Embryos were injected intraperitoneally with 20000 E13.5 GFP⁺ purified cells from UBC-GFP embryos. FL and blood were collected 3 days post-injection. (H) Representative FACS plots showing the erythroid contribution of GFP⁺ cells in fetal liver and blood after injection of P2 or LK cells and quantification (I) (P2 n=3, LK n=4, 4 independent experiments). Data are represented as mean $\pm$ standard deviation.

P1/P2 PROGENITORS REQUIRE C-MYB EXPRESSION

The transcription factor *c-Myb* is essential for definitive hematopoiesis and *Myb*^{-/-} embryos have impaired definitive hematopoiesis that affects all lineages and are not viable after E15. Only primitive YS-derived erythropoiesis, primitive megakaryocytes (Tober et al., 2008) and tissue-resident macrophages (Schulz et al., 2012) are found in *c-Myb* mutants. Ter119⁺ cells were drastically decreased in frequency and numbers in *Myb*^{-/-} FLs when compared to heterozygous littermates (Figure 21A-B). P1 cells were undetectable in *Myb*^{-/-} while P3 cells were present, albeit in reduced numbers (Figure 21B). CD24⁺ cells in *Myb*^{-/-} FL expressed high levels of *Afp* and *Alb* indicating their hepatic cell affiliation (Supplementary Figure 5D). *c-Myb* was reported to regulate Kit expression (Ratajczak et al., 1998). To assess whether P1 and P2 cells, although unable to express Kit, were nonetheless present in the *Myb*^{-/-} FL we analyzed the expression of erythroid genes in CD24⁻, CD24⁺ and Ter119⁺ cells from *Myb*^{+/-} and *Myb*^{-/-} FLs. *Epor*, *Tal1* and *Klf1* were not detected in CD24⁻ and CD24⁺*Myb*^{-/-}, compared to *Myb*^{+/-} cells (Figure 21C). Only Ter119⁺ cells expressed detectable levels of *Epor* and *Tal1* together with high levels of *Hbb-y* indicating they represent primitive erythrocytes. Of note, *Klf1*, a transcription activator of the β -globin promoter, was not expressed in primitive Ter119⁺*Myb*^{-/-} cells (Figure 21C). These results demonstrated that differentiation and/or survival of CD45⁺Kit⁺ erythroid progenitors required the transcription factor *c-Myb*.

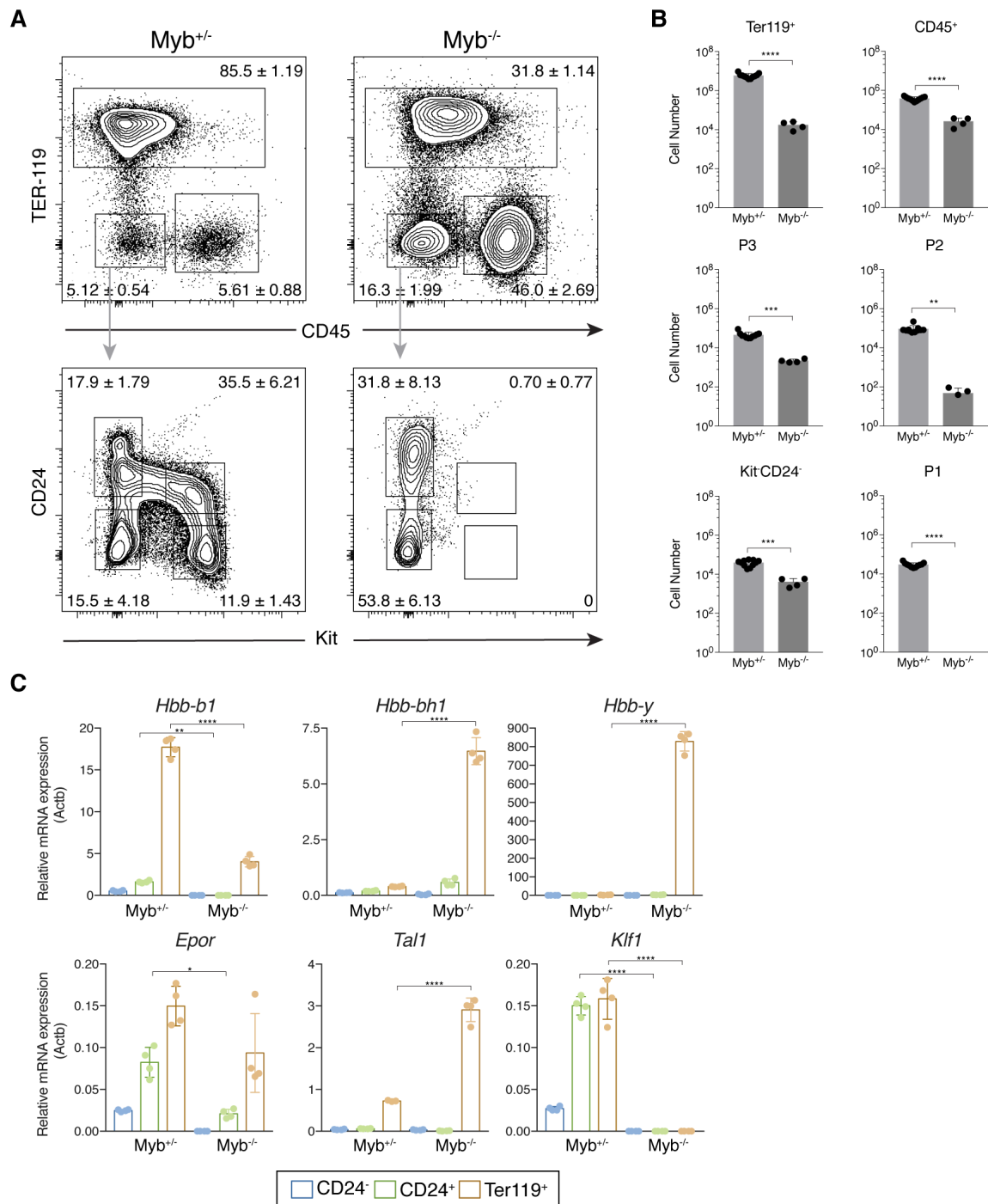


FIGURE 21 P1/P2 PROGENITORS REQUIRE C-MYB EXPRESSION

(A) Representative FACS plots showing percentages of Ter119 and CD45 (top panel) and Kit and CD24 (lower panel) expressing cells in *c-Myb*^{+/+} and *c-Myb*^{-/-} E14.5 FL and corresponding absolute numbers (B). (C) Expression of hemoglobin (*Hbb-b1*, *Hbb-bh1* and *Hbb-y*) and key erythroid genes (*Epor*, *Tal1* and *Klf1*) in CD24⁻, CD24⁺ and Ter119⁺ cells were analyzed by qPCR. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparison test **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001. Data are represented as mean ± standard deviation from 2 independent experiments.

P1/P2 CELLS ORIGINATE FROM YOLK SAC AND ARE MAJOR CONTRIBUTORS TO EMBRYONIC ERYTHROPOIESIS

P1/P2 (CD45⁺Kit⁺) cells were not detected in the adult BM, suggesting that they represent a transient hematopoietic population. To assess the origin of P1/P2 cells we analyzed FL from Csf1r^{MeriCreMer}Rosa26^{YFP} pregnant females pulsed with a single dose of OH-TAM at E8.5 (**Figure 22A**) that marks tissue-resident macrophages but virtually no HSC-derived progenitors (**Gomez-Perdiguero et al., 2015**). At E11.5, P1 and P2 cells were labeled to comparable levels of those in the microglia, taken as positive control (**Figure 22B**; **Supplementary Figure 6D**). In subsequent days the frequency of YFP⁺ cells in more differentiated P3 and erythroblast (Lin⁺CD71⁺) increased whereas that of more immature P1 decreased. In line with previous reports YFP⁺ LSK cells were undetectable (**Figure 22B**; **Supplementary Figure 6**). The dynamic of YFP labeled erythroid progenitors is consistent with a progression in erythroid differentiation and indicates a lineage relationship between the 3 subsets. Moreover, the frequency of YFP labeled FL erythroblasts decreases from E12.5 until E14.5 while it increases in blood erythrocytes (**Figure 22C**). The frequency of YFP labeled P1 and P2 decreases between E11.5 and E13.5, a dynamic that reflects a fast differentiation progression, culminating with the exit of erythroblasts from FL into circulation.

To test whether YFP⁺ and YFP⁻ cells represented two distinct populations we performed single-cell multiplex gene expression analysis in FL P1, P2 and P3 cells of Csf1r^{MeriCreMer}Rosa26^{YFP} embryos pulsed at E8.5. Unsupervised hierarchical clustering did not segregate YFP⁺ from YFP⁻ cells for the expression of progenitor, erythroid and myeloid transcripts indicating that they have a similar profile and therefore do not represent two divergent progenitor populations (**Figure 22D**). Cluster I and IV contained P1 cells characterized by the expression of *Gata1*, *Lmo2* and *c-Myb*. Cluster I differed from Cluster IV by the high frequency of cells expressing *Epor*, *Tal1*, *Klf1* and *Ki-67*. Interestingly, some cells in this cluster also co-expressed the myeloid factors *Runx1*, *Gata2*, *Zfp1* and *Mpl*, suggesting a broad myeloid transcriptional priming, consistent with data from *in vitro* differentiation assays (**Figure 20B**). High expression of *Ki-67* also indicates that Cluster I in contrast to Cluster IV are proliferating. Few cells segregated from all other in Cluster II defined by expression of *Csf3r*, *Ly6c* and *Runx2* in the absence of erythroid associated transcripts. Cluster III comprises a majority of P2 cells, expressing high levels of erythroid genes and low levels of hemoglobin, thus defining a transitional erythroid population. Cluster V contains P3 cells that express high levels of hemoglobin in the absence of *c-Kit* or *c-Myb*.

To analyze the differentiation trajectory between the 3 populations we generated a diffusion map and obtained a trajectory in which P1 cells progress through a P2 and subsequently a P3 stages (**Figure 22E**). This differentiation trajectory is in line with the gene

expression data (**Figure 18G**), with the imaging flow cytometry results (**Figure 19A**) and with the clonal differentiation assays (**Figure 20B**). YFP⁺ and YFP⁻ cells have similar trajectories indicating they do not represent different progenitor subsets (**Figure 22E**).

The Csflr^{MerCreMer}Rosa26^{YFP} lineage-tracing model induced at E8.5 labels Csflr expressing EMPs and macrophages. To complement the results above and minimize the possibility of disregarding the contribution of Csflr⁻ YS-progenitors, we used the Cdh5^{CreERT2}Rosa26^{YFP} model that, with a single dose of OH-TAM at E7.5, efficiently labels YS hematopoiesis (**Zovein et al., 2008; Gentek et al., 2018**) (**Figure 22F; Supplementary Figure 7B**). P2, P3 and erythroblasts are labeled at the same frequency as microglia at E12.5 and E14.5 and decrease thereafter (**Figure 22G**), similar to the results obtained with Csflr^{MerCreMer}Rosa26^{YFP} embryos. While YFP⁺ FL erythroblasts decrease from E12.5 to E18.5, blood erythroblasts increase (**Figure 22H**), indicating that they exit the FL and enter circulation. Moreover, the frequency of YFP⁺ blood erythrocytes is similar to that found in microglia (**Figure 22I**), establishing that circulating erythrocytes of EMP YS origin dominate the compartment up until E18.5.

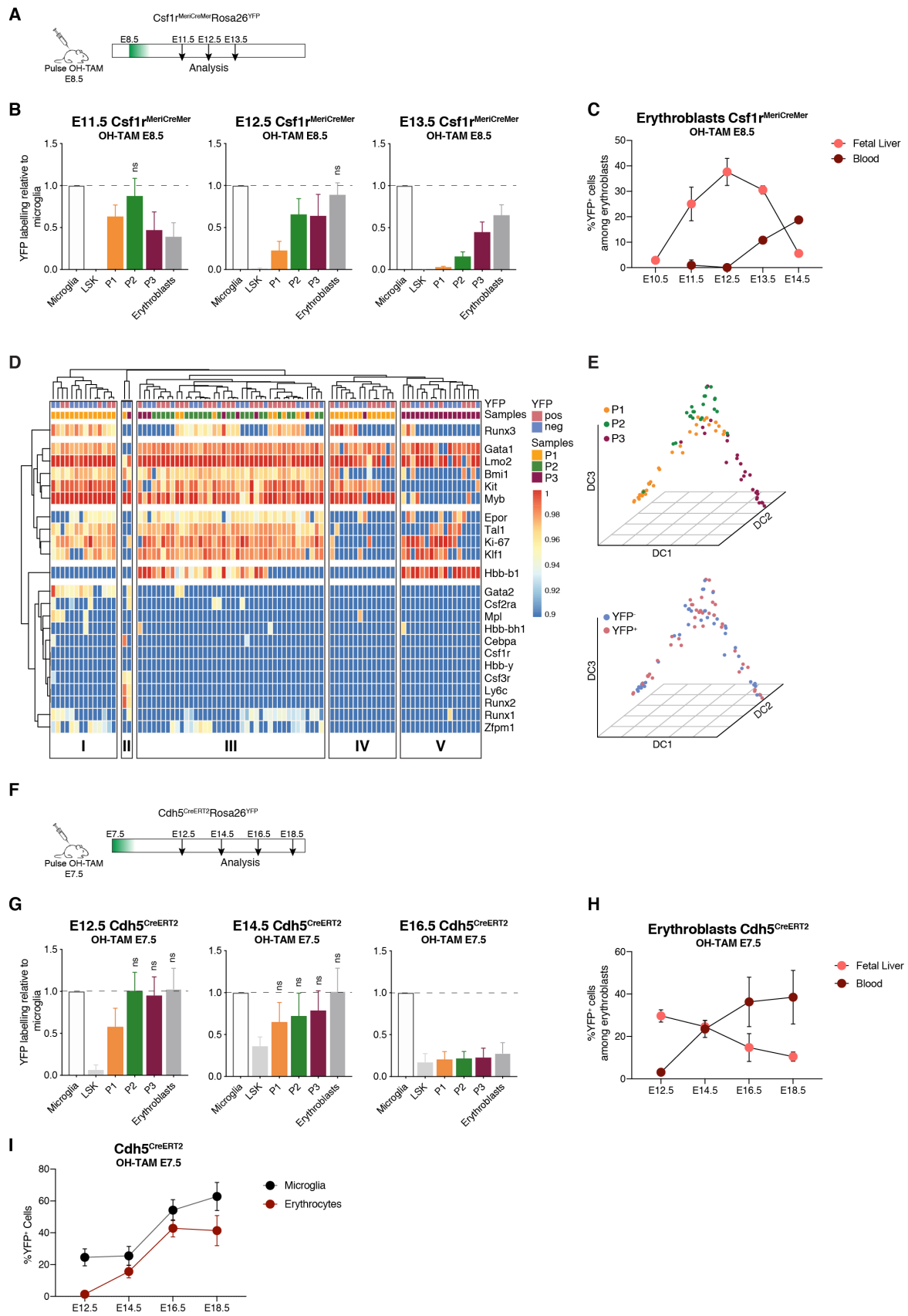


FIGURE 22 P1/P2 CELLS ORIGINATE FROM YOLK SAC AND ARE MAJOR CONTRIBUTORS TO EMBRYONIC ERYTHROPOIESIS

(A) Experimental design for lineage tracing experiments using the $Csf1^{MerCreMer}Rosa26^{YFP}$. Cre recombinase expression was induced in $Csf1^{MerCreMer}Rosa26^{YFP}$ pregnant females with a single injection of OH-TAM at E8.5 and embryos were analyzed 3, 4 and 5 days after injection. (B) Ratio of YFP⁺ LSK cells and the different erythroid subsets over that in microglia to at E11.5, E12.5 and E13.5 after a pulse of OH-TAM at E8.5 (E11.5 n=12, E12.5 n=11, E13.5 n=7). (C) Frequency of YFP⁺ erythroblasts in Fetal Liver (pink) and Blood (red) from E10.5 to E14.5 after a pulse of OH-TAM at E8.5 (E10.5 n= 3, E11.5 n=12, E12.5 n=11, E13.5 n=7, E14.5 n=2). (D) Heatmap of single-cell qPCR in sorted cells from $Csf1^{MerCreMer}$ E13.5 FL pulsed with OH-TAM at E8.5. Each column represents a single-cell and its color-coded according to YFP expression (YFP⁺ vs YFP⁻) and cell type (P1, P2, P3). Gene expression was normalized to *Actb* and *Gapdh* and unsupervised hierarchical clustering was performed. (E) Diffusion map of the 3 populations based on single-cell gene expression. Top panel is color-coded according to cell type and bottom panel according to YFP expression. (A total of 87 cells were analyzed). (F) Experimental design for lineage tracing experiments using the $Cdh5^{CreERT2}Rosa26^{YFP}$. Cre recombinase expression was induced in $Cdh5^{CreERT2}Rosa26^{YFP}$ pregnant females with a single injection of OH-TAM at E7.5 and embryos were analyzed 5, 7 and 9 days after injection. (G) Ratio of YFP⁺ LSK and erythroid cells over those in microglia at E12.5, E14.5 and E16.5 after a pulse of OH-TAM at E7.5 (E12.5 n=4, E14.5 n=5, E16.5 n=6). (H) Percentage of YFP⁺ erythroblasts in Fetal Liver (pink) and Blood (red) at E12.5, E14.5 and E16.5 after a pulse of OH-TAM at E7.5 (I) YFP⁺ Microglia and Blood Erythrocytes at E12.5, E14.5, E16.5 and E18.5 after a pulse of OH-TAM at E7.5. Microglia (CD45⁺F4/80⁺CD11b⁺) cells were used as controls (E12.5 n=4, E14.5 n=5, E16.5 n=11, E18.5 n=4). Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparison test. Data are represented as mean $\pm$ standard deviation. See also **Supplementary Figure 6** and **Supplementary Figure 7**.

HSCS DO NOT CONTRIBUTE TO ERYTHROPOIESIS UP UNTIL BIRTH

To evaluate HSC contribution to fetal erythropoiesis we analyzed $Flt3^{Cre}Rosa26^{YFP}$ embryos where HSC-derived multipotent progenitors (MPP $Flt3^{+}$) and their progeny are YFP⁺ (**Benz et al., 2008; Buza-Vidas et al., 2011**) in addition to a transient population of $Flt3^{+}$ progenitors recently identified (**Beaudin et al., 2016**) (**Figure 22E**). Less than 2% of microglial cells are YFP⁺ at all time-points analyzed. LSK were increasingly labeled with >30% of YFP⁺ cells at E16.5 and reaching >40% at E18.5 (**Supplementary Figure 8D**). Both CMP and GMP compartments exhibited similar levels of YFP expression to those in LSK (**Figure 23B**). By contrast, P1+P2 or P3 cells were minimally labeled with YFP by E18.5. YFP⁺ erythroblasts were virtually undetectable at E16.5 and were less than 20% of that found in LSK by E18.5, indicating that HSC are not contributing to mature erythrocytes up until one day before birth. MEP showed a delayed profile, reaching 50% of LSK labeling only around birth.

Most erythrocytes are derived from MEP that differentiate from FLT3 expressing MPP. However, recent evidence indicated that a fraction of megakaryocytes might bypass the MPP stage and differentiate directly from a progenitor phenotypically indistinguishable from HSC (**Carrelha et al., 2018**). These results raised the possibility that also erythrocytes may bypass the MPP stage because they originate from a common MEP progenitor. Using the $Flt3^{Cre}Rosa26^{YFP}$ mouse model we could therefore underestimate the contribution of HSC to the erythrocyte compartment. To address this potential problem, we analyzed

Cdh5^{CreERT2}Rosa26^{YFP} embryos induced at E10.5, thus specifically labeling the AGM-hemogenic endothelium and all HSC-derived hematopoiesis (**Zovein et al., 2008**) (**Figure 23C, Supplementary Figure 9B**). We obtained similar results with E10.5 induced Cdh5^{CreERT2}Rosa26^{YFP} as with Flt3^{Cre}Rosa26^{YFP} with a ratio of 1 YFP labeled CMP over LSKs at E16.5, in contrast to ratios of 0.5 labeled MEPs or P1+P2 cells and 0.1 in erythroblasts. Analysis at E18.5 showed ratios of CMPs, GMPs, MEPs or P1+P2 cells close to 1, while erythroblasts were still below 0.5. Further comparison of embryos pulsed with OH-TAM at E7.5 vs E10.5 showed similar contribution to non-erythroid lineages (CD45⁺Kit⁻ cells) of YS and HSC, at E18.5 (**Figure 23F**), whereas the erythroid compartment is mostly derived from YS hematopoiesis (**Figure 23E-F**). Taken together these observations indicated that neither HSC-derived nor any other FLT3 expressing progenitor contribute significantly to erythropoiesis throughout fetal life.

FL stroma produces Epo, essential for erythrocyte production, albeit at lower concentrations than kidney, the adult source of Epo (**Suzuki et al., 2011**). Embryonic progenitors have been reported to react to lower concentrations of Epo than their adult counterparts (**Rich et al., 1980**). We compared erythrocyte production from P2 of YS origin with that from CD45⁺ MEP of HSC origin from the same FL (**Figure 24A**). HSC-derived MEP were about two-fold less efficient in generating erythrocytes than YS-derived P2 at limiting levels of Epo and required more than ten-fold higher concentrations to reach 50% of erythrocyte colony formation. P2 cells exhibited higher sensitivity to Epo and both P1 and P2 cells express higher levels of Epo receptor than CD45⁺ CMPs or MEPs of HSC origin from the same FL (**Figure 24B-C**). *Gata1*, *Sp1* and *Tal1* transcripts that regulate *Epor* expression (**Zon et al., 1991; Feng et al., 2005; Rogers et al., 2012**), were increased in P1 and P2 when compared to CD45⁺ CMPs and CD45⁺ MEPs, respectively (**Figure 24B-C**). These results provide experimental evidence for the mechanism controlling the selection of YS-derived over HSC-derived progenitors in fetal erythropoiesis.

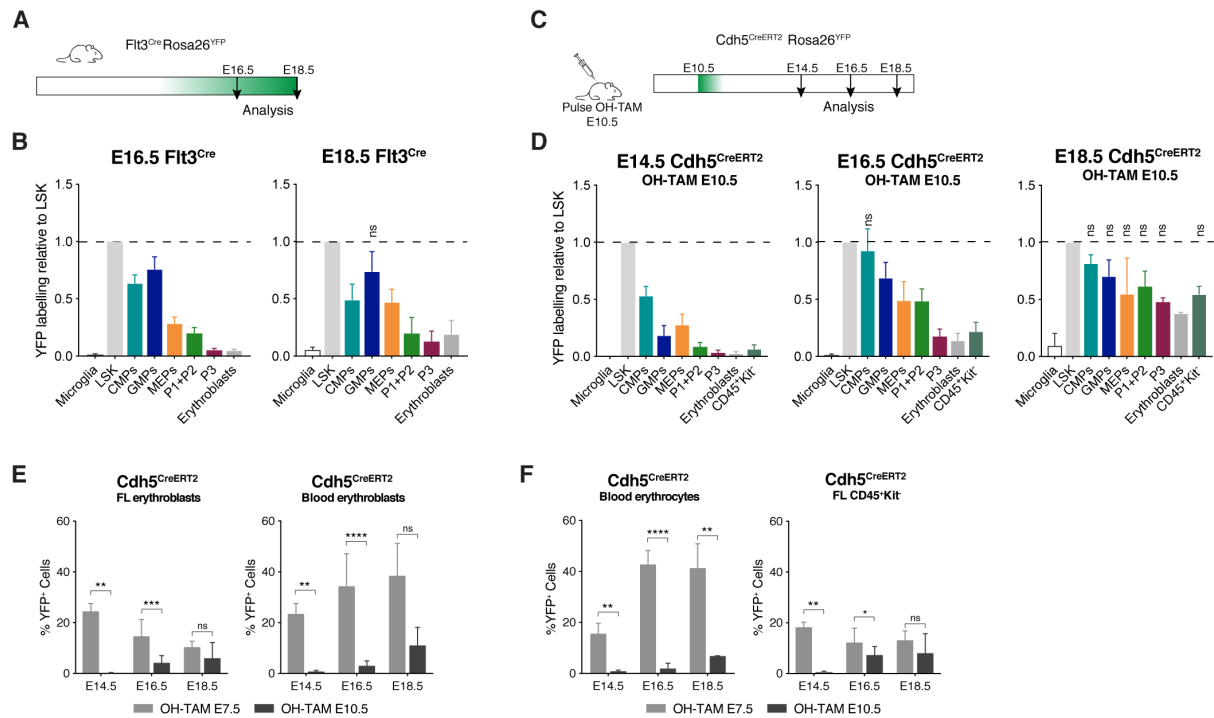


FIGURE 23 HSCs DO NOT CONTRIBUTE TO ERYTHROPOIESIS UP UNTIL BIRTH

(A) Experimental design for lineage tracing experiments using the $Flt3^{Cre}Rosa26^{YFP}$ mice model. Embryos were analyzed at E16.5 and E18.5. **(B)** Ratio of YFP⁺ designated hematopoietic subsets over those in LSK at E16.5 and E18.5 (E16.5 n=3, E18.5 n=4). **(C)** Experimental design for lineage tracing experiments using the $Cdh5^{CreERT2}Rosa26^{YFP}$ mouse model. Cre recombinase expression was induced in $Cdh5^{CreERT2}Rosa26^{YFP}$ pregnant females with a single injection of OH-TAM at E10.5 and embryos were analyzed 4, 6 and 8 days after injection. **(D)** Ratio of YFP⁺ hematopoietic subsets over those in LSK at E14.5, E16.5 and E18.5. **(E)** Percentage of YFP⁺ erythroblasts in Fetal Liver (left) and Blood (right) at E14.5, E16.5 and E18.5 after a pulse of OH-TAM at E7.5 or E10.5. **(F)** Percentage of YFP⁺ erythrocytes in Blood (left) and CD45⁺Kit⁺ cells in Fetal Liver (right) at E14.5, E16.5 and E18.5 after a pulse of OH-TAM at E7.5 or E10.5. (OH-TAM E7.5: E14.5 n=5, E16.5 n=11, E18.5 n=4; OH-TAM E10.5: E14.5 n=7, E16.5 n=9, E18.5 n=2). LSK (Lin⁺CD45⁺Sca1⁺Kit⁺) cells were used as controls. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparison test or Mann-Whitney test when appropriate, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are represented as mean ± standard deviation. See also **Supplementary Figure 8** and **Supplementary Figure 9**.

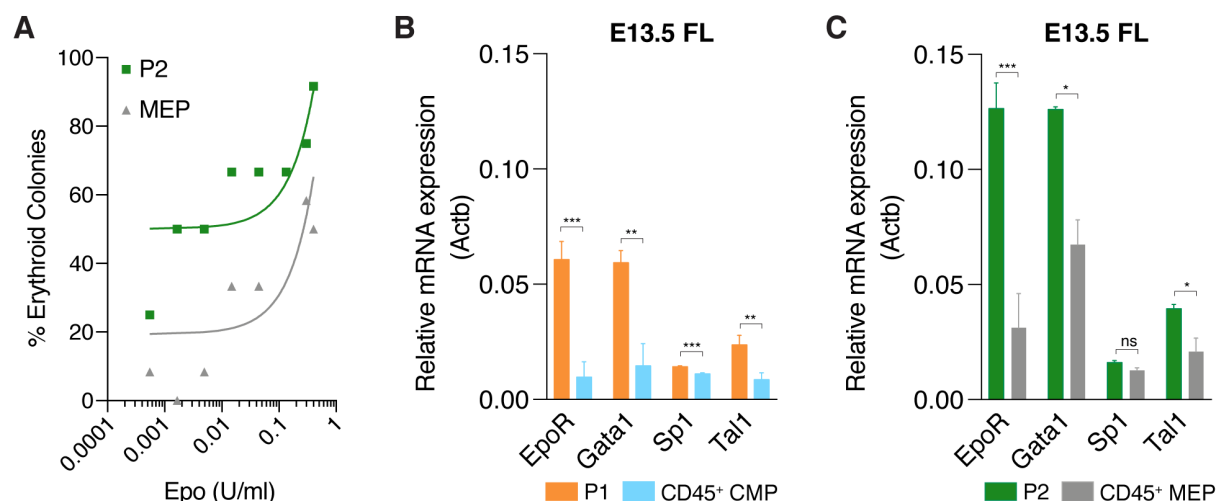


FIGURE 24 P1/P2 CELLS HAVE HIGHER SENSITIVITY TO ERYTHROPOIETIN THAN HSC DERIVED PROGENITORS

(A) Frequency of erythroid colonies in P2 and MEP (CD45⁺) cells from E13.5 FL using serial dilutions of erythropoietin. Representative plot of 3 independent experiments. Curves represent the linear regression of the data. (B) Quantitative RT-PCR analysis of the expression levels of *EpoR* and its regulators *Gata1*, *Sp1* and *Tal1* in P1 and CD45⁺ CMPs (n=3). (C) Quantitative RT-PCR analysis of the expression levels of *EpoR* and its regulators *Gata1*, *Sp1* and *Tal1* in P2 and CD45⁺ MEPs (n=3). In each experiment, cells were sorted from the same embryos. Statistical significance was assessed using paired t-tests, *p<0.05, **p<0.01, ***p<0.001. Data are represented as mean ± standard deviation.

DISCUSSION

Here we describe a population of CD45⁺Kit⁺ (P1/P2) hematopoietic cells unique to FL, found from E11.5 up until birth and that, at its peak (around E14.5), represent a major population comprising more than 70% of the CD45⁺Ter119⁺ FL cells and 10-15 % of total FL cells. *In vitro* analysis of their differentiation potential showed that they give rise to erythroid colonies (50-70%) at higher frequencies than adult BM MEPs (~15%) (**Akashi et al., 2000**). The majority of CD45⁺Kit⁺ cells express CD24, a marker found in immature hematopoietic and other cell types and mature erythrocytes, and up-regulated concomitantly with CD71, the transferrin receptor, a marker only found in erythroid progenitors (**Dong et al., 2011**).

Gene expression analysis and *in vitro* assays indicated a developmental progression where P1 cells further develop into P2 and later into P3 cells before acquisition of Ter119 expression. Both P1 and P2 cells contained high frequency of CFU-E and had increasing expression of the erythroid genes *Gata1* and *Epor*. By contrast, P3 cells did not generate colonies *in vitro* and expressed the erythroid transcripts *Gata1*, *Lmo2*, *Klf1* and *Epor* at levels similar to Ter119⁺ cells, stage at which they also express *Hbb-b1*, low levels of *Hbb-bh1* and undetectable *Hbb-y* transcripts, indicating they are within the definitive erythroid lineage. Of notice, P1 cells are among the most actively proliferative FL progenitors indicating that they can considerably expand before terminal differentiation.

YS-derived primitive erythrocytes and megakaryocytes (**Tober et al., 2008**) and tissue-resident macrophages are *c-Myb* independent, and so is EMP generation (**Schulz et al., 2012**). Accordingly, CD45⁺ cells present in the FL of *c-Myb* mutants representing tissue-resident macrophages were not affected (**Schulz et al., 2012**). Ter119⁺ cells, however, were drastically decreased in the FL of *c-Myb* mutants and P1 cells were undetectable (**Schulz et al., 2012**). Ter119⁺ cells in the *c-Myb*^{-/-} FL expressed embryonic globins ($\epsilon\gamma$ and $\beta h1$), consistent with their primitive origin and did not express *Klf1*, a transcriptional activator of the β -globin promoter essential for the transition from embryonic to adult hemoglobin expression (**Perkins et al., 1995, 2016**). *c-Myb* induces proliferation of erythroid progenitors and Kit expression and therefore HSC-independent erythroid cells are affected by *c-Myb* mutations (**Vegiopoulos et al., 2006**). Recently *c-Myb* was shown to control the expression of *Klf1* and *Lmo2* required for erythropoiesis, offering an explanation for the differential impact of *c-Myb* inactivation in the progeny of YS derived EMP (**Bianchi et al., 2010**). Single injections of OH-TAM at E8.5 in Csf1^{MerCreMer}Rosa26^{YFP} mark exclusively YS-derived cells and their progeny, among which is the microglia (**Gomez-Perdiguerro et al., 2015**). In these mice P1 and P2 cells are marked at levels similar to the microglia, three days after OH-TAM, indicating they are the progeny of YS EMPs. Consistent with the lineage relationship previously established, the frequency of labeled P1/P2 cells decreased with time after injection whereas the frequency of

labeled erythroblasts in FL and later in blood, increased. EMPs emerge in the YS between E8.5-10.5 and, a single injection of OH-TAM at E8.5, will reach the highest circulating levels of the drug 6 hours later, rapidly decreasing thereafter (**Zovein et al., 2008**). Only a fraction of EMP or already differentiated myeloid progeny that maintain *Csf1r* expression will be labeled with YFP. By contrast, differentiation into erythroid progenitors results in the loss of *Csf1r* expression, thus explaining the decreasing frequency of labeled immature erythroid progenitors with time. P1, P2, P3 and erythroblasts are labeled at similar levels to those found in microglia up until E14.5, timepoint after which labeling of erythroblasts and erythrocytes in blood increases. Similar results were obtained analyzing *Cdh5^{CreERT2}Rosa26^{YFP}* embryos, in which a pulse with OH-TAM at E7.5 labels the hemogenic endothelium giving rise to YS progenitors but not that generating HSCs (**Gentek et al., 2018**).

A kinetic that mirror images the one described above is found in *Flt3^{Cre}Rosa26^{YFP}* mice where *Flt3* expressing cells and their progeny are permanently labeled with YFP. By E16.5, where equivalent frequencies of LSKs, CMPs and GMPs are YFP⁺, only a small frequency of erythroid progenitors including MEPs and virtually undetectable frequencies of P3 or erythroblasts are labeled. By E18.5 MEPs were labeled at similar levels to those of CMPs although the erythroblast compartment still shows a modest contribution of *Flt3* expressing progenitors. Because lymphoid progenitors persistently express *Flt3* after commitment they are the highest labeled population in this model and were excluded from the analysis. It has been recently described that megakaryocyte/erythrocyte progenitors can bypass the stage of *Flt3* expressing MPP and would therefore be undetected in this model (**Carrelha et al., 2018**). *Cdh5^{CreERT2}Rosa26^{YFP}* embryos pulsed with OH-TAM at E10.5 where HSC-derived hematopoiesis is labeled (**Zovein et al., 2008**), exhibited a minor fraction of YFP⁺ erythroblasts or erythrocytes at any timepoint. By contrast, non-erythroid CD45⁺Kit⁺ mature populations in FL do not appear to be preferentially originated from one of the sources. We thus observed consistent results in both models and excluded the possibility of underestimating the contribution of HSCs to fetal erythrocytes due to the bypass of *Flt3*⁺ progenitors by megakaryocyte/erythrocyte progenitors.

HSCs in FL expand but also differentiate giving rise to multilineage progeny that comprise CMPs, GMPs, and lymphoid progenitors. However, our data shows that despite a rapid differentiation of FL HSCs, they do not contribute significantly to the erythroid compartment before birth and therefore *in vivo* embryonic HSC differentiation is skewed. The FL stromal microenvironment can sustain erythropoiesis and FL HSCs can differentiate into erythrocytes *in vitro*. We show that the low levels of Epo available in FL before the kidney is competent to produce adult levels of this hormone modulate the differentiation pattern of HSCs that do not produce MEPs and do not contribute to erythropoiesis (**Zanjani et al., 1981**).

Consistently, low levels of the EpoR regulator *Gata1*, result in embryonic anemia but normal adult erythropoiesis (**McDevitt et al., 1997**). Our observation that YS-derived erythrocyte progenitors express higher levels of *Gata1*, *Tal1* and consequently *Epor* is also consistent with an advantage of embryonic versus adult erythroid progenitors. Large numbers of expanding YS-derived erythrocyte progenitors efficiently outcompete HSC progeny in an environment where resources for erythroid differentiation are limiting.

These results reinforce the notion that in contrast to what has been accepted, YS hematopoiesis is not only devoted to providing oxygen to the embryo before HSCs differentiate in FL but rather sustain embryonic survival until birth. FL was shown to be a less efficient environment for cytokine production than BM. Recently, low levels of Interleukin 7 in FL were identified as the selective mechanism that resulted in autoreactive properties of B1 B cells (**Wong et al., 2019**). It is therefore tempting to speculate that low cytokine production is a general characteristic of FL cells with important implications for fetal hematopoiesis. In humans, kidney-derived Epo is also only produced after birth offering an explanation for why premature neonates develop severe anemia (**Widness, 2008**).

Consistently, a recent report analyzing human fetal liver hematopoiesis indicates that all cells in the erythrocyte lineage, similar to the observation in the mouse reported here, do not express CD45 at stages ranging from 7-17 weeks post-conception (**Popescu et al., 2019**).

Our observations may also help clarify why patients with mutations in *Epo* that alter the kinetics of receptor binding only show postnatal anemia (**Kim et al., 2017**). These observations suggest that fetal erythropoiesis originates in the YS, also in humans and will impact our understanding of embryonic hematopoiesis in general and in the pathogenesis of infant erythrocyte abnormalities.

MATERIALS AND METHODS

Mice

C57BL/6J mice were purchased from Envigo, Ubiquitin–GFP (**Schaefer et al., 2001**) mice used for transplantation experiments were a kind gift from P. Bousso (Pasteur Institute), $Myb^{-/-}$, $Csf1r^{MeriCreMer}$, $Flt3^{Cre}$ and $Rosa26^{YFP}$ and $Cdh5^{CreERT2}$ mice have been previously described (**Gomez-Perdiguero et al., 2015; Gentek et al., 2018**). 6-8-week-old mice or timed pregnant females were used. Timed-pregnancies were generated after overnight mating, the following morning females with vaginal plug were considered to be at E0.5. Recombination in $Csf1r^{MeriCreMer}Rosa26^{YFP}$ was induced by single injection at E8.5 of 75 µg per g (body weight) of 4-hydroxytamoxifen (OH-TAM) (Sigma, Cat# H7904), supplemented with 37.5 µg per g (body weight) progesterone (Sigma, Cat# P3972) as described (**Gomez-Perdiguero et al., 2015**). Recombination in $Cdh5^{CreERT2}$ was induced by single injection at E7.5 or E10.5 of 1.2mg of OH-TAM, supplemented with 0.6mg progesterone as described (**Gentek et al., 2018**). All animal manipulations were performed according to the ethic charter approved by the French Agriculture ministry and to the European Parliament Directive 2010/63/EU.

Cell suspension

E11.5-E18.5 FLs were dissected under a binocular magnifying lens. FLs were recovered in HBSS (Gibco, Cat# 24020-091) supplemented with 1% FCS (Eurobio, Cat# CVFSVF000U) and passed through a 26-gauge needle of a 1-ml syringe to obtain single-cell suspensions. Before staining, cell suspensions were filtered with a 100 µm cell strainer.

Flow cytometry and cell sorting

For sorting, FL were depleted ($Ter119^{+}CD45^{+}$) using MACS Columns (Miltenyi Biotec). Cell suspensions were stained for 20-30 min at 4°C in the dark with antibodies listed in Supplementary Table 2. Biotinylated antibodies were detected by incubation for 15 min at 4°C in the dark with streptavidin. Antibodies to lineage markers included anti-Ter119, anti-Gr1, anti-CD19, anti-CD3, anti-CD4, and anti-CD8, anti-NK1.1, anti-Il7r, anti-TCR $\alpha\beta$, anti-TCR $\gamma\delta$ and anti-CD11c (all identified in **Supplementary Table 6**). Stained cells were analyzed on a custom BD LSR Fortessa or BD FACSymphony and were sorted with a BD FACS Aria III (BD Biosciences) according to the guidelines for the use of flow cytometry and cell sorting (**Cossarizza et al., 2019**). Data were analyzed with FlowJo software (v.10.5.3, BD Biosciences) or R packages as described in “Bioinformatic Analysis”.

Gene expression by RT-PCR

Cells were sorted directly into lysis buffer and mRNA was extracted (RNeasy Micro Kit (Qiagen), reverse-transcribed (PrimeScript RT Reagent Kit (Takara Bio), and quantitative PCR with Power SYBR Green PCR Master Mix (Applied Biosystems) (see **Supplementary**

Table 7 for primers). qPCR reactions were performed on a Quantstudio3 thermocycler (Applied Biosystems), gene expression was normalized to that of *Actb* and relative expression was calculated using the $2^{-\Delta Ct}$ method.

Imaging Flow Cytometry Analysis

E13.5 FL cells were stained with the surface markers CD45 BV605 (1:50 dilution), Ter119 Biotin (1:100 dilution) followed by incubation with Streptavidin PE-Cy7 (1:100 dilution), CD71 PE (1:100 dilution), CD24 BV510 (1:50 dilution) and Kit Pacific Blue (1:20 dilution) and the RNA Dye Thiazole Orange (TO). Prior to acquisition, nuclei were stained with 20 μ M DRAQ5 (Biostatus) and filtered with 100 μ m mesh. Data acquisition was performed using an ImageStream^x Mark II Imaging Flow Cytometer (Amnis, Luminex Corporation) using 405 nm, 488 nm, 561 nm and 642 nm excitation lasers and the 40 $\times$ magnification collection optic. Laser powers were set in order to maximize signal resolution but minimize any saturation of the CCD camera with bright-field images collected in channels 1 and 9. A minimum of 100,000 cell events were collected per sample. In order to calculate spectral compensation, single-stained cells were acquired with the bright-field illumination turned off. Spectral compensation and data analysis were performed using the IDEAS analysis software (v.6.2.64, Luminex Corp).

EdU incorporation and cell cycle analysis

EdU detection was done using the Click-iT EdU pacific blue flow cytometry assay kit (Invitrogen, Cat# C10418). Cell cycle was analyzed after fixation with Fixation/Permeabilization kit (Invitrogen, Cat#72-5775-40) and staining with Ki67 (SolA15). DAPI (4,6-diamidino-2-phenylindole) was added 7 min before analysis.

***In vitro* liquid and semi-solid cultures**

For limiting dilution analysis sorted cells were plated in 1:3 diluting densities starting at 27 cells/well until 1 cell/well in complete medium OPTI-MEM with 10% FCS, penicillin (50 units/ml), streptomycin (50 μ g/ml) and β -mercaptoethanol (50 μ M) supplemented with a saturating amount of the following cytokines: macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), Kit ligand (Kitl), Erythropoietin (Epo) (R&D Systems, Cat# 959-ME) and Thrombopoietin (Tpo) (R&D Systems, Cat# 488-TO) for myeloid and erythroid differentiation. Except stated otherwise, cytokines were obtained from the supernatant of myeloma cell lines (provided by F. Melchers) transfected with cDNA encoding those cytokines. After 5-7 days wells were assessed for the presence of hematopoietic colonies. Cell frequencies, determined with ELDA software ('extreme limiting-dilution analysis') from the Walter and Eliza Hall Institute Bioinformatics Division, are presented as the number of positive wells and the number of total tested wells (Hu et al., 2009). For colony-forming assays sorted cells were plated at 100 cells/35 mm culture dishes

in duplicates in Methocult M3434 (StemCell Technologies, Cat# 03434) as described by the manufacturer. CFU-E were assessed at 3 days and remaining colonies at 7 days.

***In vivo* analysis of lineage potential**

Pregnant WT females were anesthetized by intraperitoneal injection of a solution of Ketamine 10 mg/ml + Xylazine 1 mg/ml diluted in PBS: (50 to 100 μ l per 10 g body weight), using a 1 ml syringe. E13.5 GFP⁺ Kit⁺CD24⁺ or CD45⁺LK FL cells from UBC-GFP embryos were purified and injected intraperitoneally into recipient E13.5 WT embryos (20000 cells/embryo) of anesthetized females. Sham controls were performed. The FL and the fetal blood of injected and control embryos were analyzed at E16.5.

Multiplex single-cell qPCR

Single cells were sorted directly into 96-well plates loaded with RT-STA Reaction mix (CellsDirect™ One-Step qRT-PCR Kit, Invitrogen, according to the manufacturer's procedures) and 0.2x specific TaqMan® Assay mix (see **Supplementary Table 8** for the TaqMan® assays list) and were kept at -80 °C at least overnight. For each subset analyzed, a control-well with 20 cells was also sorted. Pre-amplified cDNA (20 cycles) was obtained according to manufacturer's note and was diluted 1:5 in TE buffer for qPCR. Multiplex qPCR was performed using the microfluidics Biomark HD system for 40 cycles (Fluidigm) as previously described (Chea et al., 2016). The same TaqMan probes were used for both RT/pre-amp and qPCR. Only single cells for which at least 2 housekeeping genes could be detected before 20 cycles were included in the analysis.

Bioinformatic Analysis

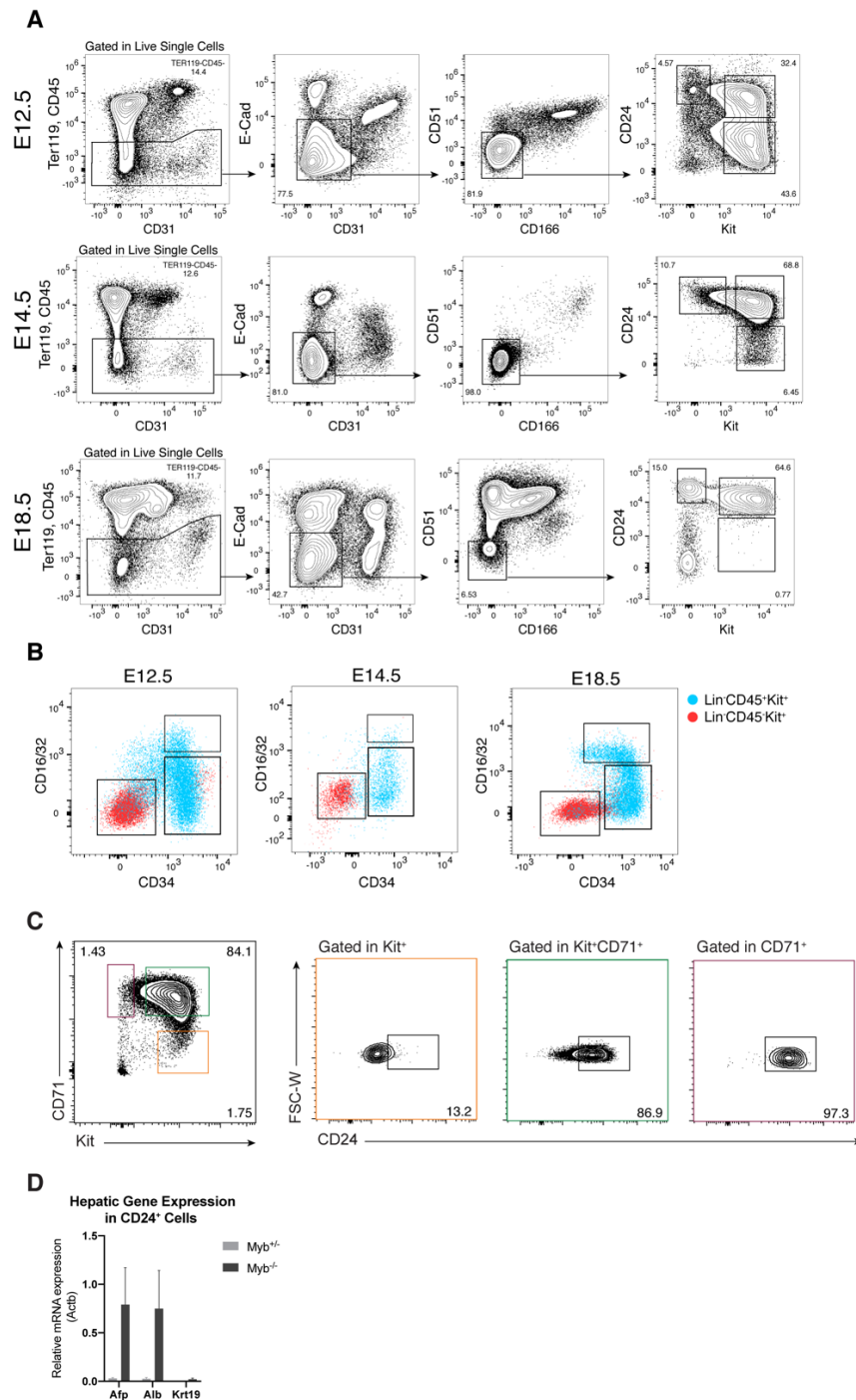
Flow cytometry data analysis was performed in FCS files of live CD45⁺Ter119⁻ cell fraction using R packages "Rtsne", "Rphenograph" and "pheatmap" using Rv3.5.0. Gene expression raw data (BioMark™, Fluidigm) of single cells was normalized with Gapdh and Actb. Heatmaps and hierarchical clustering were generated using R packages "pheatmap" and "Rphenograph" (Levine et al., 2015).

Quantification and Statistical Analysis

All results are shown as mean $\pm$ standard deviation (SD). Statistical significance was determined using one-way ANOVA followed by Tukey multiple comparison test where a P value of <0.05 was considered significant and a P value >0.05 was considered not significant.

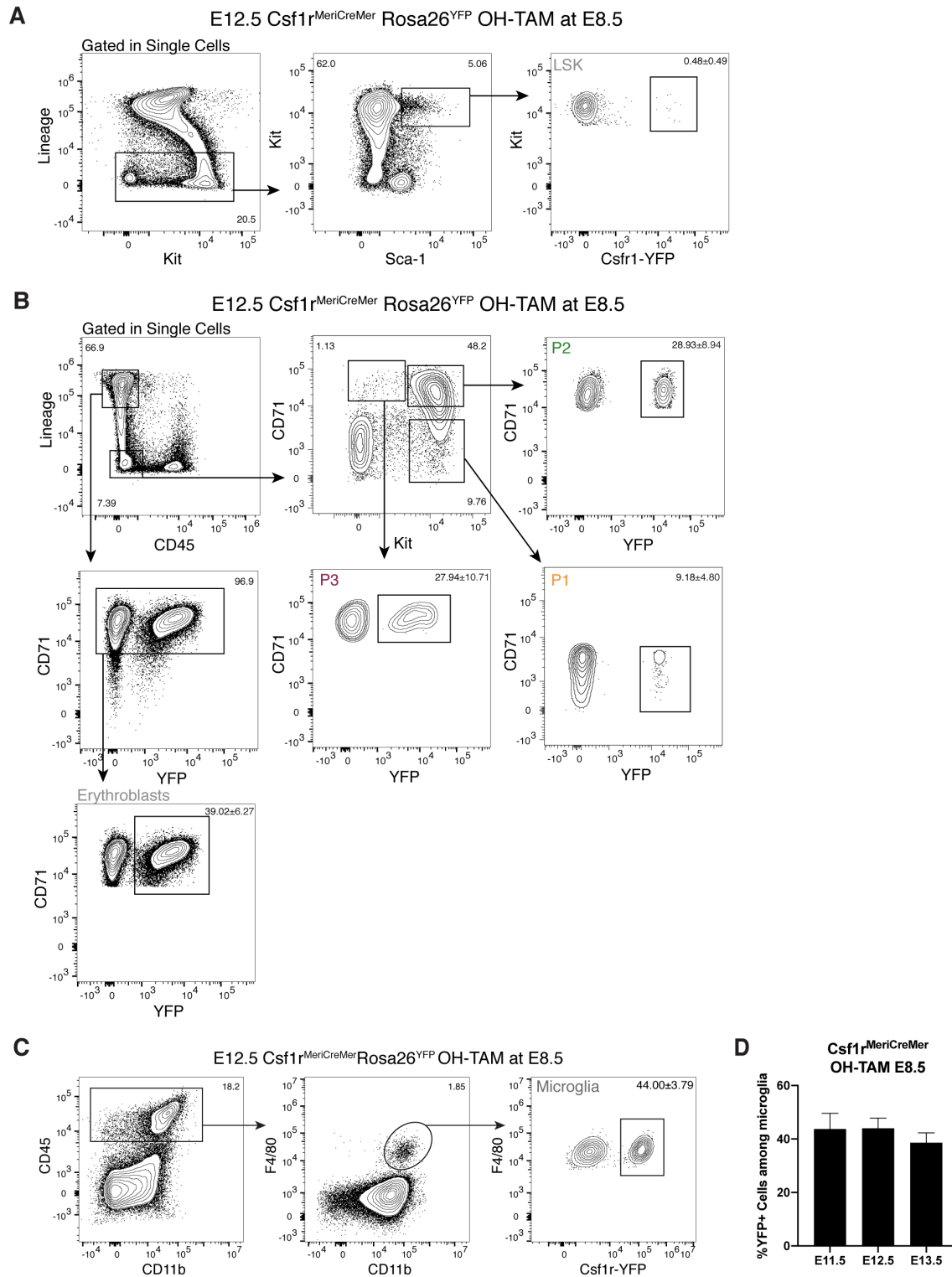
SUPPLEMENTARY INFORMATION

SUPPLEMENTARY FIGURES



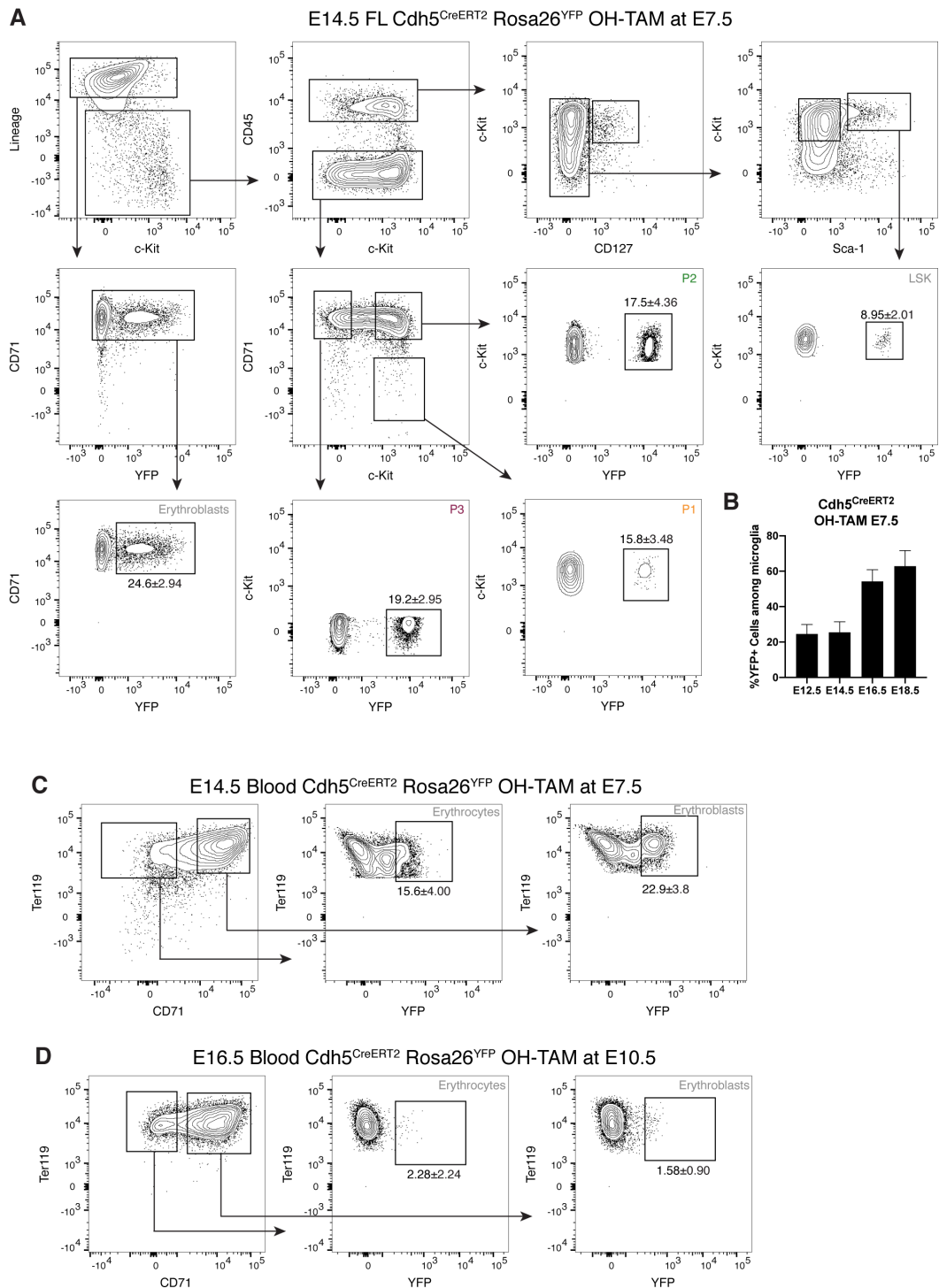
SUPPLEMENTARY FIGURE 5 PHENOTYPE OF E12.5, E14.5 AND E18.5 FETAL LIVER AND ADULT BONE MARROW POPULATIONS

(A) Flow cytometry analysis of E12.5, E14.5 and E18.5 fetal liver cells using Ter119, CD45, E-Cadherin, CD31, CD51 and CD166. Viable Ter119⁺CD45⁺E-Cad⁺CD31⁺CD51⁺CD166⁺ cells can be subdivided into 3 populations according to expression of CD24 and Kit. **(B)** Flow cytometry profile of E12.5, E14.5 and E18.5 fetal liver Lin⁺CD45⁺Kit⁺ (CD45⁺LK) (blue) and CD45⁺Kit⁺ (red) cells according to CD16/32 and CD34 expression. **(C)** Flow cytometry analysis of CD24 expression in P3 (CD71⁺Kit⁺), P2 (CD71⁺Kit⁺) and P1 (CD71⁺Kit⁺) cells in E13.5 FL cells.



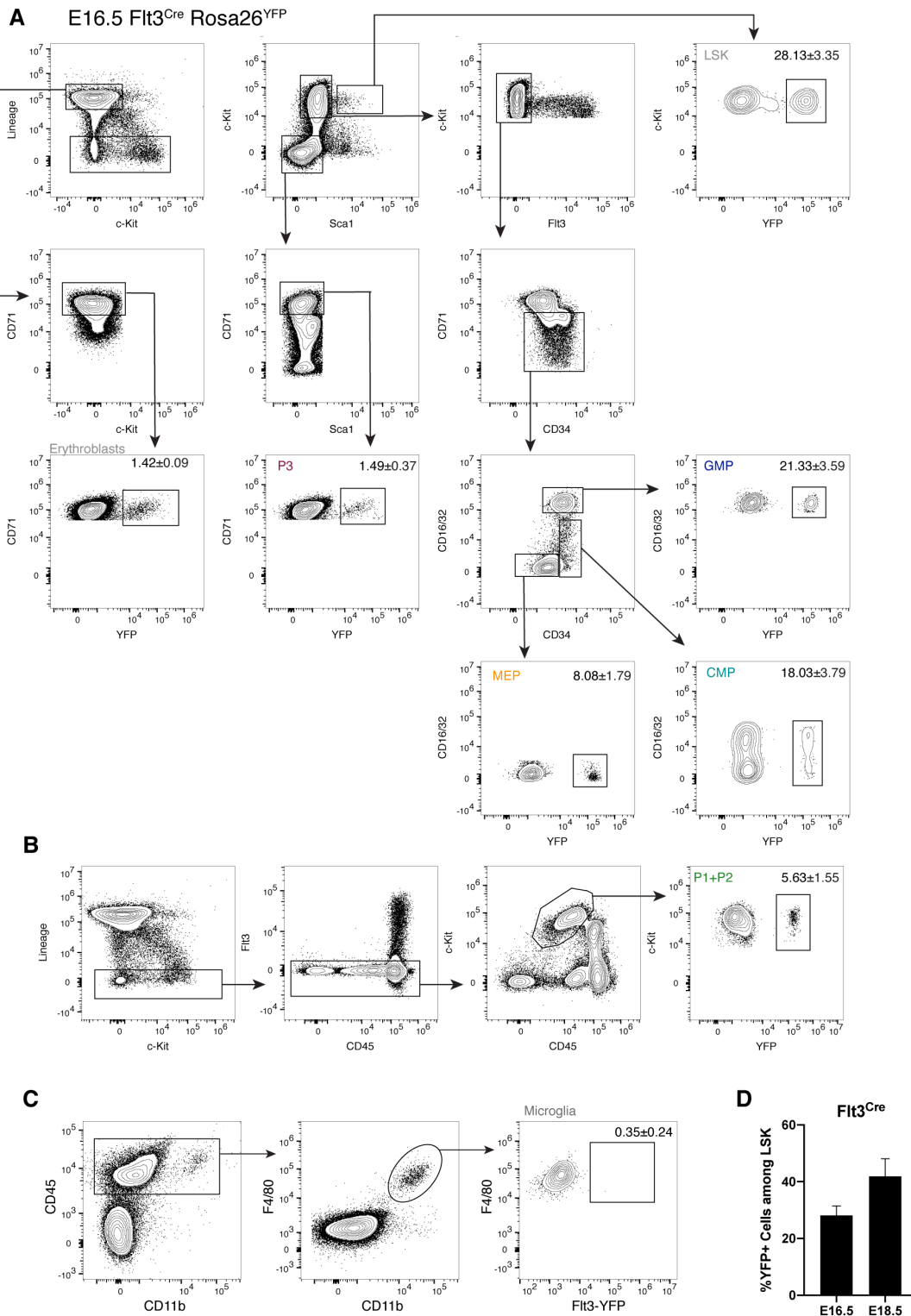
SUPPLEMENTARY FIGURE 6 GATING STRATEGIES USED TO DETERMINE YFP LABELED HEMATOPOIETIC POPULATIONS IN THE $Csf1^{MerCreMer}Rosa26^{YFP}$ LINEAGE TRACING MODEL (RELATED TO FIGURE 22)

(A) Gating strategy for the analysis of YFP expression in Lin⁻Kit⁺Sca1⁺ (LSK) cells. (B) Gating strategy for the analysis of YFP expression in P1 (Lin⁻CD45⁻Kit⁺CD71⁻), P2 (Lin⁻CD45⁻Kit⁺CD71⁺), P3 (Lin⁻CD45⁻Kit⁺CD71⁺) and erythroblast (Lin⁺CD71⁺) cells. (C) Gating strategy for the analysis of YFP expression in microglia (CD45⁺F4/80⁺CD11b⁺) cells. Data representative of E12.5 YFP⁺ embryos. (D) Percentage of YFP⁺ cells among microglia at E11.5, E12.5 and E13.5.



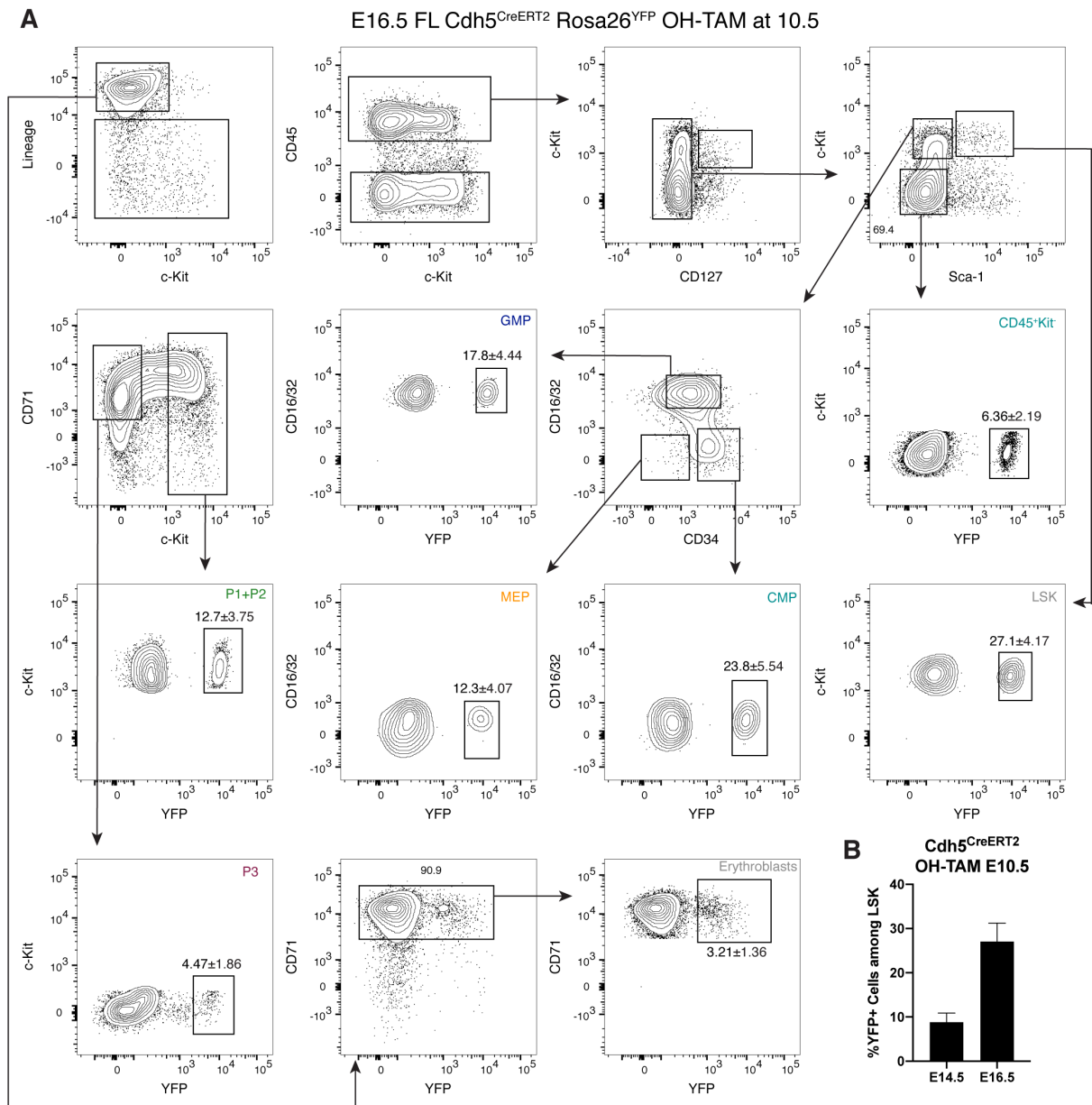
SUPPLEMENTARY FIGURE 7 GATING STRATEGIES USED TO DETERMINE YFP LABELED HEMATOPOIETIC POPULATIONS IN THE $CDH5^{CreERT2}Rosa26^{YFP}$ FATE-MAPPING MODEL (RELATED TO FIGURE 23)

(A) Gating strategy for the analysis of YFP expression in Lin⁺Kit⁺Sca1⁺ (LSK), P1 (Lin⁺CD45⁺Kit⁺CD71⁻), P2 (Lin⁺CD45⁺Kit⁺CD71⁺), P3 (Lin⁺CD45⁺Kit⁺CD71⁺) and erythroblast (Lin⁺CD71⁺) cells. Data representative of E14.5 YFP⁺ embryos. (B) Percentage of YFP⁺ cells among microglia at E12.5, E14.5 and E16.5. (C) Gating strategy for the analysis of YFP expression in blood erythrocytes (Ter119⁺CD71⁻) and erythroblasts (Ter119⁺CD71⁺) after OH-TAM pulse at E7.5. (D) Gating strategy for the analysis of YFP expression in blood erythrocytes (Ter119⁺CD71⁻) and erythroblasts (Ter119⁺CD71⁺) after OH-TAM pulse at E10.5.



SUPPLEMENTARY FIGURE 8 GATING STRATEGIES USED TO DETERMINE YFP LABELED HEMATOPOIETIC POPULATIONS IN THE FLT3^{CRE}ROSA26^{YFP} FATE-MAPPING MODEL (RELATED TO FIGURE 23)

(A) Gating strategy for the analysis of YFP expression in Lin⁺Kit⁺Sca1⁺ (LSK), Common Myeloid Progenitors (CMP), Granulocyte-Monocyte Progenitors (GMP), Megakaryocyte Erythrocyte Progenitors (MEP), P3 and erythroblasts. (B) Gating strategy for the analysis of YFP expression in P1+P2. (C) Gating strategy for the analysis of YFP expression in microglia cells. Data representative of E16.5 YFP⁺ embryos. (D) Percentage of YFP⁺ cells among LSK at E16.5 and E18.5.



SUPPLEMENTARY FIGURE 9 GATING STRATEGIES USED TO DETERMINE YFP LABELED HEMATOPOIETIC POPULATIONS IN THE $CDH5^{CreERT2}Rosa26^{YFP}$ FATE-MAPPING MODEL (RELATED TO FIGURE 23)

(A) Gating strategy for the analysis of YFP expression in Lin⁺Kit⁺Sca1⁺(LSK), Common Myeloid Progenitors (CMP), Granulocyte-Monocyte Progenitors (GMP), Megakaryocyte Erythrocyte Progenitors (MEP), P1+P2, P3, erythroblast and CD45⁺Kit⁻ cells. (B) Percentage of YFP⁺ cells among LSK at E14.5 and E16.5.

SUPPLEMENTARY TABLES

SUPPLEMENTARY TABLE 6 LIST OF ANTIBODIES FOR FLOW CYTOMETRY

Protein	Clone	Fluorochrome	Manufacturer	Catalog #
CD11b	M1/70	APC-Cy7	SONY	1106130
CD16/32	93	PerCP	SONY	1106620
CD16/32	2.4G2	PE	BD Biosciences	553145
CD19	6D5	Biotin	SONY	1177520
CD19	1D3	Pacific Blue	BD Biosciences	562701
CD24	M1/69	BV510	SONY	1109155
CD24	M1/69	PE-Cy7	BD Biosciences	560536
CD31	MEC13.3	BUV737	BD Biosciences	565097
CD31	390	PerCP-Cy5.5	Biolegend	102420
CD324	DECMA-1	PE-Cy7	SONY	1336545
CD34	RAM34	APC	eBioscience	50-0341-82
CD34	RAM34	BV421	BD Biosciences	562608
CD41	MWReg30	PE	BD Biosciences	561850
CD45	30-F11	Biotin	SONY	1115520
CD45	104	BV650	SONY	1149180
CD54	YN1/1.7.4	Pacific Blue	Biolegend	116116
CD71	C2	PE	BD Biosciences	553267
Gr1	RB6-8C5	FITC	Biolegend	108406
Gr1	RB6-8C5	Biotin	BD Biosciences	553125
Gr1	RB6-8C5	BV510	BD Biosciences	563040
Il7ra	A7R34	Biotin	eBioscience	13-1271-85
Ki-67	SolA15	FITC	eBioscience	11-5698-82
Kit	2B8	APC-Cy7	SONY	1129130
Kit	2B8	APC	SONY	1129060
Kit	2B8	Pacific Blue	Biolegend	105827
NK1.1	PK136	Biotin	BD Biosciences	553163

Sca1	D7	BV711	SONY	1140655
Ter119	TER-119	Biotin	SONY	1181020
Ter119	TER-119	PE-Cy7	SONY	1181110
Streptavidin	-	BV786	SONY	2626245
Streptavidin	-	APC	SONY	2626035
Streptavidin	-	PE-Cy7	SONY	2626030

SUPPLEMENTARY TABLE 7 LIST OF PRIMERS FOR QRT-PCR

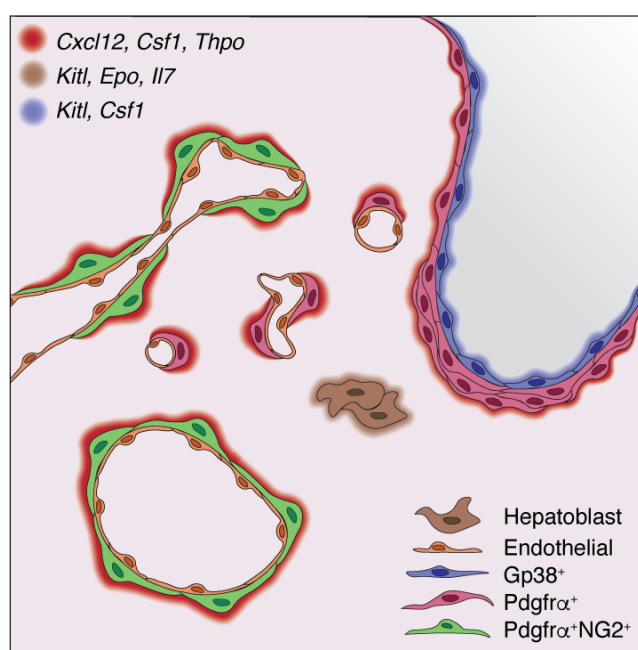
Gene	Gene ID	Primer FW	Primer RV	PrimerBank ID	SOURCE
Actb	11461	GCTTCTTTGCAGCTCCT TCGT	ATCGTCATCCATGGCGA ACT	-	
Bmi1	12151	ATCCCCACTTAATGTGT GTCCT	CTTGCTGGTCTCCAAGT AACG	192203a1	
Epor	13857	GGGCTCCGAAGAACTT CTGTG	ATGACTTTCGTGACTCA CCCT	116292185c1	
Gata1	14460	ATCAGCACTGGCCTACT ACAGAG	GAGAGAAGAAAGGACT GGGAAAG	-	(Kozhemyaki na et al., 2014)
Hbb-b1	15129	GCACCTGACTGATGCTG AGAA	TTCATCGGCGTTCACCT TTCC	31982300a1	
Hbb-bh1	15132	TGGACAACCTCAAGGA GACC	TGCCAGTGTACTGGAAT GGA	-	(Otsuka et al., 2016)
Hbb-y	15135	TGGCCTGTGGAGTAAG GTCAA	GAAGCAGAGGACAAGT TCCCA	6680177a1	
Klf1	16596	AGACTGTCTTACCCTCC ATCAG	GGTCCTCCGATTTACAGA CTCAC	6754454a1	
Lmo2	16909	ATGTCCTCGGCCATCGA AAG	CGGTCCCCTATGTTCTG CTG	6678702a1	
Myb	17863	AGACCCCGACACAGCA TCTA	CAGCAGCCCATCGTAG TCAT	19526459a1	
Runx3	12399	CAGGTTCAACGACCTTC GATT	GTGGTAGGTAGCCACTT GGG	9789899a1	

SUPPLEMENTARY TABLE 8 LIST OF TAQMAN PROBES FOR SINGLE-CELL MULTIPLEX GENE EXPRESSION

Gene	Gene ID	Taqman Assay ID
Bmi1	12151	Mm00776122_gH
Cebpa	12606	Mm00514283_s1
Csf1r	12978	Mm01266652_m1
Csf2ra	12982	Mm00438331_g1
Csf3r	12986	Mm00432735_m1
Epor	13857	Mm00833882_m1
Gata1	14460	Mm01352636_m1
Gata2	14461	Mm00492301_m1
Hbb-b1	15129	Mm01611268_g1
Hbb-bh1	15132	Mm00433932_g1
Hbb-y	15135	Mm00433936_g1
mKi-67	17345	Mm01278617_m1
Kit	16590	Mm00445212_m1
Klf1	16596	Mm00516096_m1
Lmo2	16909	Mm01281680_m1
Ly6c	17067 and 100041546	Mm03009946_m1
Mpl	17480	Mm00440310_m1
Myb	17863	Mm00501741_m1
Runx1	12394	Mm01213404_m1
Runx2	12393	Mm03003491_m1
Runx3	12399	Mm00490666_m1
Tal1	21349	Mm01187033_m1
Zfp1	22761	Mm00494336_m1
Actb	11461	Mm01205647_g1
Gapdh	14433	Mm99999915_g1
Hprt	15452	Mm03024075_m1

CHAPTER V – HEMATOPOIETIC CYTOKINE-EXPRESSING CELLS IN THE DEVELOPING LIVER

*The developing fetal liver supports the development of the hematopoietic stem cells that will maintain the adult hematopoietic system. In this organ, the conditions for stem cell expansion and differentiation to distinct lineages are met, either due to cell-autonomous mechanisms of newly generated progenitors and/or due to specific environmental cues. However, the precise signals and/or cell types involved in such decisions are unclear. Here, we analyzed the non-hematopoietic compartment of the fetal liver in search for the hematopoietic cytokine-expressing cells. We found 5 distinct subsets of stromal cells based on the expression of the surface markers CD324, CD31, CD54, Gp38 and PDGFR α . Besides endothelial cells, all subsets express different levels of hematopoietic cytokines, with Cxcl12 being mostly produced by PDGFR α ⁺ cells. Epo and Il7 mRNA transcripts are mostly found within hepatoblasts. We investigated the *in situ* localization of the distinct subsets and found that hepatoblasts are spread throughout the tissue, Gp38⁺ cells are found in the mesothelium and PDGFR α ⁺ cells can be subdivided into NG-2⁺ and NG-2⁻, with the former found in association with the major vessels, while the latter associated with sinusoids. These results bring us one step further in the characterization of the fetal liver environment and will serve as basis for further studies.*



Keywords: fetal liver; niche; hematopoiesis; cytokine signaling

This chapter includes parts of the following manuscript in preparation:

Soares-da-Silva F*, Peixoto M*, Pinto-do-Ó P#, Cumano A#. **The interplay of fetal liver niche cells in embryonic hematopoiesis.** *these authors contributed equally #senior authorship

FOREWORD

The developing liver is the main hematopoietic organ during embryonic development. It supports hematopoiesis from E10.5 to E16.5, period after which HSPCs start migrating to the fetal BM (Johnson et al., 1975; Palis et al., 1999; Christensen et al., 2004; Gekas et al., 2005). The proliferation or differentiation of HSPCs is the result of intrinsic and extrinsic factors, being hematopoietic cytokines part of the latter. Distinct cytokine signaling pathways have been described to have a role in BM HSC survival, maintenance or commitment to distinct blood lineages (Table 4). Whether the same cytokines are implicated in HSPC proliferation in the FL is unclear. Various populations of FL cells have been described to play a role in embryonic hematopoiesis. Fetal hepatic progenitors characterized by the expression of *DLK1* are able to maintain LT-HSCs in culture over a 3-week period when hematopoietic cytokines are used as supplement (Chou et al., 2013). Stellate cells express low levels of *Kitl*, *Epo*, *Csf1* and *Flt3l* and could therefore also interact with the developing hematopoietic progenitors (Tan et al., 2017b). NG2⁺Nestin⁺ pericytes express *Kitl*, *Cxcl12*, *Angptl2* transcripts and have been implicated in the maintenance of HSCs, as selective elimination of these cells results in moderate reduction of HSC numbers and a reduction in the proliferation rate of the remaining HSCs (Khan et al., 2016).

Here, we analyzed the entire non-hematopoietic compartment to directly evaluate the hematopoietic cytokine-expressing cells. We used a minimal panel of 8 surface markers that allows the discrimination of 5 major populations based on surface expression of CD324, CD31, CD54, Gp38 and PDGFR α . By evaluating the expression of hematopoietic cytokines in the 5 subsets along embryonic development we found that with the exception of endothelial cells that do not seem to produce any particular cytokine, all the 4 remaining subsets produce distinct levels of *Kitl*, *Thpo*, *Epo*, *Il7*, *Csf1* and *Cxcl12*. Of note, single-cell multiplex gene expression analysis showed that the majority of hepatoblasts produce *Cxcl12* and *Kitl*. Moreover, we explored the *in situ* localization of these subsets along ontogeny in cryosections. Gp38⁺ cells are found lining the hepatic lobes and are most probably mesothelial cells. PDGFR α ⁺ cells are spread throughout the parenchyma lining the sinusoids but can also be found in the sub-mesothelial region. Hepatoblasts are dispersed in the tissue since early time-points, and originate hepatocytes and bile duct cells that can be observed after E16.5.

HEMATOPOIETIC CYTOKINES ARE PRODUCED BY DISTINCT STROMAL POPULATIONS

CHARACTERIZATION OF THE STROMAL SUBSETS

We analyzed, by flow cytometry, expression of the surface markers described in **Table 5** that distinguished stromal populations, namely CD324, CD31, CD54, CD146, Gp38, PDGFR α , NG2 and ALCAM in Ter119⁻CD45⁻Kit⁻CD71⁻ (non-hematopoietic) E14.5 FL cells. Endothelial cells were identified based on the expression of CD31, CD324⁺CD54⁺ cells represent hepatoblasts and, within the CD31⁻CD324⁻ fraction, Gp38 and PDGFR α defined three more subsets (**Figure 25**).

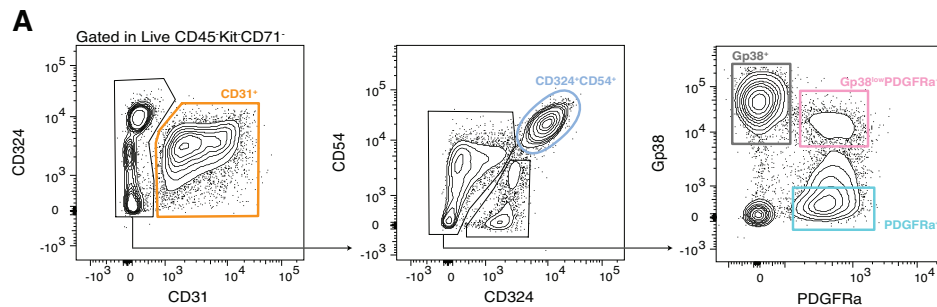


FIGURE 25 FLOW CYTOMETRY ANALYSIS OF TER119⁻CD45⁻KIT⁻CD71⁻ E14.5 FETAL LIVER CELLS

(A) Flow cytometry analysis of E14.5 Ter119⁻CD45⁻Kit⁻CD71⁻ cells with the endothelial marker CD31, the adhesion molecule CD54, the epithelial marker CD324, the mesenchymal marker PDGFR α and the mesothelial marker Gp38.

Next, we analyzed the gene expression profile of liver and mesenchymal transcripts in the five cell subsets (**Figure 26**). *Krt19*, which is associated to cholangiocytes of the intra-hepatic bile ducts but is also expressed in the mesothelium (**Tanaka et al., 2009**), is mostly expressed by Gp38⁺PDGFR α ⁻ cells. The cholangiocyte transcription factor *Onecut1* (**Clotman et al., 2002**) is expressed by hepatoblasts but not by Gp38⁺PDGFR α ⁻ cells. All cell subsets with the exception of the endothelium expressed *Dlk1*, a marker used to identify hepatoblasts in the FL (**Tanimizu et al., 2003; Tanaka et al., 2009**). Smooth muscle actin gene *Acta2* and stellate cell marker *Ngfr* are mostly expressed by Gp38⁺PDGFR α ⁺ cells. High levels of *Nestin* are found in PDGFR α expressing cells (Gp38^{+/low}PDGFR α ⁺ and Gp38⁻PDGFR α ⁺). CD31⁺ and Gp38⁺PDGFR α ⁻ cells express low levels of this transcript. *Desmin*, associated with sub-mesothelial and hepatic stellate cells, is expressed by the two PDGFR α ⁺ subsets. The same can be observed for *Lhx2*, another hepatic stellate cell marker (**Tan et al., 2017a**). To distinguish hepatic stellate cells from pericytes, we analyzed the expression of *Kcnj8*, a pericyte specific marker (**Bondjers et al., 2006**), which is expressed by both PDGFR α ⁺ subsets (**Figure 26**).

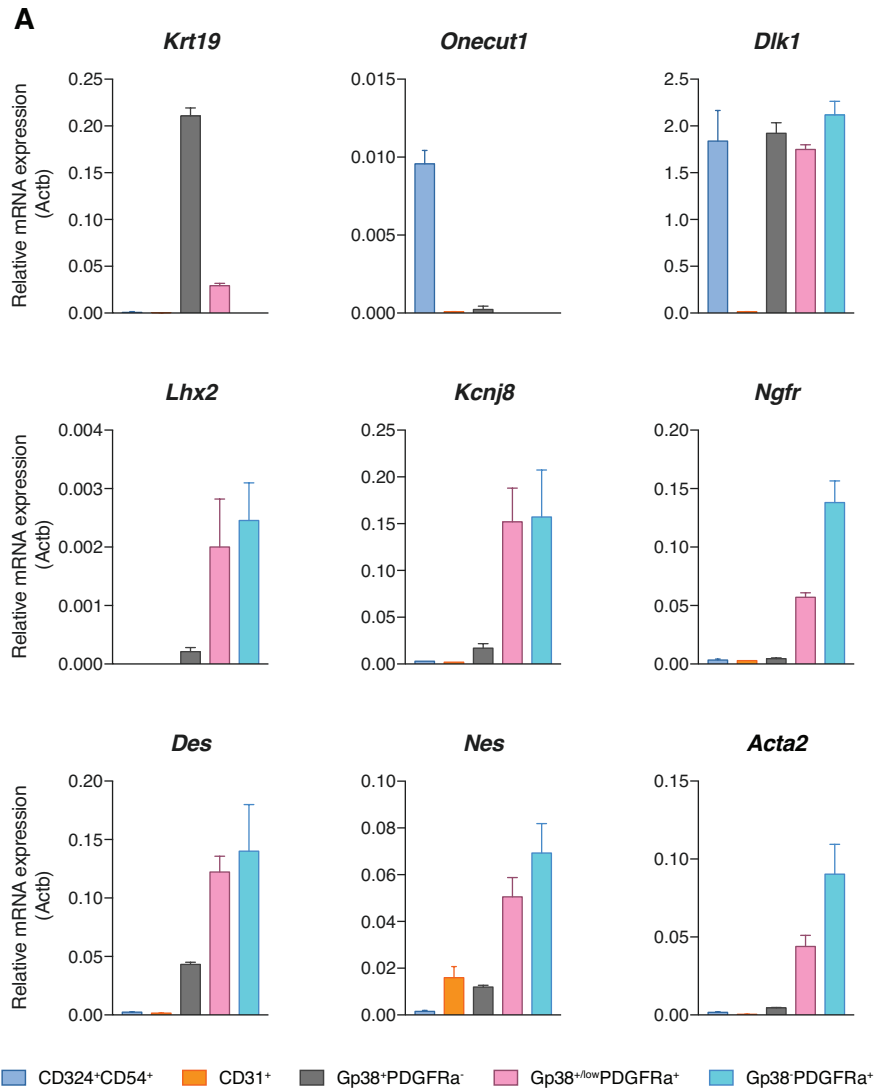


FIGURE 26 GENE EXPRESSION ANALYSIS OF THE STROMAL SUBSETS

(A) Gene expression analysis of the cholangiocyte (*Hnf6*, *Krt19*), hepatoblast (*Dlk1*) and mesenchymal (*Lhx2*, *Kcnj8*, *Ngfr*, *Des*, *Nes*, *Acta2*) transcripts of the sorted cells. Relative mRNA expression was compared to that of *Actb*.

HEMATOPOIETIC CYTOKINE-EXPRESSING CELLS

To evaluate which populations contributed with cytokines to the development of the hematopoietic system we analyzed the expression of cytokine transcripts in the five cell subsets from E14.5 to E18.5 (**Supplementary Figure 10**). The role of the analyzed cytokines in hematopoiesis is described in **Table 4**. At E14.5, Gp38⁺PDGFR α ⁻ cells and hepatoblasts are the major producers of *Kitl* (**Figure 27A**). The chemoattractant *Cxcl12* is mostly expressed by PDGFR α ⁺ subsets and at low levels by hepatoblasts. *Thpo* and *Csf1* have a similar pattern of expression, being mostly produced by PDGFR α ⁺ subsets and Gp38⁺PDGFR α ⁻ cells, but also at low levels by hepatoblasts. *Epo* is exclusively expressed by hepatoblasts, whereas *Il7* is mostly expressed by hepatoblasts but also by Gp38⁺PDGFR α ⁻ cells at low levels (**Figure**

27A). We also analyzed the expression of *Kitl*, *Epo* and *Il7* in hepatoblasts at earlier time-points (**Figure 27B-D**). Both *Kitl* and *Epo* are expressed since early FL development (E10.5) (**Figure 27B-C**). *Kitl* expression is maintained throughout all analyzed timepoints and decreases after E16.5 (**Figure 27B**), while *Epo* expression drastically decreases after E12.5 (**Figure 27C**). *Il7* levels remain low during development, with a peak of expression at E16.5 (**Figure 27D**).

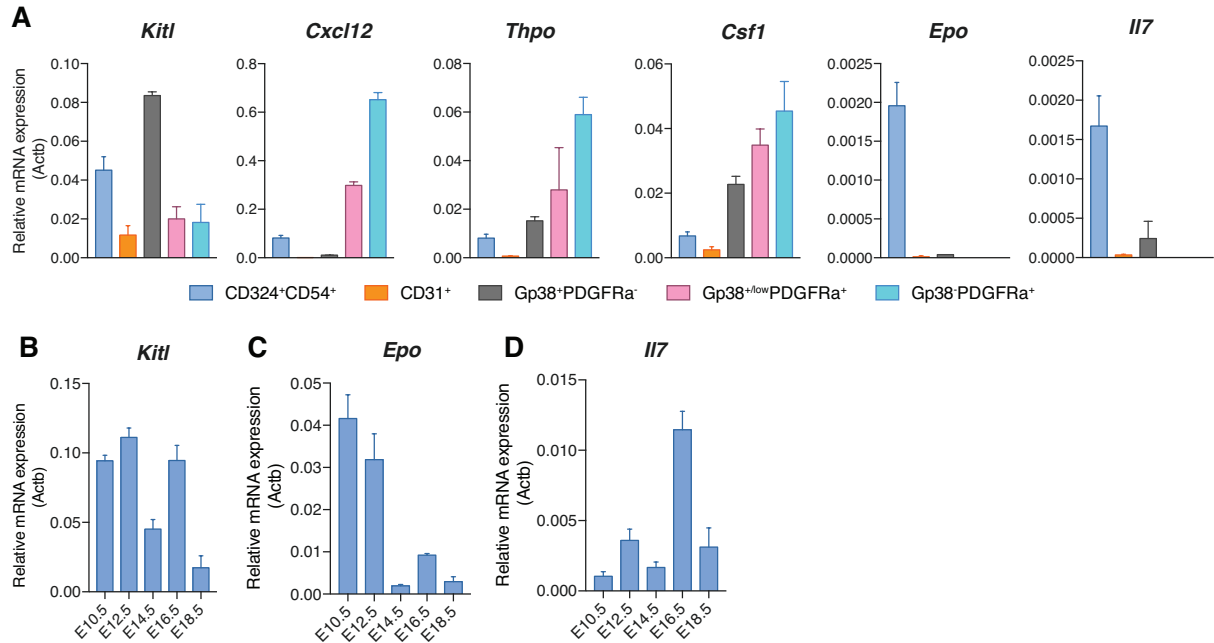


FIGURE 27 CYTOKINE EXPRESSION IN FL STROMAL SUBSETS

(A) Gene expression analysis of the hematopoietic cytokines *Kitl*, *Cxcl12*, *Thpo*, *Csf1*, *Epo* and *Il7* at E14.5 FL. **(B-D)** Gene expression analysis of the hematopoietic cytokines *Kitl*, *Epo* and *Il7* in hepatoblasts from E12.5 to E18.5. Relative mRNA expression was compared to that of *Actb*.

Next, to understand the heterogeneity of the cytokine expressing subsets, we performed single-cell multiplex gene expression analysis (**Figure 28**). As expected, all hepatoblasts expressed transcripts of both hepatocyte (*Afp*, *Alb*, *Hnf4a*, *Krt18*) and cholangiocyte (*Hnf6/Onecut1*) lineages and the analysis of the selected genes renders these cells as a homogeneous population (**Figure 28A**). Both *Kitl* and *Cxcl12* were expressed by 96%, *Csf1* by more than 50%, *Thpo* by 40% and *Il7* by 12% of a total of 26 hepatoblasts analyzed. Mesenchymal transcripts such as *Nes*, *Des*, *Lepr*, *Acta2* or *Lhx2* were not expressed.

A similar analysis was performed in Gp38⁺PDGFR α ⁻ and Gp38⁺PDGFR α ⁺ cells (**Figure 28B**). The two cells subsets can be easily distinguished by the expression of cytokeratins (*Krt18* and *Krt19*) and of mesenchymal markers (*Acta2*, *Lhx2*, *Nes* and *Lepr*). As observed by bulk gene expression analysis (**Figure 27A**), Gp38⁺PDGFR α ⁺ cells are the major producers of *Cxcl12* (**Figure 28B**). Gp38⁺PDGFR α ⁻ cells segregate in two different clusters based on the differential expression of *Des* and *Cxcl12*. Of note, *Cxcl12* expression was minimal when

Gp38⁺PDGFR α ⁻ cells were analyzed in bulk, possibly due to a dilution of non-expressing cells. *Kitl* is expressed by all Gp38⁺PDGFR α ⁻ and around 70% of Gp38⁺PDGFR α ⁺ cells. *Il7* and *Thpo* are not expressed in the cells analyzed and *Csf1* is expressed by more than 70% of all cells. As observed in the bulk gene expression analysis, Gp38⁺PDGFR α ⁻ cells do not express the cholangiocyte marker *Onecut1* and are therefore associated to the mesothelial lineage (Figure 26A and Figure 28B). None of the cells expressed *Epo* transcript.

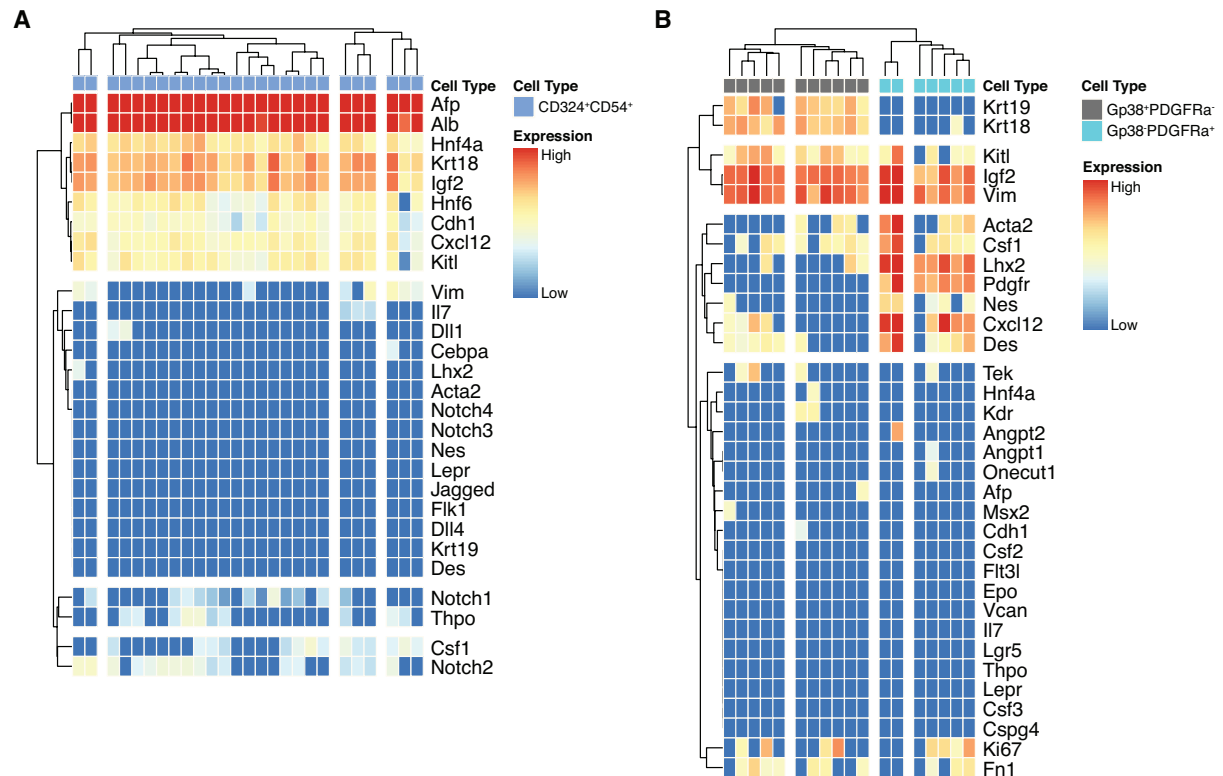


FIGURE 28 SINGLE-CELL GENE EXPRESSION ANALYSIS OF E14.5 FL STROMAL SUBSETS

(A,B) Heatmap of single-cell qPCR in sorted cells from E14.5 FL. Each column represents a single-cell and its color-coded according to cell type ((A) CD324⁺CD54⁺ hepatoblasts, (B) Gp38⁺PDGFR α ⁻ and Gp38⁺PDGFR α ⁺ cells). Gene expression was normalized to *Actb* and *Gapdh* and unsupervised hierarchical clustering was performed.

IN SITU ANALYSIS OF STROMAL SUBSETS

HEPATOBLASTS AND ENDOTHELIAL CELLS

Hepatoblasts and endothelial cells are the most frequent non-hematopoietic cell types in the FL. We analyzed the distribution of these cells along ontogeny by immunostaining of FL cryosections from E12.5 until P7. Hepatoblasts were identified by the nuclear staining of Hnf4 α and endothelial cells by the expression of CD31. At E12.5, both HNF4 α ⁺ and CD31⁺ cells can be found throughout the tissue, with CD31 labeling vessel lining endothelial cells but also a large frequency of round cells (**Figure 29A**). At E14.5, CD31 is expressed in cells that are mostly present around the sinusoids and larger vessels, but also in some round multinucleated large cells (**Figure 29B**). Similar to E12.5, at E14.5 hepatoblasts are distributed evenly across the tissue. By E16.5 alterations in the frequency and distribution of Hnf4 α ⁺ cells are already evident as these cells start to arrange around the vessels (**Figure 29C**). Hnf4 α is expressed in liver progenitor cells from E8.5 and continues to be expressed in hepatocytes throughout life (**Duncan et al., 1994**). At E16.5, Hnf4a expressing cells might represent more mature hepatocytes. From E12.5 to E16.5, all Hnf4 α expressing cells also express the epithelial marker E-cadherin. At E16.5, E-cadherin^{bright} cells can be observed around the vessels where bile ducts are starting to form (**Figure 29D**), structures that can be clearly noticed by E18.5 (**Figure 29E**). Hexagonal shaped hepatocytes can be observed from E18.5 onwards by fainter expression of E-Cadherin, as the architecture of the liver changes (**Figure 29E**). At post-natal stage P7, bile ducts are clearly visible by the expression of EpCAM (**Figure 29F**).

MESOTHELIAL AND SUB-MESOTHELIAL CELLS

Next, we aimed at identifying the location of the other three subsets of stromal cells identified in **Figure 25A**. For that, we stained FL cryosections at distinct time-points with Gp38, PDGFR α , CD146 and ALCAM. NG2 cells, that co-label with Gp38 cells (**Supplementary Figure 11**) are found in the outermost cell layer of the liver, in what is assumed to be the mesothelial layer (**Figure 30A-B,D**). Mesothelial cells are described as Gp38⁺ (**Lua et al., 2016**), but NG2 expression has not been reported. These cells co-express CD146 (**Figure 30B**) but no PDGFR α as observed by flow cytometry (**Supplementary Figure 11**). PDGFR α ⁺ and ALCAM⁺ cells are detected in sub-mesothelial cells as described in the literature (**Asahina et al., 2011; Lua et al., 2016**).

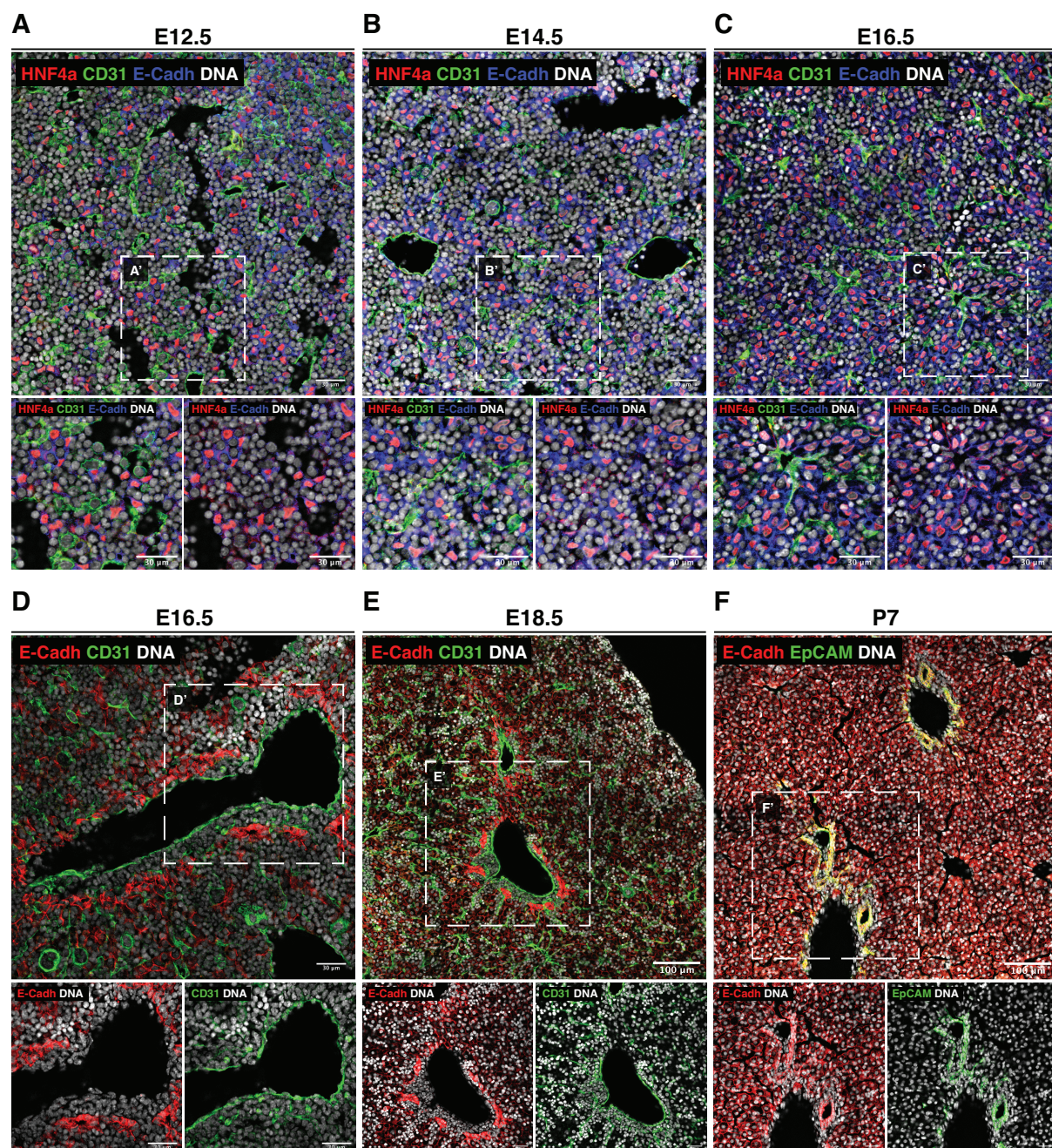


FIGURE 29 HEPATOBLASTS AND ENDOTHELIAL CELLS

(A-C) Immunostaining of E12.5 (A), E14.5 (B) and E16.5 (C) liver cryosections with the hepatic marker Hnf4 α (red), the endothelial marker CD31 (green) and the epithelial marker E-Cadherin (blue). Scale bar 30 μ m. (D-E) Immunostaining of E16.5 (D) and E18.5 (E) liver cryosections with the epithelial marker E-Cadherin (red) and endothelial marker CD31 (green). Scale bar (D) 30 μ m, (E) 100 μ m, (E') 30 μ m. (F) Immunostaining of post-natal day 7 (P7) liver cryosection with the epithelial marker E-Cadherin (red) and bile duct marker EpCAM (green). Scale bar (F) 100 μ m, (F') 30 μ m. Nuclei are shown in white. Fields are representative of the tissue section.

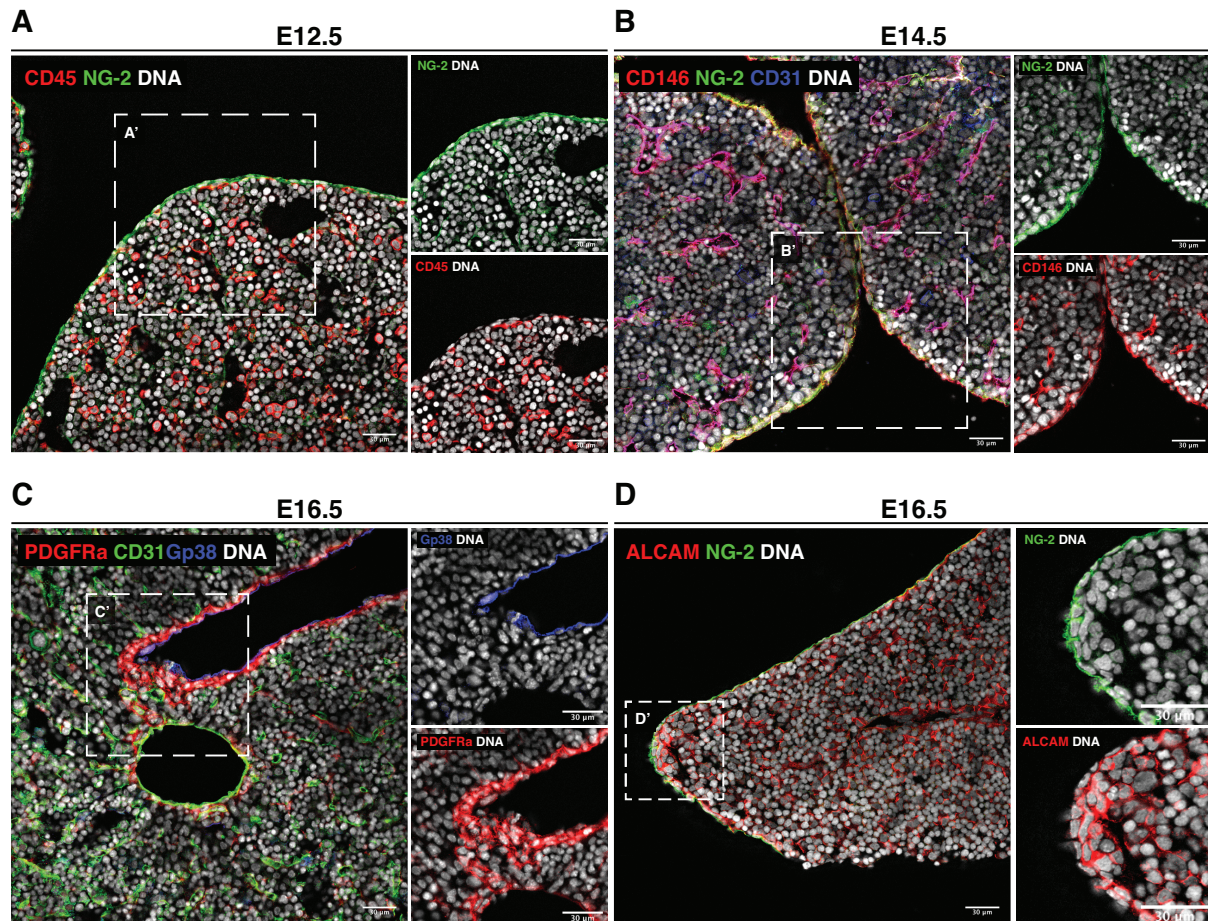


FIGURE 30 MESOTHELIAL AND SUB-MESOTHELIAL CELLS

(A) Immunostaining of E12.5 liver cryosections with the hematopoietic marker CD45 (red) and the pericyte marker NG2 (green). (B) Immunostaining of E14.5 liver cryosections with the cell adhesion molecule CD146 (red), the pericyte marker NG2 (green) and endothelial marker CD31 (blue). (C) Immunostaining of E16.5 liver cryosections with the mesenchymal marker PDGFR α (red), endothelial marker CD31 (green) and mesothelial marker Gp38 (blue). (D) Immunostaining of E16.5 liver cryosections with the cell adhesion molecule ALCAM (red) and the pericyte marker NG2 (green). Nuclei are shown in white. Scale bar 30 μ m. Fields are representative of the tissue section.

MESENCHYMAL CELLS

To establish the location of the mesenchymal cells we analyzed the distribution of PDGFR α and NG2 cells in the parenchyma at E12.5, E14.5 and E16.5 (**Figure 31A-C**). In all time-points, PDGFR α ⁺ cells appear in close contact with CD31⁺ endothelial cells, in a location where smooth muscle cells and pericytes (mural cells) are found. NG2 expression pattern is distinct across ontogeny. At E12.5, NG2⁺ cells are distributed along the parenchyma (**Figure 31A**) but become more concentrated around large vessels at E14.5 and E16.5 (**Figure 31B-C**). At E18.5, NG2⁺ are also associated with large vessels and can be found forming a single cell layer beneath CD31⁺ endothelial cells (**Figure 31D'**) or clustering around large caliber vessels (**Figure 31D''**).

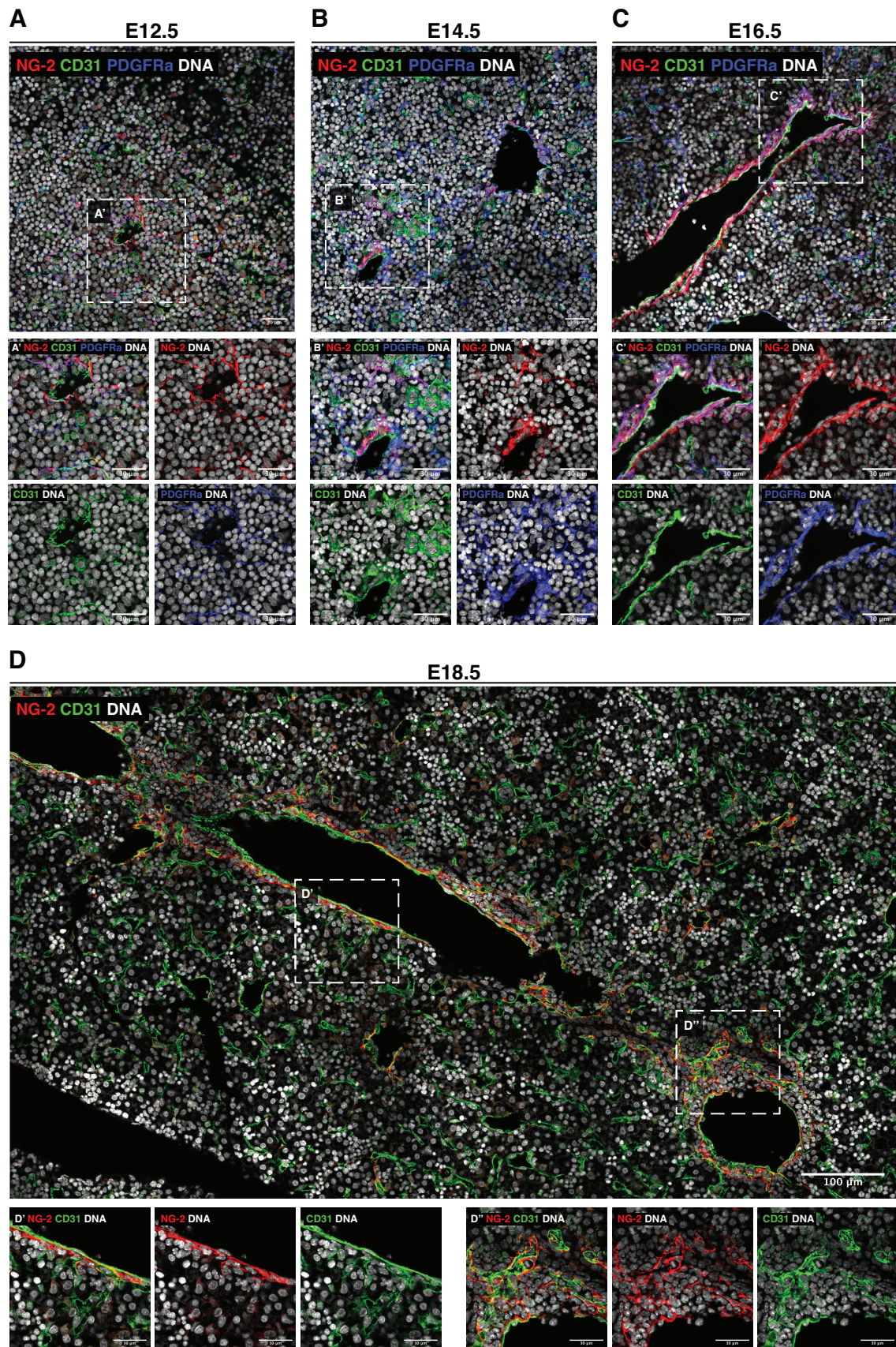


FIGURE 31 MESENCHYMAL CELLS

(A-C) Immunostaining of E12.5 (A), E14.5 (B) and E16.5 (C) liver cryosections with the pericyte marker NG2 (red), the endothelial marker CD31 (green) and the mesenchymal marker PDGFR α (blue). Scale

bar 30 μm (**D**) Immunostaining of E18.5 liver cryosections with the pericyte marker NG2 (red) and the endothelial marker CD31 (green). Scale bar (**D**) 100 μm , (**D'** and **D''**) 30 μm . Nuclei are shown in white. Fields are representative of the tissue section.

IN SITU LOCALIZATION OF HEMATOPOIETIC CELLS

In order to understand whether hematopoietic cells have a preferential distribution across the FL, we analyzed expression of the erythroid marker Ter119 and the pan-hematopoietic marker CD45. At early stages, the FL parenchyma is packed with Ter119⁺ erythroid cells (**Figure 32A-B**), that become scarce and localized to the interior of the vessel by the end of gestation (**Figure 32E-F**). Remarkably, CD45⁺ cells that are few at E12.5 (**Figure 32A**), become localized to the periphery of the FL in close association with the mesothelium (**Figure 32C**) and form clusters around major vessels by E16.5 (**Figure 32D**). This phenomenon is particularly evident at E18.5 (**Figure 32E-F**).

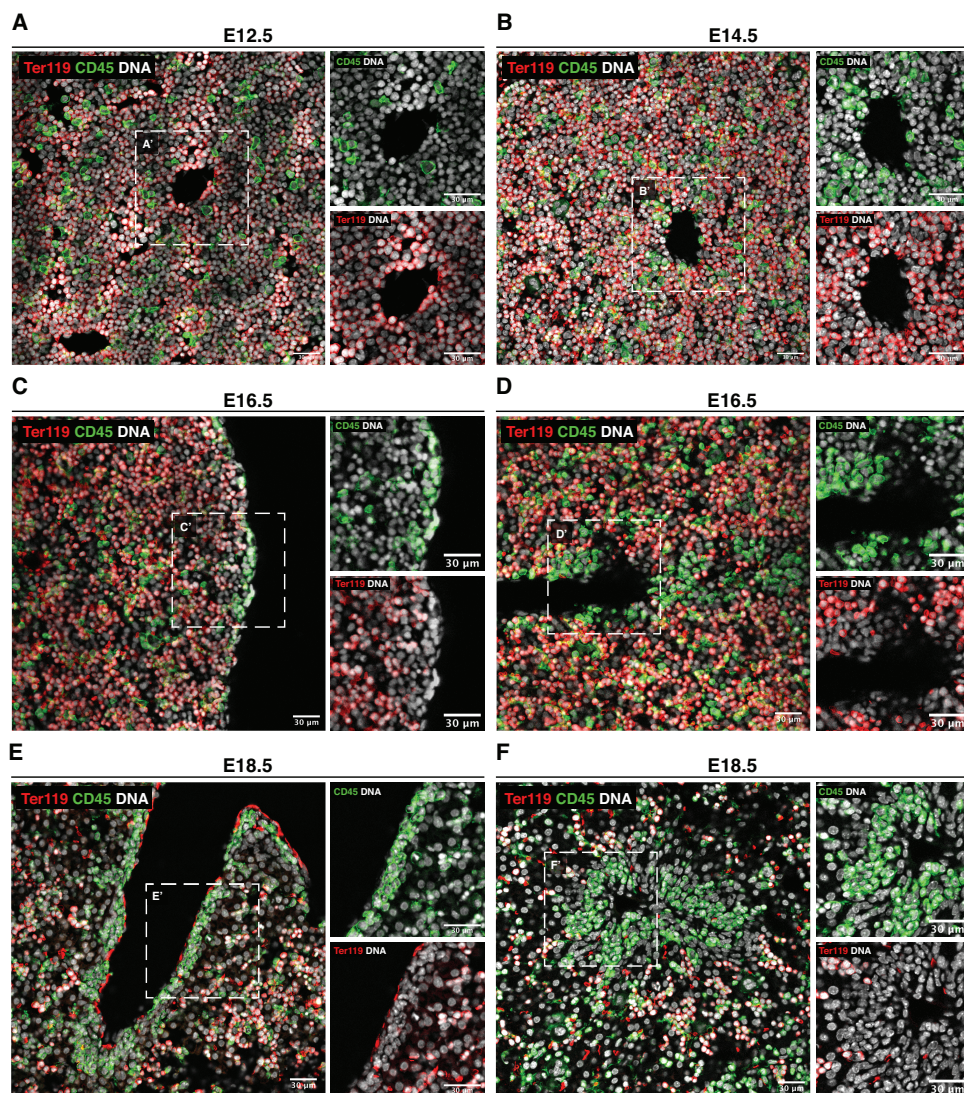


FIGURE 32 DISTRIBUTION OF HEMATOPOIETIC CELLS IN FETAL LIVER

(**A-F**) Immunostaining of E12.5 (**A**), E14.5 (**B**), E16.5 (**C-D**) and E18.5 (**E-F**) liver cryosections with the erythroid marker Ter119 (red) and the pan-hematopoietic marker CD45 (green). Scale bar 30 μm .

To investigate the localization of HSPCs, several surface markers are necessary. Current microscopy techniques usually allow imaging of up to 4 different markers, with the exception of recent protocols that allow visualization of eight (Coutu et al., 2017). To overcome this limitation, we used imaging mass cytometry that allowed the detection of 15 distinct markers in a single tissue section. Analysis of EpCAM expression allows the identification of newly formed bile ducts (**Figure 33A, top panel**). Ter119⁺ cells that are distributed along the tissue at E16.5 become restricted to vessels (**Figure 33A, middle panel**) as observed previously (**Figure 32**), and CD45⁺ cells accumulate around the vessels at E18.5 (**Figure 33A, middle panel**). These cells do not express the macrophage marker F4/80 but express Ly6c and Gr1, most likely representing monocytes and granulocytes (**Figure 33A, bottom panel**). Of note, accumulation of myeloid subsets can be observed at P7 in close association with EpCAM⁺ bile ducts (**Figure 33A, top and bottom panel**).

Remarkably, this technique allowed detection of phenotypic LT-HSCs that do not express lineage markers, are CD45⁺, Kit⁺, CD150⁺ and CD48⁻ (**Figure 33B**).

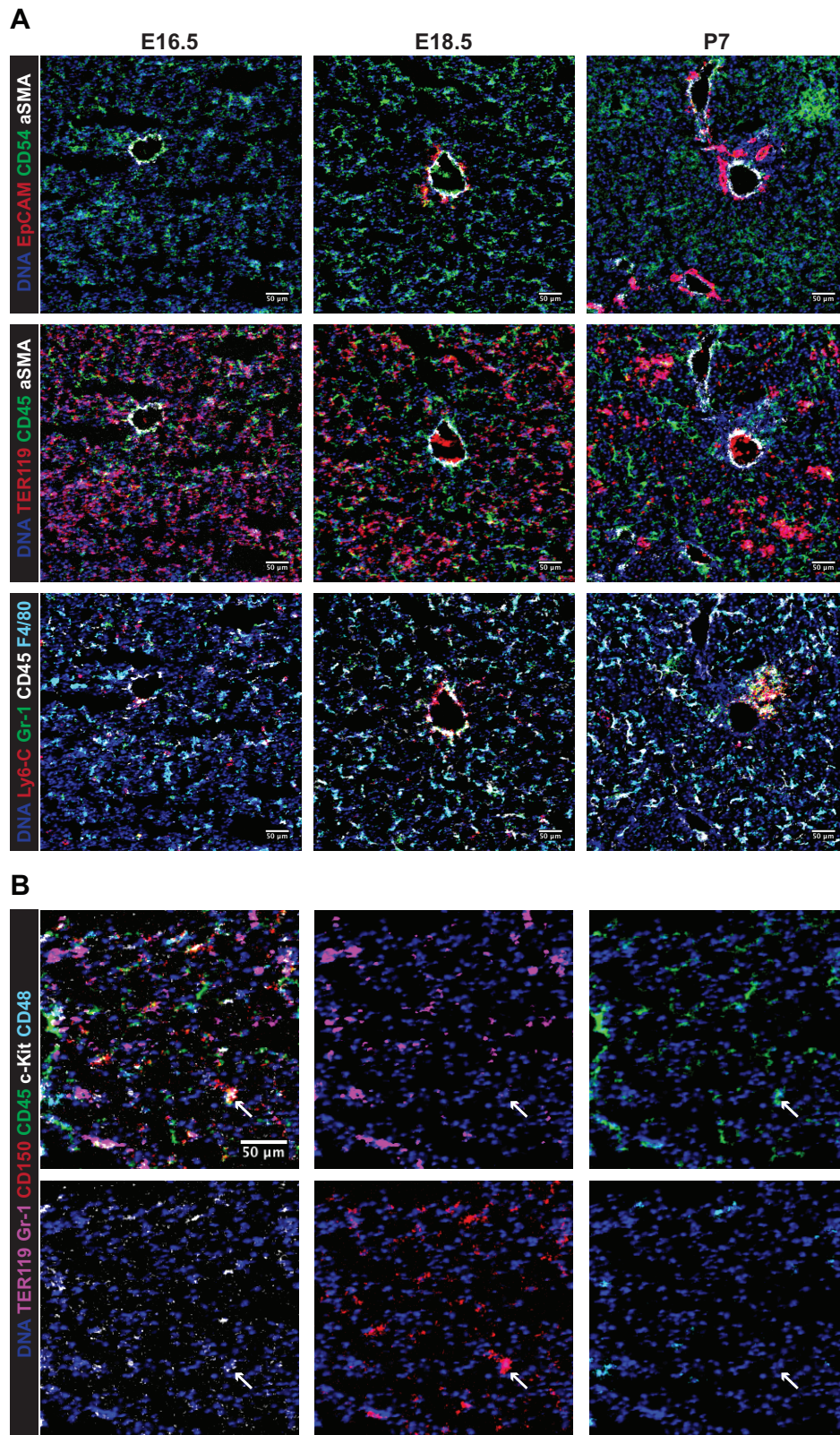


FIGURE 33 IMAGING MASS CYTOMETRY OF THE FL AT E16.5, E18.5 AND P7

(A) Imaging mass cytometry analysis of E16.5, E18.5 and post natal day (P)7 cryosections. Each row represents a single tissue section. In the same section it is possible to visualize the expression of CD326, CD54, α SMA, TER119, CD45, Ly6C, Ly6C/G (Gr-1) and F4/80. (B) Phenotypic hematopoietic

stem cells (HSCs) identified through their characteristic expression profile of CD45, CD150, Kit and absence of CD48 (arrow). Scale bar 50 μ m.

FUNCTIONAL ANALYSIS OF STROMAL SUBSETS

All stromal subsets analyzed with the exception of endothelial cells appear as promising regulators of the hematopoietic niche in the FL as hepatoblasts, Gp38⁺PDGFR α ⁻ and Gp38⁻PDGFR α ⁺ populations have a distinct cytokine expression pattern. Hepatoblasts, besides expressing *Kitl* and low levels of *Cxcl12*, express *Il7* and are the unique producers of *Epo*. To analyze the *in vivo* functional role of these cells a mouse model that allows early and specific targeting is needed. We analyzed three distinct transgenic mouse models that express *Cre* recombinase under the promoter of albumin: Tg(Alb1-Cre)1Khk, Tg(Alb1-Cre)21Mgn and Tg(Alb1-Cre)7Gsc (**Postic et al., 1999; Kellendonk et al., 2000; Parviz et al., 2002**). We crossed the three transgenic mice to Rosa26-RFP mice to analyze the level of recombination at E12.5 and E14.5 (**Supplementary Figure 12**). Both Tg(Alb1-Cre)1Khk and Tg(Alb1-Cre)21Mgn showed low levels of recombination (less than 10%) at these stages. On the other hand, Tg(Alb1-Cre)7Gsc mice showed that by E12.5, around 70% of hepatoblasts express RFP, making this a suitable model for further studies (**Supplementary Figure 12**).

DISCUSSION

Here, we performed a global description of the non-hematopoietic (Ter119⁻CD45⁻Kit⁻CD71⁻) populations present in the FL, based on the expression of CD324, CD31, Gp38 and PDGFR α , by gene expression and immunofluorescence analysis. The stromal compartment encompasses 3% of total FL cells, from which more than 80% are either CD324⁺ hepatoblasts or CD31⁺ endothelial cells, rendering rare subsets difficult to analyze. Within non-hematopoietic, non-hepatoblast and non-endothelial cells, 3 subsets were identified based on expression of Gp38, associated with bile ducts or mesothelial cells (**Schacht et al., 2005; Asahina et al., 2010**), and PDGFR α associated with mesenchymal cells (**Farahani et al., 2015**).

Cytokine gene expression analysis indicated that all subsets analyzed, with the apparent exception of endothelial cells, contribute with different signals to the hematopoietic compartment. While the chemoattractant *Cxcl12* was mostly produced by PDGFR α ⁺ cells as previously reported (**Khan et al., 2016**), *Kitl* was mostly expressed in Gp38⁺PDGFR α ⁻ cells, that have not been described to play a role in hematopoiesis up until now. Mesothelial cells, characterized by the expression of Gp38 and cytokeratins (**Asahina et al., 2009, 2011; Lua et al., 2016**), can be distinguished from cholangiocytes by the absence of the bile duct specific marker *Onecut1* (**Clotman et al., 2002**). Accordingly, Gp38⁺PDGFR α ⁻ cells present at E14.5 lack expression of *Onecut1* at both bulk and single-cell level and localize at the outermost layer of the FL.

In addition to the expression of *Kitl*, hepatoblasts are the only producers of *Epo* and *Il7*. Erythropoietin is mostly produced at early stages of FL development, E10.5 and E12.5, in agreement with the need of this hormone for the development of the erythroid compartment that shifts from the yolk sac to the FL (**Palis et al., 1999; McGrath et al., 2015**). *Il7* is produced at low levels in early time-points and reaches a peak of expression at E16.5, correlating with the need of a low IL-7 environment for the generation of the FL CLPs that give rise to the first wave of thymic seeding progenitors (TSP) (**Berthault et al., 2017**).

The vasculature is composed of endothelial cells that are surrounded by mural cells, which can be classified as smooth muscle cells or pericytes, the latter being referred to as hepatic stellate cells in the liver ("Hepatic stellate cell nomenclature.," **1996; Armulik et al., 2005**). PDGFR α ⁺ subsets (encompassing both Gp38^{+/low}PDGFR α ⁺ and Gp38⁻PDGFR α ⁺ cells) express the pericyte, *Kcnj8*, and the stellate-specific, *Lhx2*, *Ngfr* and *Desmin*, markers. Pericytes are characterized by the expression of NG2 and are associated with arterioles, venules and capillaries (**Ozerdem et al., 2001**). We found NG2 expression in cells surrounding

the major vessels in FL as described previously (**Khan et al., 2016**), but also in the mesothelium.

Hematopoietic cells are the major cell types found in FL until E18.5. While Ter119⁺ cells are evenly distributed throughout the FL parenchyma and around vessels, CD45⁺ cells form large clusters around major vessels. This phenomenon could explain the migration of HSPCs from the FL to the fetal BM and requires further addressing.

The observations herein described reinforce the hypothesis that the hematopoietic microenvironment in the FL is composed of various cell types, and suggest a regulatory circuit where multiple cell subsets contribute to hematopoiesis.

MATERIALS AND METHODS

Mice

C57BL/6J mice were purchased from Envigo. 6-8-week-old mice or timed pregnant females were used. Timed-pregnancies were generated after overnight mating, the following morning females with vaginal plug were considered to be at E0.5. Transgenic Tg(Alb1-Cre)^{1Khk} mice were obtained from the Mutant Mouse Regional Resource Center (MMRC, University of North Carolina), Tg(Alb1-Cre)^{21Mgn} mice were obtained from Institut Pasteur and Tg(Alb1-Cre)^{7Gsc} mice were kindly provided by Frédéric Lemaigre. All animal manipulations were performed according to the ethic charter approved by the French Agriculture ministry and to the European Parliament Directive 2010/63/EU.

Cell suspension

E14.5 FLs were dissected under a binocular magnifying lens. FLs were recovered in Hanks' balanced-salt solution (HBSS) supplemented with 1% fetal calf serum (FCS) (Gibco), cut in 1 mm² pieces and incubated with 0,05 mg/ml Liberase TH (Roche) for 6-8 minutes at 37°C. Enzymatic treatment was stopped by addition of cold HBSS + 1% FCS followed by centrifugation. FL were depleted of hematopoietic cells (Ter119⁺CD45⁺Kit⁺CD71⁺) using MACS columns (Miltenyi Biotec) according to manufacturer instructions. Briefly, for MACS columns depletion, cells were incubated with biotinylated Ter119, CD45, Kit and CD71 antibodies for 20-30 min at 4°C, washed, incubated with anti-biotin microbeads (Miltenyi Biotec) for 20 min at 4 °C and passed through a LS column (Miltenyi Biotec). The flow-through was collected and used for cell surface staining.

Flow cytometry and cell sorting

Cell suspensions were stained for 20-30 min at 4°C in the dark with antibodies listed in **Supplementary Table 9**. Dead cells were excluded by incorporation of Propidium Iodide. Stained cells were analyzed on a BD SYMPHONY (BD Biosciences) and were sorted with a BD FACSAria III (BD Biosciences) according to the guidelines for the use of flow cytometry and cell sorting (**Cossarizza et al., 2019**). Data were analyzed with FlowJo software (v.10.5.3, BD Biosciences).

Gene expression by RT-PCR

Cells were sorted directly into lysis buffer and mRNA was extracted (RNeasy Micro Kit (Qiagen), reverse-transcribed (PrimeScript RT Reagent Kit (Takara Bio), and quantitative PCR was performed with SYBR Master Mix (Applied Biosystems)(see **Supplementary Table 10** for primers). qPCR reactions were performed on a Quantstudio3 thermocycler (Applied Biosystems), gene expression was normalized to that of *Actb* and relative expression was calculated using the 2^{-ΔCt} method.

Multiplex single-cell qPCR

Single cells were sorted directly into 96-well plates loaded with RT-STA Reaction mix (CellsDirect™ One-Step qRT-PCR Kit, Invitrogen, according to the manufacturer's procedures) and 0.2x specific TaqMan® Assay mix (see Supplementary Table 4 for the TaqMan® assays list) and were kept at -80 °C at least overnight. For each subset analyzed, a control-well with 20 cells was also sorted. Pre-amplified cDNA (20 cycles) was obtained according to manufacturer's note and was diluted 1:5 in TE buffer for qPCR. Multiplex qPCR was performed using the microfluidics Biomark HD system for 40 cycles (Fluidigm) as previously described (Chea et al., 2016). The same TaqMan probes were used for both RT/pre-amp and qPCR (see **Supplementary Table 11** for probes). Only single cells for which at least 2 housekeeping genes could be detected before 20 cycles were included in the analysis.

Tissue collection for cryopreservation

E12.5, E14.5, E16.5, E18.5, P7 and P14 livers were collected directly into 2%PFA fixative buffer and kept at 4°C overnight. After washing, tissues were incubated with 0.12M Phosphate buffer with 15% sucrose overnight at 4°C, and embedded in 0.12M Phosphate buffer with 15% sucrose and 7.5% gelatin, at 37°C. Gelatin embedded tissues were frozen in 2-methylbutane cooled in liquid nitrogen and stored at -80°C until sectioning.

Histological processing and immunofluorescence staining

30µm frozen sections were placed in PBS and heated at 37°C for 5-7 min, to remove the gelatin remnants and then rinsed in PBS. When targeting cytoplasmic or nuclear antigens, permeabilization with 0.2 or 1% Triton X (Sigma-Aldrich) was performed, respectively, for 20 min. When the primary antibody used was biotinylated, blocking with Streptavidin/Biotin Blocking kit (SP-2002, Vector Laboratories) was performed. For primary antibodies raised in mouse, the Vector M.O.M.™ kit (BMK-2202, Vector Laboratories) was used to block endogenous mouse immunoglobulins, following manufacturer's recommendations. For antibodies not raised in mouse, blocking was performed with 10% donkey serum, 0.05% Tween-20 in PBS for 1h at Room Temperature (RT). Sections were incubated with primary antibodies overnight at 4°C and incubated with secondary antibodies for 1 hour at RT (see **Supplementary Table 12** for antibodies). For biotinylated primary antibodies, a 15 min incubation with streptavidin was performed. Lastly, sections were incubated with Hoechst (Cat. 5664877, BD Pharmingen) for 45 min and mounted with Fluoroshield™ (F6182, Sigma-Aldrich). Between each incubation step, sections were rinsed 3 times with 0.05% Tween-20 in PBS. Images were acquired on a Leica TCS SP5 using a 40x oil or water immersion lens or 63x oil or glycerol immersion lens. Scans were acquired at 200Hz, bidirectional mode, with z spacing of ranging from 1-2.5µm. Images were acquired at 8- or 12-bits and 1024×1024

resolution. Images were then analyzed using FIJI. Gamma treatment (range 0.3-0.5) and background subtraction were performed.

Hyperion

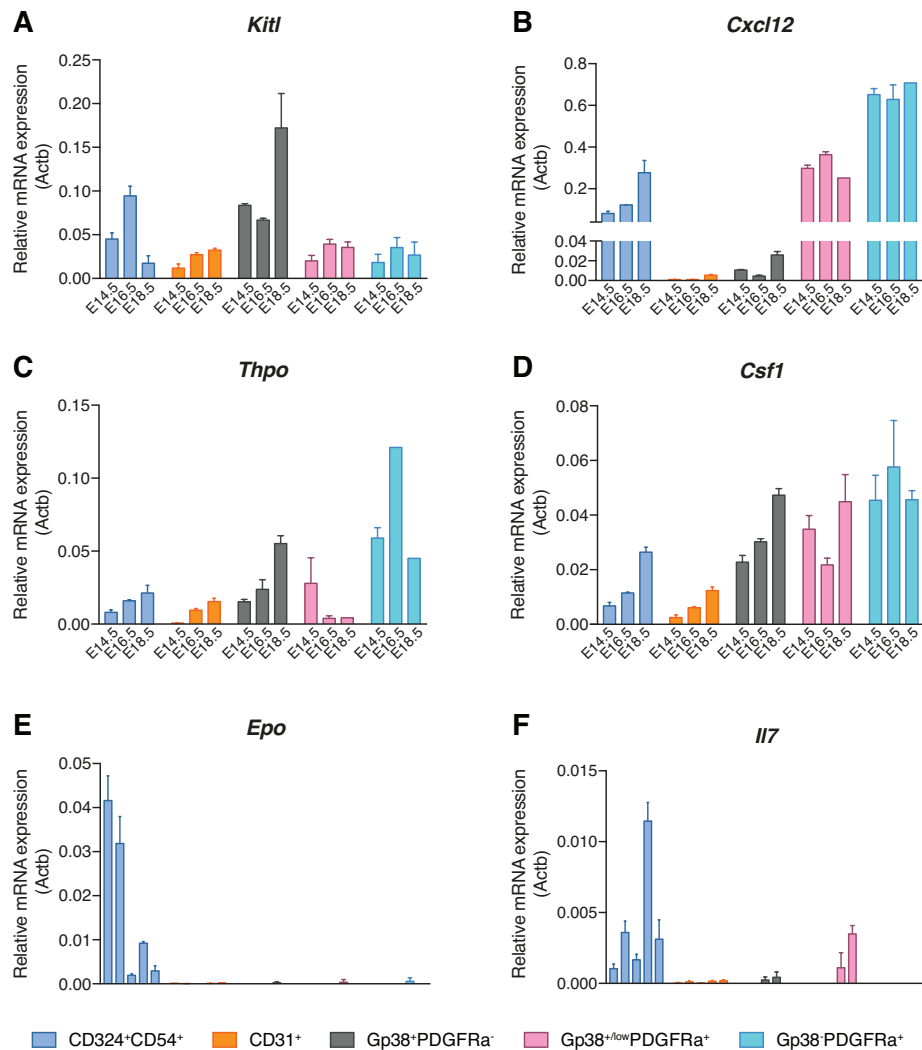
8µm frozen sections were rinsed 3 times in DPBS for 5 min at RT, followed by blocking with 3% BSA in DPBS for 45 min at RT. Sections were then incubated with the antibody cocktail, diluted in 0.5% BSA in PBS, at 4°C overnight (see **Supplementary Table 13** for antibodies). After incubation with antibodies, sections were rinsed twice in 0.1% Triton-X in DPBS for 8 min, followed by two washes in DPBS for 8 min on a shaker. Sections were stained with Ir-Intercalator for 30 min at RT, followed by a rinse in water for 5 min. For IMC image acquisition, immunostained and dried tissue slides were inserted into the ablation chamber of the Hyperion Imaging System. Regions of interest were selected based on hematoxylin and eosin stains performed on consecutive tissue sections after which areas of 3,000 × 2,300 µm were ablated and acquired at 200 Hz. Data was exported as MCD files and visualized using the Fluidigm MCD™ viewer. In order to better separate antibody signal and noise, each marker was visually inspected and a minimum signal threshold of 1 or 2 dual counts was set in the Fluidigm MCD™ viewer. Individual TIFF files were exported and analyzed using FIJI.

Bioinformatic Analysis

Gene expression raw data (BioMark™, Fluidigm) of single cells was normalized with *Gapdh* and *Actb*. Heatmaps and hierarchical clustering were generated using R packages “pheatmap” and “Rphenograph” (**Levine et al., 2015**).

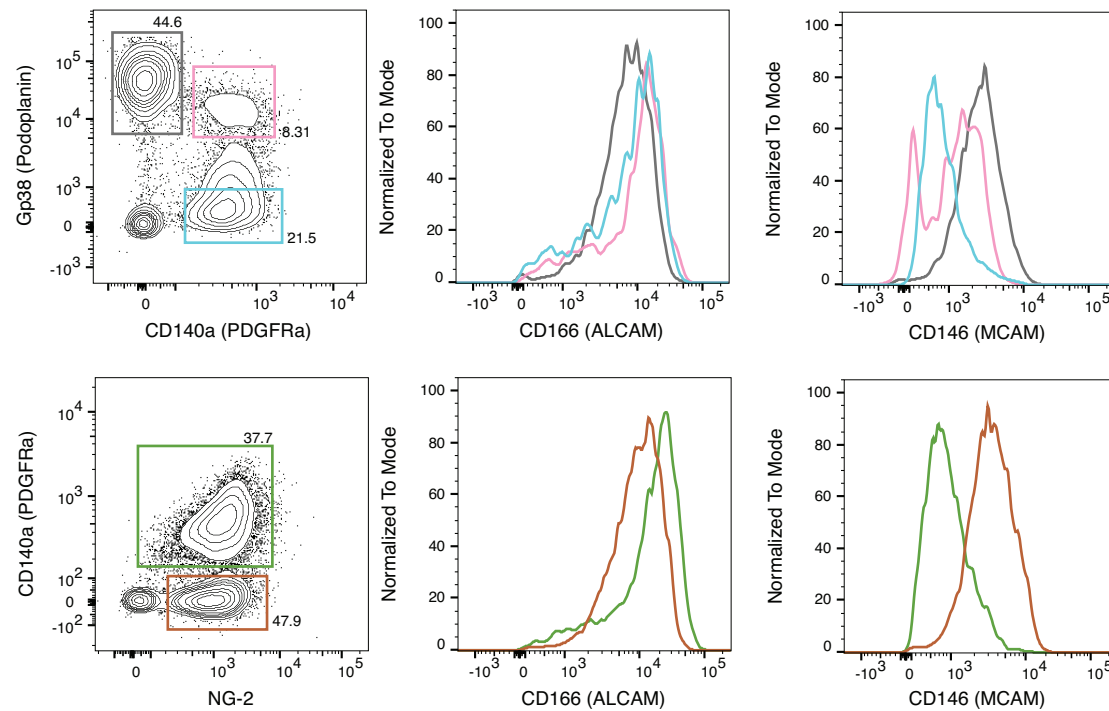
SUPPLEMENTARY INFORMATION

SUPPLEMENTARY FIGURES



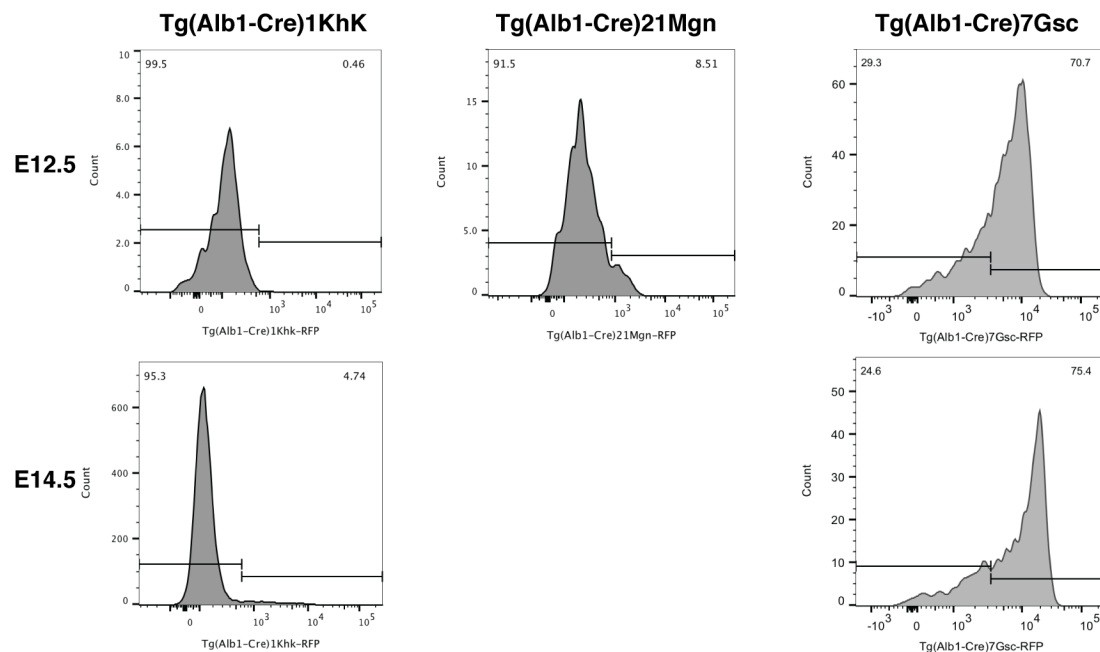
SUPPLEMENTARY FIGURE 10 CYTOKINE EXPRESSION IN FL STROMAL SUBSETS

(A-F) Gene expression analysis of the hematopoietic cytokines *Kitl* (A), *Cxcl12* (B), *Thpo* (C), *Csf1* (D), *Epo* (E) and *Il7* (F) at E14.5, E16.5 and E18.5 FL stages. Relative mRNA expression was compared to that of *Actb*.



SUPPLEMENTARY FIGURE 11 FLOW CYTOMETRY ANALYSIS OF E14.5 FL STROMA

Flow cytometry analysis of E14.5 FL. Top panel shows ALCAM and CD146 expression in Gp38⁺PDGFRα⁻, Gp38^{+/low}PDGFRα⁺ and Gp38⁻PDGFRα⁺ cells. Bottom panel shows ALCAM and CD146 expression in NG2⁺PDGFRα⁺ and NG2⁺PDGFRα⁻ cells. NG2⁺PDGFRα⁻ (brown) most likely represent Gp38⁺PDGFRα⁻ cells as they share the same ALCAM and CD146 profile.



SUPPLEMENTARY FIGURE 12 RFP EXPRESSION IN ALB-CRE ROSA26-RFP MICE

Flow cytometry analysis of E12.5 and E14.5 FL showing RFP expression in CD324⁺ cells using Tg(Alb1-Cre)1Khk, Tg(Alb1-Cre)21Mgn and Tg(Alb1-Cre)7Gsc mice crossed to Rosa26-RFP.

SUPPLEMENTARY TABLES

SUPPLEMENTARY TABLE 9 LIST OF ANTIBODIES USED FOR FLOW CYTOMETRY

Protein	Clone	Fluorochrome	Manufacturer	Catalog #
CD140a	eBioALC48	PE-Cy7	eBioscience	17-1661-82
CD140b	APB5	APC	Biolegend	136007
CD144	BV13	BV421	SONY	1290065
CD146	ME-9F1	FITC	Miltenyi	130-102-230
CD146	ME-9F1	BV510	BD Biosciences	740200
CD166	eBioALC48	APC	Invitrogen	25-1401-82
CD24	M1/69	BV510	SONY	1109155
CD24	M1/69	PE-Cy7	BD Biosciences	560536
CD31	MEC13.3	BUV737	BD Biosciences	565097
CD31	390	PerCP-Cy5.5	Biolegend	102420
CD31	MEC13.3	AF488	Biolegend	102513
CD324	DECMA-1	PE-Cy7	SONY	1336545
CD324	DECMA-1	FITC	Invitrogen	53-3249-82
CD324	DECMA-1	PE-Cy7	SONY	1336545
CD326	G8.8	APC	BD Biosciences	563478
CD51	RMV-7	BV421	BD Biosciences	740062
CD54	YN1/1.7.4	Pacific Blue	Biolegend	116116
CD54	YN1/1.7.4	PE	Biolegend	116108
Gp38	8.1.1	PE	BD Biosciences	566390
Gp38	8.1.1	APC-Cy7	SONY	1237090
Gp38	8.1.1	APC	eBioscience	50-5381-82
Kit	2B8	APC-Cy7	SONY	1129130
Kit	2B8	APC	SONY	1129060
Kit	2B8	Pacific Blue	Biolegend	105827
NG2	1E6.4	PE	Miltenyi	130-100-468
Sca1	D7	BV711	SONY	1140655
Streptavidin	-	BV786	SONY	2626245

SUPPLEMENTARY TABLE 10 LIST OF PRIMERS FOR QRT-PCR

Gene	Gene ID	Primer FW	Primer RV	PrimerBank ID
Acta2	11475	GTCCCAGACATCAGGGAGTAA	TCGGATACTTCAGCGTCAGGA	6671507a1
Csf1	12977	GTGTCAGAACACTGTAGCCAC	TCAAAGGCAATCTGGCATGAAG	166064045c1
Cxcl12	20315	TGCATCAGTGACGGTAAACCA	TTCTTCAGCCGTGCAACAATC	7305465a1
Des	13346	GTGGATGCAGCCACTCTAGC	TTAGCCGCGATGGTCTCATAC	33563250a1
Epo	13856	ACAAAGCCATCAGTGGTCTACG	TCTGGAGGCGACATCAATTCC	113931667c2
Il7	16196	TTCCTCCACTGATCCTTGTCT	AGCAGCTTCCTTTGTATCATCAC	6680433a1
Kcnj8	16523	CTCTTCGCTATCATGTGGTGG	AACGCAGACGTGAATGACCTG	145966749c3
Kitl	17311	GAATCTCCGAAGAGGCCAGAA	GCTGCAACAGGGGGTAACAT	198596a1
Krt19	16669	GACCTAGCCAAGATCCTGAGT	TCAGCTCCTCAATCCGAGCA	55741635c2
Lhx2	16870	CTGTTCCACAGTCTGTCGGG	CAGCAGGTAGTAGCGGTCAG	6754538a1
Nes	18008	AGAGTCAGATCGCTCAGATCC	GCAGAGTCCTGTATGTAGCCAC	50363231c1
Ngfr	18053	CTAGGGGTGTCCTTTGGAGGT	CAGGGTTCACACACGGTCT	15082265a1
Onecut1	15379	GCCCTGGAGCAAACCTCAAGT	TTGGACGGACGCTTATTTTCC	118130856c2
Thpo	21832	AACCCAGACGGAACAGAGC	GTGCTGCCATCACTCCCTC	291045236c3
Actb	11461	GCTTCTTTGCAGCTCCTTCGT	ATCGTCATCCATGGCGAACT	

SUPPLEMENTARY TABLE 11 LIST OF TAQMAN PROBES FOR SINGLE-CELL MULTIPLEX GENE EXPRESSION

Gene	Gene ID	Taqman Assay ID
Acta2	11475	Mm01546133_m1
Afp	11576	Mm00431715_m1
Angpt1	11600	Mm00456503_m1
Angpt2	11601	Mm00545822_m1
Cdh1	12550	Mm01247357_m1
Csf1	12977	Mm00432686_m1
Csf2	12981	Mm01290062_m1
Csf3	12985	Mm00438334_m1
Cspg4	121021	Mm00507257_m1
Cxcl12	20315	Mm00445553_m1
Des	13346	Mm00802455_m1
Epo	13856	Mm01202755_m1
Flt3l	14256	Mm00442801_m1
Fn1	14268	Mm01256744_m1
Hnf4a	15378	Mm01247712_m1
Igf2	16002	Mm00439564_m1
Il7	16196	Mm00434291_m1
Kdr	16542	Mm01222421_m1
Kitl	17311	Mm00442972_m1
Krt18	16668	Mm01601704_g1
Krt19	16669	Mm00492980_m1
Lepr	16847	Mm00440181_m1
Lgr5	14160	Mm00438890_m1
Lhx2	16870	Mm00839783_m1
mKi-67	17345	Mm01278617_m1
Msx2	17702	Mm00442992_m1
Nes	18008	Mm00450205_m1
Onecut1	15379	Mm00839394_m1
Pdgfra	18595	Mm00440701_m1
Runx1	12394	Mm01213404_m1
Tek	21687	Mm00443243_m1
Thpo	21832	Mm00437040_m1
Vcan	13003	Mm01283063_m1
Vim	22352	Mm01333430_m1
Actb	11461	Mm01205647_g1
Gapdh	14433	Mm99999915_g1
Hprt	15452	Mm03024075_m1

SUPPLEMENTARY TABLE 12 LIST OF ANTIBODIES FOR IHC

Antibody	Conjugation	Clone	Catalog #	Company	Species
aSMA	-	1A4	A5228	Sigma	Mouse
Kit	FITC	2B8	11-1171-82	eBioscience™	Rat
CD105	PE	MJ7/18	120408	BioLegend	Rat
CD106	PE	429	105713	BioLegend	Rat
CD11b	Biotin	M1/70	103203	BioLegend	Rat
CD11b	APC	M1/70	17-0112-82	eBioscience™	Rat
CD11c	Biotin	N418	108703	BioLegend	Hamster
CD140a	-	APA5	16-1410-82	eBioscience™	Rat
CD140a	-	APA5	AF1062	R&D	Goat
CD140b	PE	APB5	136005	BioLegend	Rat
CD144	APC	eBioBV13	17-1441-80	eBioscience™	Rat
CD146	-	poly	AF6106	R&D	Sheep
CD166	-	eBioALC48	14-1661-81	eBioscience™	Rat
CD166	FITC	eBioALC48	11-1661-80	eBioscience™	Rat
CD166	APC	eBioALC48	17-1661-80	eBioscience™	Rat
CD19	Biotin	6D5	108403	BioLegend	Rat
CD29	-	KM16	14-0292	Sigma-Aldrich	Rat
CD31	-	MEC13.3	553370	BD Pharmingen	Rat
CD3e	Biotin	145-2-c11	103103	BioLegend	Hamster
CD45	-	poly	AF114	R&D	Goat
CD45	Biotin	30-F11	103103	BioLegend	Rat
CD45R	Biotin	RA3-6B2	100303	BioLegend	Rat
CD45R	FITC	RA3-6B2	103205	BioLegend	Rat
CD51	PE	RMV-7	104105	BioLegend	Rat
CD71	APC	RI7217	113820	BioLegend	Rat
CK19	-	Ks19.1	MA5-12663	ThermoFischer	Mouse
ColIV	-	poly	AB769	Millipore	Goat
E-Cad	-	24E10	3195	Cell Signaling	Rabbit
F4/80	FITC	BM8	11-4801-82	eBioscience™	Rat
Fibronectin	-	poly	F3648	Fibronectin	Rabbit
FLK1	PE	Avas12a1	555308	DB Pharmingen	Rat
Gp38	-	8.1.1	NB600-1015	Novus Biologicals	Hamster
Gp38	eFluor-660	eBio8.1.1	50-5381-82	eBioscience™	Hamster
Gr-1	Biotin	RB6-8C5	108403	BioLegend	Rat

Gr-1	PE	RB6-8C5	108408	BioLegend	Rat
HNF4a	-	K9218	MA1-199	ThermoFischer	Mouse
HNF6	-	poly	PA5-41478	ThermoFischer	Mouse
Laminin	-	poly	F9393	Sigma-Aldrich	Rabbit
NG2	-	poly	AB5320	Millipore	Rabbit
NG2	PE	1E6.4	130-100-468	Milteny Biotec	Rat
NK1.1	Biotin	PK136	101203	BioLegend	Mouse
Sca1	-	E12-161.7	553333	BD Pharmingen	Rat
Sca1	PE	D7	12-5981-81	eBioscience™	Rat
Ter119	-	Ter119	550565	BD Pharmingen	Rat
Ter119	Biotin	Ter119	116203	BioLegend	Rat
Vimentin	-	-	MS-129-P	ThermoFischer	Mouse

SUPPLEMENTARY TABLE 13 LIST OF ANTIBODIES USED FOR IMAGING MASS CYTOMETRY

Cyttof Metal	Element	Marker	Clone	Vendor	Catalogue Number
89	Y	Anti-Mouse CD45	30-F11	FLDM	3089005B
141	Pr	alphaSMA	1A4	FLDM	3141017D
145	Nd	CD31	TLD - 3A12	NOVUS	NB100-647965
146	Nd	F4/80	BM8	FLDM	3146008B
148	Nd	CD11b	M1/70	FLDM	3143015B
150	Nd	Ly-6C	HK1.4	FLDM	3150010B
152	Sm	CD48	polyclonal	Origene	TA351055
153	Eu	Wnt3a	3A6	Abcam	ab81614
157	Gd	CD150	9D1	Bio-Rad	MCA2274GA
158	Gd	CD34	QBEND/10	NOVUS	NB120-963
162	Dy	TER-119	TER-119	FLDM	3162003B
163	Dy	CD54 (ICAM-1)	YN1/1.7.4	FLDM	2311506
164	Dy	Ly-6A/E (Sca1) 7D3	D7	FLDM	3164005B
164	Dy	CD49f	G0H3	FLDM	3164006B
166	Er	CD326 (EpCAM)	G8.8	FLDM	3166014B
168	Er	Ki-67	B56	FLDM	31680078
171	Yb	CD44	IM7	FLDM	3171003B
173	Yb	CD117 (Kit)	2B8	FLDM	3173004B
174	Yb	Ly-6G/C	RB6-8C5	FLDM	3174008B
191/193	Ir	DNA Content	NA	FLDM	21008B

CHAPTER VI – GENERAL DISCUSSION AND PERSPECTIVES

The FL is the major hematopoietic organ during embryonic development. It does not harbor *de novo* HSC generation and is colonized by circulating progenitors (**Johnson et al., 1975; Houssaint, 1981**). These circulating cells are retained in the FL through cell adhesion-mediated processes and/or chemoattraction where they meet the conditions necessary for proliferation and differentiation (**Hayashi et al., 2019**). Whether the extensive expansion capacity of HSPCs in FL is cell-intrinsic or influenced by the microenvironment is still unclear. A cell-intrinsic process would most likely correlate with the ability of these cells to expand in sites other than the FL, which is not the case, pointing towards a combination of cell-autonomous and extrinsic mechanisms.

The microenvironment of the FL, encompassing cellular and extracellular components, has been difficult to assess and studies analyzing the *in vivo* role of distinct cell types in HSC maintenance and proliferation have only recently been reported (**Khan et al., 2016; Lee et al., 2021**). Here, we attempted to characterize the contribution of the FL non-hematopoietic compartment at E14.5, the peak of HSC expansion (**Ema et al., 2000**). Hematopoietic cells, most of which Ter119⁺ erythrocytes, compose more than 80% of total FL cells at E14.5, rendering it difficult to identify and analyze rare stromal subsets. Unbiased analysis of the FL stromal compartment required therefore optimization of a set of procedures. Depletion of Ter119⁺ and CD45⁺ cells led to an increase from 15 to 90% of the Ter119⁻CD45⁻ compartment, without a selective loss of stromal cells. Enzymatic digestion of the FL proved essential for obtaining live cells for analysis, especially after E16.5. Ter119⁻CD45⁻ cells analyzed with cell markers previously used in FL and BM studies (**Nitou et al., 2002; Chan et al., 2009; Tanaka et al., 2009; Flygare et al., 2011; Khan et al., 2016; Kusumbe et al., 2016**), allowed the identification of epithelial, endothelial and mesenchymal cells, and three populations, P1, P2 and P3, of unidentified lineage affiliation.

Transcriptomic analysis indicated that among the highest expressed genes in P2 cells were *Klf1*, *Epor*, *Gata1*, *Runx3* and *c-Myb*, all hematopoietic genes. P1, P2 and P3 cells expressed transcripts associated to the erythroid lineage, even though they did not express the pan-hematopoietic marker CD45, the erythroid marker Ter119, nor hemoglobin. P1 and P2 cells originated erythroid progeny after *in vitro* differentiation (BFU-E and CFU-E), and P2 cells originated Ter119⁺ erythrocytes after *in utero* injections. In the adult hematopoietic system, CD45 is expressed by all hematopoietic lineages with the exception of erythrocytes and platelets (**Thomas et al., 1988**). Nevertheless, CD45 is expressed in erythroid progenitors with BFU-E and CFU-E activity, being downregulated as cells enter the erythroblast stage and upregulate the erythroid marker Ter119 (**Kina et al., 2000; Chen et al., 2009; Li et al., 2014**;

Liu et al., 2015). CD45⁺Ter119⁺ erythroid progenitors have been described in the BM, however they do not form colonies in methylcellulose and require stromal cells for their maintenance, therefore not corresponding to the population here identified in the FL (**Boulais et al., 2018**).

Analysis of c-Myb^{-/-} mice that have primitive but lack definitive erythropoiesis (**Mucenski et al., 1991**), showed that P1 and P2 cells are absent, and therefore require c-Myb. P1, P2 and P3 cells did not express *Hbb-y* and P3 cells expressed low levels of embryonic *Hbb-bh1* and adult *Hbb-b1* globins, indicating they were of definitive origin. Lineage tracing of EMP-derived hematopoiesis showed that P1, P2 and P3 cells are labeled at the same frequency as microglia, indicating they are EMP-derived. The decreasing frequency of labeled P1, P2 and P3 cells in FL and concomitant increase of erythroblast labeling in blood is consistent with a fast differentiation coupled with egression of erythroid progenitors into circulation and these cells are still detected at high levels by the end of gestation.

Lineage-tracing models showed that HSC-derived erythroblasts or erythrocytes were not observed before E18.5, suggesting that HSCs do not contribute significantly to the erythroid compartment until birth. By contrast, non-erythroid CD45⁺Kit⁺ mature FL hematopoietic cells do not appear to be preferentially originated from EMP or HSC. Consequently, this data suggests that *in vivo* embryonic HSC differentiation is skewed. Moreover, we show that the low levels of EPO in the FL modulate the differentiation pattern of HSCs. HSC-derived MEPs have more than 2-fold lower levels of *EpoR* and required 10-fold higher concentrations of EPO for differentiation when compared to YS-derived P2 cells. GATA-1 is considered the “master” regulator of erythropoiesis as it transcriptionally regulates most aspects of erythroid maturation and function, including regulation of *EpoR* expression (**Zon et al., 1991**; **Ferreira et al., 2005**). We showed that HSC-derived CMPs or MEPs have 3-fold and 2-fold lower levels of *Gata1* than P1 and P2 cells, respectively, thus providing mechanistic insights for the differential regulation of *EpoR* expression. Erythropoietin is the principal growth factor that promotes the survival, proliferation and differentiation of erythroid progenitor cells (**Koury et al., 1988**). In the absence of EPO, erythroid progenitors enter apoptosis since EPO controls the survival of these cells by inhibition of apoptosis through Bcl-X_L and Bcl-2 proteins (**Koury et al., 1990a, 1990b**; **Silva et al., 1996**). The low levels of EPO in FL and low sensitivity of HSC-derived MEPs could result in apoptosis of the latter and provide a selective advantage to erythroid progenitors of YS origin.

The FL microenvironment has been mostly studied to understand the mechanisms involved in HSC expansion, despite the evidence for a wider implication in fetal hematopoiesis. It has been recently demonstrated that low levels of IL-7, a cytokine crucial for B cell development, favors the generation of B1 cells in FL, highlighting the impact of the FL microenvironment on the preferential production of particular cell types (**Wong et al., 2019**).

The stromal compartment of the FL is however, poorly characterized. Hematopoietic cells represent roughly 97% of total FL cells at E14.5, rendering rare subsets difficult to assess. Analysis of FL non-hematopoietic (CD45⁻Ter119⁻CD71⁻Kit⁻) cells identified hepatoblasts, endothelial cells, mesothelial cells and vessel-lining mural cells. All expressed relevant but distinct patterns of hematopoietic cytokines. *Epo* and *Il7* transcripts are almost exclusively expressed by hepatoblasts, with *Epo* being mostly expressed before E14.5. It has been described that the regulation of *Epo* at early stages of development is retinoic acid-dependent and that it becomes hypoxia dependent after E11.5 (**Makita, 2001**). This differential regulation allows the early expression of this hormone important for the establishment of definitive erythropoiesis in FL, and possibly explains the drastic decrease after E12.5. *Il7* levels, in contrast, remain low until E16.5, consistent with the requirement of a low IL-7 environment for the generation of the FL CLPs that give rise to the first wave of thymic seeding progenitors (TSP) (**Berthault et al., 2017**). As described previously, we found the highest levels of the chemoattractant *Cxcl12* transcript in PDGFR α ⁺ cells (**Khan et al., 2016; Lee et al., 2021**). In the BM, *Kitl* is mostly expressed by endothelial and Lepr⁺ perivascular cells (**Ding et al., 2012; Asada et al., 2017**), while we observed little expression of *Kitl* by endothelial or PDGFR α ⁺ cells in FL. At E14.5 this cytokine is mostly expressed by Gp38⁺PDGFR α ⁻ cells and at lower levels by hepatoblasts as shown previously (**Chou et al., 2010; Sugiyama et al., 2011**). Of notice, Gp38⁺Pdgfr α ⁻ cells had not been analyzed in the context of hematopoiesis previously. Single-cell gene expression analysis confirmed *Kitl* expression by all cells and showed that although these cells express the ductal marker *Krt19*, they do not express the cholangiocyte transcription factor *Onecut1* (**Clotman et al., 2002**), and therefore did not belong to this lineage. *In situ* analysis of Gp38 expressing cells further confirmed their localization in the mesothelium, lining the hepatic lobes. Recently it has been described that *Kitl* expression in FL endothelial and hepatic stellate cells is important for the maintenance of HSCs in P0 livers, however *Kitl* depletion from these cells showed no defect up until E15.5 (**Lee et al., 2021**), suggesting they are not the major producers during embryonic development. Our analysis of CD31⁺ endothelial cells showed little contribution to cytokine production in the FL even though they constitute the most frequent non-hematopoietic population from E10.5 onward. The vasculature in the FL comprises endothelial cells that line the sinusoids and the major vessels. Whether distinct subsets of endothelial cells produce varying levels of cytokines and were diluted in our analysis needs further addressing.

Various studies showed hematopoietic cytokine production by hepatoblasts, although the role of these cells *in vivo* has not been properly addressed. Transgenic mice with specific Cre recombination in hepatocytes have been described (**Postic et al., 1999; Kellendonk et al., 2000; Parviz et al., 2002**). However, only few models allow early Cre recombination and thus

the deletion of specific factors from early development stages. The use of unsuitable transgenic mice can lead to misleading conclusions and demands careful consideration as we demonstrated for the three Alb^{Cre} mice analyzed, of which only the Tg(Alb1-Cre)7Gsc showed sufficient levels of recombination at E12.5 (around 70% of hepatoblasts). This model will be useful for accurate analysis of the roles of hepatoblasts in embryonic hematopoiesis. Another important cell type whose part will be addressed *in vivo* is the Gp38⁺ mesothelial cell population. These cells have been shown to originate hepatic stellate cells (**Asahina et al., 2011**), and their role in hematopoiesis has so far been ignored. Interestingly, we find CD45⁺ cells in close association with the mesothelium from E12.5 onwards. Accumulation of these cells in mesothelium and close to major vessels is more pronounced after E16.5, however, their lineage affiliation is still unknown. CD150⁺Lin⁻ HSPCs have been found in close association with the portal vessels and adjacent to sinusoidal endothelial cells at postnatal stages (**Khan et al., 2016; Lee et al., 2021**), but their localization in early FL stages when these cells are actively cycling has not been assessed. We show that HNF4 α ⁺ hepatoblasts are evenly distributed along the parenchyma, which is filled by sinusoids at all time-points. PDGFR α ⁺ cells are found lining the sinusoids and are therefore also present along the parenchyma, but also accumulate in major vessels, co-expressing the pericyte marker NG-2. Of note, pericytes are usually associated with the microvasculature in other organs, but in the FL these cells appear close to major vessels (**Khan et al., 2016**) and NG-2 is also expressed by the mesothelium. Our observations point to a situation where distinct cells provide signals to hematopoietic cells and reinforces the hypothesis in which multiple cell types contribute to HSPC proliferation and/or differentiation.

The *in vivo* role of the microenvironment can be addressed by distinct viewpoints. Targeted cell ablation studies can be used to demonstrate a functional role of a specific cell type. However, and because we are interested in embryonic hematopoiesis, the effects observed could result from a disturbed development of the organ and indirect effect in hematopoietic cells. Deletion of specific signals from candidate cell types appears to be a more reasonable approach as it precisely evaluates the effects of the signal deleted either directly in hematopoietic cells or through indirect mechanisms involving other cell types. The latter approach is highly dependent on the existence of a candidate signal to be deleted. For instance, the role of hepatoblasts in YS-derived definitive erythropoiesis could be assessed by deletion of Epo from these cells. On the other hand, identification of the FL niche cells and the signals that promote HSC expansion can be more difficult as we do not currently know whether the cytokines and signals described in BM are responsible for FL HSC proliferation. Understanding the mechanisms underlying HSC expansion in FL will thus require a systematic approach of distinct populations, together with insights from cell-autonomous mechanisms.

REFERENCES

- Adolfsson, J., Månsson, R., Buza-Vidas, N., Hultquist, A., Liuba, K., Jensen, C. T., Bryder, D., Yang, L., Borge, O. J., Thoren, L. A. M., Anderson, K., Sitnicka, E., Sasaki, Y., Sigvardsson, M., & Jacobsen, S. E. W. (2005). Identification of Flt3⁺ lympho-myeloid stem cells lacking erythro-megakaryocytic potential: A revised road map for adult blood lineage commitment. *Cell*, 121(2), 295–306. <https://doi.org/10.1016/j.cell.2005.02.013>
- Aisen, P. (2004). Transferrin receptor 1. *The International Journal of Biochemistry & Cell Biology*, 36(11), 2137–2143. <https://doi.org/10.1016/j.biocel.2004.02.007>
- Ajami, B., Bennett, J. L., Krieger, C., Tetzlaff, W., & Rossi, F. M. V. (2007). Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nature Neuroscience*, 10(12), 1538–1543. <https://doi.org/10.1038/nn2014>
- Akashi, K., Traver, D., Miyamoto, T., & Weissman, I. L. (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature*, 404(6774), 193–197. <https://doi.org/10.1038/35004599>
- Alexander, W. S., Roberts, A. W., Nicola, N. A., Li, R., & Metcalf, D. (1996). Deficiencies in progenitor cells of multiple hematopoietic lineages and defective megakaryocytopoiesis in mice lacking the thrombopoietic receptor c-Mpl. *Blood*, 87(6), 2162–2170. <http://www.ncbi.nlm.nih.gov/pubmed/8630375>
- Alvarez-Silva, M., Belo-Diabangouaya, P., Salaün, J., & Dieterlen-Lièvre, F. (2003). Mouse placenta is a major hematopoietic organ. *Development*, 130(22), 5437–5444. <https://doi.org/10.1242/dev.00755>
- Ara, T., Tokoyoda, K., Sugiyama, T., Egawa, T., Kawabata, K., & Nagasawa, T. (2003). Long-Term Hematopoietic Stem Cells Require Stromal Cell-Derived Factor-1 for Colonizing Bone Marrow during Ontogeny. *Immunity*, 19(2), 257–267. [https://doi.org/10.1016/S1074-7613\(03\)00201-2](https://doi.org/10.1016/S1074-7613(03)00201-2)
- Armulik, A., Abramsson, A., & Betsholtz, C. (2005). Endothelial/Pericyte Interactions. *Circulation Research*, 97(6), 512–523. <https://doi.org/10.1161/01.RES.0000182903.16652.d7>
- Asada, N., Kunisaki, Y., Pierce, H., Wang, Z., Fernandez, N. F., Birbrair, A., Ma'ayan, A., & Frenette, P. S. (2017). Differential cytokine contributions of perivascular haematopoietic stem cell niches. *Nature Cell Biology*, 19(3), 214–223. <https://doi.org/10.1038/ncb3475>
- Asahina, K. (2012). Hepatic stellate cell progenitor cells. *Journal of Gastroenterology and Hepatology*, 27(Suppl 2), 80–84. <https://doi.org/10.1111/j.1440-1746.2011.07001.x>
- Asahina, K., Tsai, S. Y., Li, P., Ishii, M., Jr, R. E. M., Henry, M., & Tsukamoto, H. (2010). Mesenchymal Origin of Hepatic Stellate Cells, Submesothelial Cells, and Perivascular Mesenchymal Cells During Mouse Liver Development. *Hepatology*, 49(3), 998–1011. <https://doi.org/10.1002/hep.22721.Mesenchymal>
- Asahina, K., Tsai, S. Y., Li, P., Ishii, M., Maxson, R. E., Sucov, H. M., & Tsukamoto, H. (2009). Mesenchymal origin of hepatic stellate cells, submesothelial cells, and perivascular mesenchymal cells during mouse liver development. *Hepatology*, 49(3), 998–1011. <https://doi.org/10.1002/hep.22721>
- Asahina, K., Zhou, B., Pu, W. T., & Tsukamoto, H. (2011). Septum transversum-derived mesothelium gives rise to hepatic stellate cells and perivascular mesenchymal cells in developing mouse liver. *Hepatology*, 53(3), 983–995. <https://doi.org/10.1002/hep.24119>
- Azzoni, E., Frontera, V., McGrath, K. E., Harman, J., Carrelha, J., Nerlov, C., Palis, J., Jacobsen, S. E. W., & Bruijn, M. F. (2018). Kit ligand has a critical role in mouse yolk sac and aorta–gonad–mesonephros hematopoiesis. *EMBO Reports*, 19(10), 1–17. <https://doi.org/10.15252/embr.201745477>
- Barber, C. L., Montecino-Rodriguez, E., & Dorshkind, K. (2011). Reduced production of B-1-specified common lymphoid progenitors results in diminished potential of adult marrow to generate B-1 cells. *Proceedings of the National Academy of Sciences*, 108(33), 13700–13704. <https://doi.org/10.1073/pnas.1107172108>
- Barker, J. E. (1994). Sl/Sld hematopoietic progenitors are deficient in situ. *Experimental*

Hematology, 22(2), 174–177.

- Baumann, C. I., Bailey, A. S., Li, W., Ferkowicz, M. J., Yoder, M. C., & Fleming, W. H. (2004). PECAM-1 is expressed on hematopoietic stem cells throughout ontogeny and identifies a population of erythroid progenitors. *Blood*, 104(4), 1010–1016. <https://doi.org/10.1182/blood-2004-03-0989>
- Beaudin, A. E., Boyer, S. W., Perez-Cunningham, J., Hernandez, G. E., Derderian, S. C., Juijavarapu, C., Aaserude, E., MacKenzie, T., & Forsberg, E. C. (2016). A Transient Developmental Hematopoietic Stem Cell Gives Rise to Innate-like B and T Cells. *Cell Stem Cell*, 19(6), 768–783. <https://doi.org/10.1016/j.stem.2016.08.013>
- Becker, A. J., McCulloch, E. A., & Till, J. E. (1963). Cytological Demonstration of the Clonal Nature of Spleen Colonies Derived from Transplanted Mouse Marrow Cells. *Nature*, 197(4866), 452–454. <https://doi.org/10.1038/197452a0>
- Benz, C., Copley, M. R., Kent, D. G., Wohrer, S., Cortes, A., Aghaeepour, N., Ma, E., Mader, H., Rowe, K., Day, C., Treloar, D., Brinkman, R. R., & Eaves, C. J. (2012). Hematopoietic stem cell subtypes expand differentially during development and display distinct lymphopoietic programs. *Cell Stem Cell*, 10(3), 273–283. <https://doi.org/10.1016/j.stem.2012.02.007>
- Benz, C., Martins, V. C., Radtke, F., & Bleul, C. C. (2008). The stream of precursors that colonizes the thymus proceeds selectively through the early T lineage precursor stage of T cell development. *The Journal of Experimental Medicine*, 205(5), 1187–1199. <https://doi.org/10.1084/jem.20072168>
- Berthault, C., Ramond, C., Burlen-Defranoux, O., Soubigou, G., Chea, S., Golub, R., Pereira, P., Vieira, P., & Cumano, A. (2017). Asynchronous lineage priming determines commitment to T cell and B cell lineages in fetal liver. *Nature Immunology*, 18(10), 1139–1149. <https://doi.org/10.1038/ni.3820>
- Bertrand, J. Y., Chi, N. C., Santoso, B., Teng, S., Stainier, D. Y. R., & Traver, D. (2010). Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature*, 464(7285), 108–111. <https://doi.org/10.1038/nature08738>
- Bertrand, J. Y., Desanti, G. E., Lo-Man, R., Leclerc, C., Cumano, A., & Golub, R. (2006). Fetal spleen stroma drives macrophage commitment. *Development*, 133(18), 3619–3628. <https://doi.org/10.1242/dev.02510>
- Bertrand, J. Y., Giroux, S., Golub, R., Klaine, M., Jalil, A., Boucontet, L., Godin, I., & Cumano, A. (2005a). Characterization of purified intraembryonic hematopoietic stem cells as a tool to define their site of origin. *Proceedings of the National Academy of Sciences of the United States of America*, 102(1), 134–139. <https://doi.org/10.1073/pnas.0402270102>
- Bertrand, J. Y., Jalil, A., Klaine, M., Jung, S., Cumano, A., & Godin, I. (2005b). Three pathways to mature macrophages in the early mouse yolk sac. *Blood*, 106(9), 3004–3011. <https://doi.org/10.1182/blood-2005-02-0461>
- Bessis, M. (1958). [Erythroblastic island, functional unity of bone marrow]. *Revue d'hématologie*, 13(1), 8–11. <http://www.ncbi.nlm.nih.gov/pubmed/13555228>
- Bianchi, E., Zini, R., Salati, S., Tenedini, E., Norfo, R., Tagliafico, E., Manfredini, R., & Ferrari, S. (2010). c-myb supports erythropoiesis through the transactivation of KLF1 and LMO2 expression. *Blood*, 116(22), e99–e110. <https://doi.org/10.1182/blood-2009-08-238311>
- Bogue, C. W., Ganea, G. R., Sturm, E., Ianucci, R., & Jacobs, H. C. (2000). Hex expression suggests a role in the development and function of organs derived from foregut endoderm. *Developmental Dynamics*, 219(1), 84–89. [https://doi.org/10.1002/1097-0177\(2000\)9999:9999::AID-DVDY1028>3.0.CO;2-5](https://doi.org/10.1002/1097-0177(2000)9999:9999::AID-DVDY1028>3.0.CO;2-5)
- Boisset, J.-C., van Cappellen, W., Andrieu-Soler, C., Galjart, N., Dzierzak, E., & Robin, C. (2010). In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. *Nature*, 464(7285), 116–120. <https://doi.org/10.1038/nature08764>
- Bondjers, C., He, L., Takemoto, M., Norlin, J., Asker, N., Hellström, M., Lindahl, P., Betsholtz, C., Bondjers, C., He, L., Takemoto, M., Norlin, J., Asker, N., Hellström, M., Lindahl, P., & Betsholtz, C. (2006). Microarray analysis of blood microvessels from PDGF-B and PDGF-R β mutant mice identifies novel markers for brain pericytes. *The FASEB Journal*, 20(10), 1703–1705. <https://doi.org/10.1096/fj.05-4944fje>

- Borge, O. J., Ramsfjell, V., Cui, L., & Jacobsen, S. E. (1997). Ability of early acting cytokines to directly promote survival and suppress apoptosis of human primitive CD34+CD38- bone marrow cells with multilineage potential at the single-cell level: key role of thrombopoietin. *Blood*, 90(6), 2282–2292. <http://www.ncbi.nlm.nih.gov/pubmed/9310479>
- Bort, R., Signore, M., Tremblay, K., Barbera, J. P. M., & Zaret, K. S. (2006). Hex homeobox gene controls the transition of the endoderm to a pseudostratified, cell emergent epithelium for liver bud development. *Developmental Biology*, 290(1), 44–56. <https://doi.org/10.1016/j.ydbio.2005.11.006>
- Boulais, P. E., Mizoguchi, T., Zimmerman, S., Nakahara, F., Vivié, J., Mar, J. C., van Oudenaarden, A., & Frenette, P. S. (2018). The Majority of CD45- Ter119- CD31- Bone Marrow Cell Fraction Is of Hematopoietic Origin and Contains Erythroid and Lymphoid Progenitors. *Immunity*, 49(4), 627–639.e6. <https://doi.org/10.1016/j.immuni.2018.08.019>
- Bowie, M. B., McKnight, K. D., Kent, D. G., McCaffrey, L., Hoodless, P. A., & Eaves, C. J. (2006). Hematopoietic stem cells proliferate until after birth and show a reversible phase-specific engraftment defect. *Journal of Clinical Investigation*, 116(10), 2808–2816. <https://doi.org/10.1172/JCI28310>
- Briggs, J. A., Weinreb, C., Wagner, D. E., Megason, S., Peshkin, L., Kirschner, M. W., & Klein, A. M. (2018). The dynamics of gene expression in vertebrate embryogenesis at single-cell resolution. *Science*, 360(6392), eaar5780. <https://doi.org/10.1126/science.aar5780>
- Brouard, N., Jost, C., Matthias, N., Albrecht, C., Egard, S., Gandhi, P., Strassel, C., Inoue, T., Sugiyama, D., Simmons, P. J., Gachet, C., & Lanza, F. (2017). A unique microenvironment in the developing liver supports the expansion of megakaryocyte progenitors. *Blood Advances*, 1(21), 1854–1866. <https://doi.org/10.1182/bloodadvances.2016003541>
- Buenrostro, J. D., Corces, M. R., Lareau, C. A., Wu, B., Schep, A. N., Aryee, M. J., Majeti, R., Chang, H. Y., & Greenleaf, W. J. (2018). Integrated Single-Cell Analysis Maps the Continuous Regulatory Landscape of Human Hematopoietic Differentiation. *Cell*, 173(6), 1535–1548.e16. <https://doi.org/10.1016/j.cell.2018.03.074>
- Busch, K., Klapproth, K., Barile, M., Flossdorf, M., Holland-Letz, T., Schlenner, S. M., Reth, M., Höfer, T., & Rodewald, H. R. (2015). Fundamental properties of unperturbed haematopoiesis from stem cells in vivo. *Nature*, 518(7540), 542–546. <https://doi.org/10.1038/nature14242>
- Buza-Vidas, N., Woll, P., Hultquist, A., Duarte, S., Lutteropp, M., Bouriez-Jones, T., Ferry, H., Luc, S., & Jacobsen, S. E. W. (2011). FLT3 expression initiates in fully multipotent mouse hematopoietic progenitor cells. *Blood*, 118(6), 1544–1548. <https://doi.org/10.1182/blood-2010-10-316232>
- Cabezas-Wallscheid, N., Klimmeck, D., Hansson, J., Lipka, D. B., Reyes, A., Wang, Q., Weichenhan, D., Lier, A., von Paleske, L., Renders, S., Wünsche, P., Zeisberger, P., Brocks, D., Gu, L., Herrmann, C., Haas, S., Essers, M. A. G., Brors, B., Eils, R., ... Trumpp, A. (2014). Identification of Regulatory Networks in HSCs and Their Immediate Progeny via Integrated Proteome, Transcriptome, and DNA Methylome Analysis. *Cell Stem Cell*, 15(4), 507–522. <https://doi.org/10.1016/j.stem.2014.07.005>
- Cantor, A. B., & Orkin, S. H. (2002). Transcriptional regulation of erythropoiesis: an affair involving multiple partners. *Oncogene*, 21(21), 3368–3376. <https://doi.org/10.1038/sj.onc.1205326>
- Cao, H., Cao, B., Heazlewood, C. K., Domingues, M., Sun, X., Debele, E., McGregor, N. E., Sims, N. A., Heazlewood, S. Y., & Nilsson, S. K. (2019). Osteopontin is An Important Regulative Component of the Fetal Bone Marrow Hematopoietic Stem Cell Niche. *Cells*, 8(9), 985. <https://doi.org/10.3390/cells8090985>
- Carrelha, J., Meng, Y., Kettyle, L. M., Luis, T. C., Norfo, R., Alcolea, V., Boukarabila, H., Grasso, F., Gambardella, A., Grover, A., Högstrand, K., Lord, A. M., Sanjuan-Pla, A., Woll, P. S., Nerlov, C., & Jacobsen, S. E. W. (2018). Hierarchically related lineage-restricted fates of multipotent haematopoietic stem cells. *Nature*, 554(7690), 106–111. <https://doi.org/10.1038/nature25455>
- Cascio, S., & Zaret, K. S. (1991). Hepatocyte differentiation initiates during endodermal-

- mesenchymal interactions prior to liver formation. *Development*, 113(1), 217–225.
- Castilla, L. H., Wijmenga, C., Wang, Q., Stacy, T., Speck, N. A., Eckhaus, M., Marín-Padilla, M., Collins, F. S., Wynshaw-Boris, A., & Liu, P. P. (1996). Failure of Embryonic Hematopoiesis and Lethal Hemorrhages in Mouse Embryos Heterozygous for a Knocked-In Leukemia Gene CBFβ-MYH11. *Cell*, 87(4), 687–696. [https://doi.org/10.1016/S0092-8674\(00\)81388-4](https://doi.org/10.1016/S0092-8674(00)81388-4)
- Challen, G. A., Boles, N. C., Chambers, S. M., & Goodell, M. A. (2010). Distinct Hematopoietic Stem Cell Subtypes Are Differentially Regulated by TGF-β1. *Cell Stem Cell*, 6(3), 265–278. <https://doi.org/10.1016/j.stem.2010.02.002>
- Chan, C. K. F., Chen, C. C., Luppen, C. A., Kim, J. B., DeBoer, A. T., Wei, K., Helms, J. A., Kuo, C. J., Kraft, D. L., & Weissman, I. L. (2009). Endochondral ossification is required for haematopoietic stem-cell niche formation. *Nature*, 457(7228), 490–494. <https://doi.org/10.1038/nature07547>
- Charbord, P., & Moore, K. A. (2005). Gene Expression in Stem Cell-Supporting Stromal Cell Lines. *Annals of the New York Academy of Sciences*, 1044(1), 159–167. <https://doi.org/10.1196/annals.1349.020>
- Charbord, P., Tavian, M., Humeau, L., & Peault, B. (1996). Early ontogeny of the human marrow from long bones: an immunohistochemical study of hematopoiesis and its microenvironment [see comments]. *Blood*, 87(10), 4109–4119. <https://doi.org/10.1182/blood.V87.10.4109.bloodjournal87104109>
- Chea, S., Schmutz, S., Berthault, C., Perchet, T., Petit, M., Burlen-Defranoux, O., Goldrath, A. W., Rodewald, H.-R. R., Cumano, A., & Golub, R. (2016). Single-Cell Gene Expression Analyses Reveal Heterogeneous Responsiveness of Fetal Innate Lymphoid Progenitors to Notch Signaling. *Cell Reports*, 14(6), 1500–1516. <https://doi.org/10.1016/j.celrep.2016.01.015>
- Chen, E. Y., Tan, C. M., Kou, Y., Duan, Q., Wang, Z., Meirelles, G., Clark, N. R., & Ma'ayan, A. (2013). Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC Bioinformatics*, 14(1), 128. <https://doi.org/10.1186/1471-2105-14-128>
- Chen, K., Liu, J., Heck, S., Chasis, J. A., An, X., & Mohandas, N. (2009). Resolving the distinct stages in erythroid differentiation based on dynamic changes in membrane protein expression during erythropoiesis. *Proceedings of the National Academy of Sciences of the United States of America*, 106(41), 17413–17418. <https://doi.org/10.1073/pnas.0909296106>
- Chen, M. J., Li, Y., De Obaldia, M. E., Yang, Q., Yzaguirre, A. D., Yamada-Inagawa, T., Vink, C. S., Bhandoola, A., Dzierzak, E., & Speck, N. A. (2011). Erythroid/myeloid progenitors and hematopoietic stem cells originate from distinct populations of endothelial cells. *Cell Stem Cell*, 9(6), 541–552. <https://doi.org/10.1016/j.stem.2011.10.003>
- Chhabra, A., Lechner, A. J., Ueno, M., Acharya, A., Van Handel, B., Wang, Y., Iruela-Arispe, M. L., Tallquist, M. D., & Mikkola, H. K. A. (2012). Trophoblasts Regulate the Placental Hematopoietic Niche through PDGF-B Signaling. *Developmental Cell*, 22(3), 651–659. <https://doi.org/10.1016/j.devcel.2011.12.022>
- Chou, S., Flygare, J., & Lodish, H. F. (2013). Fetal hepatic progenitors support long-term expansion of hematopoietic stem cells. *Experimental Hematology*, 41(5), 479–490. <https://doi.org/10.1016/j.exphem.2013.02.003>
- Chou, S., & Lodish, H. F. (2010). Fetal liver hepatic progenitors are supportive stromal cells for hematopoietic stem cells. *Proceedings of the National Academy of Sciences*, 107(17), 7799–7804. <https://doi.org/10.1073/pnas.1003586107>
- Christensen, J. L., Wright, D. E., Wagers, A. J., & Weissman, I. L. (2004). Circulation and chemotaxis of fetal hematopoietic stem cells. *PLoS Biology*, 2(3), 368–377. <https://doi.org/10.1371/journal.pbio.0020075>
- Clotman, F., Lannoy, V. J., Reber, M., Cereghini, S., Cassiman, D., Jacquemin, P., Roskams, T., Rousseau, G. G., & Lemaigre, F. P. (2002). The onecut transcription factor HNF6 is required for normal development of the biliary tract. *Development*, 129(8), 1819–1828.
- Collardeau-Frachon, S., & Scoazec, J. Y. (2008). Vascular development and differentiation during human liver organogenesis. *Anatomical Record*, 291(6), 614–627.

- <https://doi.org/10.1002/ar.20679>
- Corbel, C., & Salaün, J. (2002). α IIb Integrin Expression during Development of the Murine Hemopoietic System. *Developmental Biology*, 243(2), 301–311. <https://doi.org/10.1006/dbio.2001.0553>
- Cossarizza, A., Chang, H., Radbruch, A., Acs, A., Adam, D., Adam-Klages, S., Agace, W. W., Aghaeepour, N., Akdis, M., Allez, M., Almeida, L. N., Alvisi, G., Anderson, G., Andrä, I., Annunziato, F., Anselmo, A., Bacher, P., Baldari, C. T., Bari, S., ... Zychlinsky, A. (2019). Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). *European Journal of Immunology*, 49(10), 1457–1973. <https://doi.org/10.1002/eji.201970107>
- Coşkun, S., Chao, H., Vasavada, H., Heydari, K., Gonzales, N., Zhou, X., de Crombrughe, B., & Hirschi, K. K. (2014). Development of the fetal bone marrow niche and regulation of HSC quiescence and homing ability by emerging osteolineage cells. *Cell Reports*, 9(2), 581–590. <https://doi.org/10.1016/j.celrep.2014.09.013>
- Coutu, D. L., Kokkalis, K. D., Kunz, L., & Schroeder, T. (2017). Three-dimensional map of nonhematopoietic bone and bone-marrow cells and molecules. *Nature Biotechnology*, 35(12), 1202–1210. <https://doi.org/10.1038/nbt.4006>
- Crane, G. M., Jeffery, E., & Morrison, S. J. (2017). Adult haematopoietic stem cell niches. *Nature Reviews Immunology*, 17(9), 573–590. <https://doi.org/10.1038/nri.2017.53>
- Crawford, L. W., Foley, J. F., & Elmore, S. A. (2010). Histology Atlas of the Developing Mouse Hepatobiliary System with Emphasis on Embryonic Days 9.5-18.5. *Toxicologic Pathology*, 38(6), 872–906. <https://doi.org/10.1177/0192623310374329>
- Crosse, E. I., Gordon-Keylock, S., Rybtsov, S., Binagui-Casas, A., Felchle, H., Nnadi, N. C., Kirschner, K., Chandra, T., Tamagno, S., Webb, D. J., Rossi, F., Anderson, R. A., & Medvinsky, A. (2020). Multi-layered Spatial Transcriptomics Identify Secretory Factors Promoting Human Hematopoietic Stem Cell Development. *Cell Stem Cell*, 27(5), 822–839.e8. <https://doi.org/10.1016/j.stem.2020.08.004>
- Crossley, M., Tsang, A. P., Bieker, J. J., & Orkin, S. H. (1994). Regulation of the erythroid Kruppel-like factor (EKLF) gene promoter by the erythroid transcription factor GATA-1. *The Journal of Biological Chemistry*, 269(22), 15440–15444. <http://www.ncbi.nlm.nih.gov/pubmed/8195185>
- Cumano, A., Dieterlen-Lievre, F., & Godin, I. (1996). Lymphoid potential, probed before circulation in mouse, is restricted to caudal intraembryonic splanchnopleura. *Cell*, 86(6), 907–916. [https://doi.org/10.1016/S0092-8674\(00\)80166-X](https://doi.org/10.1016/S0092-8674(00)80166-X)
- Cumano, A., Ferraz, J. C., Klaine, M., Di Santo, J. P., & Godin, I. (2001). Intraembryonic, but Not Yolk Sac Hematopoietic Precursors, Isolated before Circulation, Provide Long-Term Multilineage Reconstitution. *Immunity*, 15(3), 477–485. [https://doi.org/10.1016/S1074-7613\(01\)00190-X](https://doi.org/10.1016/S1074-7613(01)00190-X)
- Dancis, J., Jansen, V., Gorstein, F., & Douglas, G. W. (1968). Hematopoietic cells in mouse placenta. *American Journal of Obstetrics and Gynecology*, 100(8), 1110–1121. [https://doi.org/10.1016/S0002-9378\(15\)33411-6](https://doi.org/10.1016/S0002-9378(15)33411-6)
- de Bruijn, M. F. T., Ma, X., Robin, C., Ottersbach, K., Sanchez, M.-J., & Dzierzak, E. (2002). Hematopoietic Stem Cells Localize to the Endothelial Cell Layer in the Midgestation Mouse Aorta. *Immunity*, 16(5), 673–683. [https://doi.org/10.1016/S1074-7613\(02\)00313-8](https://doi.org/10.1016/S1074-7613(02)00313-8)
- de Bruijn, M. F. T. R., Speck, N., Peeters, M., & Dzierzak, E. (2000). Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. *The EMBO Journal*, 19(11), 2465–2474. <https://doi.org/10.1093/emboj/19.11.2465>
- de Sauvage, F. J., Carver-Moore, K., Luoh, S. M., Ryan, A., Dowd, M., Eaton, D. L., & Moore, M. W. (1996). Physiological regulation of early and late stages of megakaryocytopoiesis by thrombopoietin. *The Journal of Experimental Medicine*, 183(2), 651–656. <https://doi.org/10.1084/jem.183.2.651>
- Dieterlen-Lievre, par F. (1975). On the origin of haemopoietic stem cells in the avian embryo: an experimental approach. *Journal of Embryology and Experimental Morphology*, 33(3), 607 LP – 619. <http://dev.biologists.org/content/33/3/607.abstract>

- Ding, L., Saunders, T. L., Enikolopov, G., & Morrison, S. J. (2012). Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature*, 481(7382), 457–462. <https://doi.org/10.1038/nature10783>
- Dobrev, M. P., Pereira, P. N. G., Deprest, J., & Zwijsen, A. (2010). On the origin of amniotic stem cells: Of mice and men. *International Journal of Developmental Biology*, 54(5), 761–777. <https://doi.org/10.1387/ijdb.092935md>
- Dong, H. Y., Wilkes, S., & Yang, H. (2011). CD71 is Selectively and Ubiquitously Expressed at High Levels in Erythroid Precursors of All Maturation Stages. *The American Journal of Surgical Pathology*, 35(5), 723–732. <https://doi.org/10.1097/PAS.0b013e31821247a8>
- Donze, D., Townes, T. M., & Bieker, J. J. (1995). Role of Erythroid Kruppel-like Factor in Human - to -Globin Gene Switching. *Journal of Biological Chemistry*, 270(4), 1955–1959. <https://doi.org/10.1074/jbc.270.4.1955>
- Douarin, N. M. (1975). An experimental analysis of liver development. *Medical Biology*, 53(6), 427–455.
- Downs, K. M., & Rodriguez, A. M. (2020). The mouse fetal-placental arterial connection: A paradigm involving the primitive streak and visceral endoderm with implications for human development. *WIREs Developmental Biology*, 9(2). <https://doi.org/10.1002/wdev.362>
- Drake, C. J., & Fleming, P. A. (2000). Vasculogenesis in the day 6.5 to 9.5 mouse embryo. *Blood*, 95(5), 1671–1679. https://doi.org/10.1182/blood.V95.5.1671.005k39_1671_1679
- Dudas, J., Papoutsis, M., Hecht, M., Elmaouhoub, A., Saile, B., Christ, B., Tomarev, S. I., Von Kaisenberg, C. S., Schweigerer, L., Ramadori, G., & Wilting, J. (2004). The homeobox transcription factor Prox1 is highly conserved in embryonic hepatoblasts and in adult and transformed hepatocytes, but is absent from bile duct epithelium. *Anatomy and Embryology*, 208(5), 359–366. <https://doi.org/10.1007/s00429-004-0403-4>
- Duncan, S. A., Manova, K., Chen, W. S., Hoodless, P., Weinstein, D. C., Bachvarova, R. F., & Darnell, J. E. (1994). Expression of transcription factor HNF-4 in the extraembryonic endoderm, gut, and nephrogenic tissue of the developing mouse embryo: HNF-4 is a marker for primary endoderm in the implanting blastocyst. *Proceedings of the National Academy of Sciences*, 91(16), 7598–7602. <https://doi.org/10.1073/pnas.91.16.7598>
- Durand, C., Robin, C., & Dzierzak, E. (2006). Mesenchymal lineage potentials of aorta-gonad-mesonephros stromal clones. *Haematologica*, 91(9), 1172–1179. <http://www.ncbi.nlm.nih.gov/pubmed/16956815>
- Dyer, M. A., Farrington, S. M., Mohn, D., Munday, J. R., & Baron, M. H. (2001). Indian hedgehog activates hematopoiesis and vasculogenesis and can respecify prospective neuroectodermal cell fate in the mouse embryo. *Development*, 128(10), 1717–1730.
- Dykstra, B., Kent, D., Bowie, M., McCaffrey, L., Hamilton, M., Lyons, K., Lee, S.-J., Brinkman, R., & Eaves, C. (2007). Long-Term Propagation of Distinct Hematopoietic Differentiation Programs In Vivo. *Cell Stem Cell*, 1(2), 218–229. <https://doi.org/10.1016/j.stem.2007.05.015>
- Dzierzak, E., & Philipsen, S. (2013). Erythropoiesis: Development and differentiation. *Cold Spring Harbor Perspectives in Medicine*, 3(4). <https://doi.org/10.1101/cshperspect.a011601>
- Dzierzak, E., & Speck, N. A. (2008). Of lineage and legacy: the development of mammalian hematopoietic stem cells. *Nature Immunology*, 9(2), 129–136. <https://doi.org/10.1038/ni1560>
- Eaton, D. L., & de Sauvage, F. J. (1997). Thrombopoietin: the primary regulator of megakaryocytopoiesis and thrombopoiesis. *Experimental Hematology*, 25(1), 1–7. <http://www.ncbi.nlm.nih.gov/pubmed/8989900>
- Eberl, G., Marmon, S., Sunshine, M. J., Rennert, P. D., Choi, Y., & Littmann, D. R. (2004). An essential function for the nuclear receptor ROR γ t in the generation of fetal lymphoid tissue inducer cells. *Nature Immunology*, 5(1), 64–73. <https://doi.org/10.1038/ni1022>
- Elsaid, R., Meunier, S., Burlen-Defranoux, O., Soares-da-Silva, F., Perchet, T., Iturri, L., Freyer, L., Vieira, P., Pereira, P., Golub, R., Bandeira, A., Gomez Perdiguero, E., & Cumano, A. (2020a). A wave of bipotent T/ILC-restricted progenitors shapes the

- embryonic thymus microenvironment in a time-dependent manner. *Blood*. <https://doi.org/10.1182/blood.2020006779>
- Elsaid, R., Soares-da-Silva, F., Peixoto, M., Amiri, D., Mackowski, N., Pereira, P., Bandeira, A., & Cumano, A. (2020b). Hematopoiesis: a layered organization across species. *Frontiers in Cell and Developmental Biology*. <https://doi.org/10.3389/fcell.2020.606642>
- Ema, H., & Nakauchi, H. (2000). Expansion of hematopoietic stem cells in the developing liver of a mouse embryo. *Blood*, 95(7), 2284–2289.
- Ema, M., Yokomizo, T., Wakamatsu, A., Terunuma, T., Yamamoto, M., & Takahashi, S. (2006). Primitive erythropoiesis from mesodermal precursors expressing VE-cadherin, PECAM-1, Tie2, endoglin, and CD34 in the mouse embryo. *Blood*, 108(13), 4018–4024. <https://doi.org/10.1182/blood-2006-03-012872>
- Emambokus, N. R., & Frampton, J. (2003). The glycoprotein IIb molecule is expressed on early murine hematopoietic progenitors and regulates their numbers in sites of hematopoiesis. *Immunity*, 19(1), 33–45. [https://doi.org/10.1016/S1074-7613\(03\)00173-0](https://doi.org/10.1016/S1074-7613(03)00173-0)
- Emerson, S. G., Sieff, C. A., Wang, E. A., Wong, G. G., Clark, S. C., & Nathan, D. G. (1985). Purification of fetal hematopoietic progenitors and demonstration of recombinant multipotential colony-stimulating activity. *Journal of Clinical Investigation*, 76(3), 1286–1290. <https://doi.org/10.1172/JCI112087>
- Farahani, R. M., & Xaymardan, M. (2015). Platelet-Derived Growth Factor Receptor Alpha as a Marker of Mesenchymal Stem Cells in Development and Stem Cell Biology. *Stem Cells International*, 2015, 1–8. <https://doi.org/10.1155/2015/362753>
- Farrell, J. A., Wang, Y., Riesenfeld, S. J., Shekhar, K., Regev, A., & Schier, A. F. (2018). Single-cell reconstruction of developmental trajectories during zebrafish embryogenesis. *Science*, 360(6392), eaar3131. <https://doi.org/10.1126/science.aar3131>
- Feng, D., & Kan, Y. W. (2005). The binding of the ubiquitous transcription factor Sp1 at the locus control region represses the expression of -like globin genes. *Proceedings of the National Academy of Sciences*, 102(28), 9896–9900. <https://doi.org/10.1073/pnas.0502041102>
- Ferkowicz, M. J., Starr, M., Xie, X., Li, W., Johnson, S. A., Shelley, W. C., Morrison, P. R., & Yoder, M. C. (2003). CD41 expression defines the onset of primitive and definitive hematopoiesis in the murine embryo. *Development*, 130(18), 4393–4403. <https://doi.org/10.1242/dev.00632>
- Ferreira, R., Ohneda, K., Yamamoto, M., & Philipsen, S. (2005). GATA1 Function, a Paradigm for Transcription Factors in Hematopoiesis. *Molecular and Cellular Biology*, 25(4), 1215–1227. <https://doi.org/10.1128/MCB.25.4.1215-1227.2005>
- Flygare, J., Estrada, V. R., Shin, C., Gupta, S., & Lodish, H. F. (2011). HIF1 α synergizes with glucocorticoids to promote BFU-E progenitor self-renewal. *Blood*, 117(12), 3435–3444. <https://doi.org/10.1182/blood-2010-07-295550>
- Fowden, A. L., & Moore, T. (2012). Maternal-fetal resource allocation: Co-operation and conflict. *Placenta*, 33, e11–e15. <https://doi.org/10.1016/j.placenta.2012.05.002>
- Fox, N., Priestley, G., Papayannopoulou, T., & Kaushansky, K. (2002). Thrombopoietin expands hematopoietic stem cells after transplantation. *Journal of Clinical Investigation*, 110(3), 389–394. <https://doi.org/10.1172/JCI15430>
- Frame, J. M., Fegan, K. H., Conway, S. J., McGrath, K. E., Palis, J., Herman, B., Frame, J. M., Fegan, K. H., Conway, S. J., McGrath, K. E., & Palis, J. (2016). Definitive Hematopoiesis in the Yolk Sac Emerges from Wnt-Responsive Hemogenic Endothelium Independently of Circulation and Arterial Identity. *STEM CELLS*, 34(2), 431–444. <https://doi.org/10.1002/stem.2213>
- Fraser, S. T., Isern, J., & Baron, M. H. (2007). Maturation and enucleation of primitive erythroblasts during mouse embryogenesis is accompanied by changes in cell-surface antigen expression. *Blood*, 109(11), 4616–4616. <https://doi.org/10.1182/blood-2007-04-072652>
- Fraser, S. T., Ogawa, M., Yu, R. T., Nishikawa, S. I. S. S. I. S., Yoder, M. C., & Nishikawa, S. I. S. S. I. S. (2002). Definitive hematopoietic commitment within the embryonic vascular endothelial-cadherin⁺ population. *Experimental Hematology*, 30(9), 1070–1078.

- [https://doi.org/10.1016/S0301-472X\(02\)00887-1](https://doi.org/10.1016/S0301-472X(02)00887-1)
- Freire, A. G., & Butler, J. M. (2020). Blood making: learning what to put into the dish. *F1000Research*, 9, 38. <https://doi.org/10.12688/f1000research.21245.1>
- Fujita, J., Onoue, H., Ebi, Y., Nakayama, H., & Kanakura, Y. (1989). In vitro duplication and in vivo cure of mast-cell deficiency of Sl/Sld mutant mice by cloned 3T3 fibroblasts. *Proceedings of the National Academy of Sciences*, 86(8), 2888–2891. <https://doi.org/10.1073/pnas.86.8.2888>
- Fujiwara, Y., Browne, C. P., Cunliffe, K., Goff, S. C., & Orkin, S. H. (1996). Arrested development of embryonic red cell precursors in mouse embryos lacking transcription factor GATA-1. *Proceedings of the National Academy of Sciences of the United States of America*, 93(22), 12355–12358. <https://doi.org/10.1073/pnas.93.22.12355>
- Gao, X., Xu, C., Asada, N., & Frenette, P. S. (2018). The hematopoietic stem cell niche: from embryo to adult. *Development*, 145(2), dev139691. <https://doi.org/10.1242/dev.139691>
- Garcia, M. D., & Larina, I. V. (2014). Vascular development and hemodynamic force in the mouse yolk sac. *Frontiers in Physiology*, 5 AUG(August), 1–10. <https://doi.org/10.3389/fphys.2014.00308>
- Gekas, C., Dieterlen-Lièvre, F., Orkin, S. H., & Mikkola, H. K. A. (2005). The placenta is a niche for hematopoietic stem cells. *Developmental Cell*, 8(3), 365–375. <https://doi.org/10.1016/j.devcel.2004.12.016>
- Gentek, R., Ghigo, C., Hoeffel, G., Bulle, M. J., Msallam, R., Gautier, G., Launay, P., Chen, J., Ginhoux, F., & Bajénoff, M. (2018). Hemogenic Endothelial Fate Mapping Reveals Dual Developmental Origin of Mast Cells. *Immunity*, 48(6), 1160–1171.e5. <https://doi.org/10.1016/j.immuni.2018.04.025>
- Georgopoulos, K., Bigby, M., Wang, J.-H., Molnar, A., Wu, P., Winandy, S., & Sharpe, A. (1994). The ikaros gene is required for the development of all lymphoid lineages. *Cell*, 79(1), 143–156. [https://doi.org/10.1016/0092-8674\(94\)90407-3](https://doi.org/10.1016/0092-8674(94)90407-3)
- Gerlach, J. C., Over, P., Turner, M. E., Thompson, R. L., Foka, H. G., Chen, W. C. W., Péault, B., Gridelli, B., & Schmelzer, E. (2012). Perivascular Mesenchymal Progenitors in Human Fetal and Adult Liver. *Stem Cells and Development*, 21(18), 3258–3269. <https://doi.org/10.1089/scd.2012.0296>
- Gerlach, J. C., Thompson, R. L., Gridelli, B., & Schmelzer, E. (2019). Effects of delta-like noncanonical Notch ligand 1 expression of human fetal liver hepatoblasts on hematopoietic progenitors. *Stem Cells International*, 2019. <https://doi.org/10.1155/2019/7916275>
- Godin, I., Dieterlen-Lièvre, F., & Cumano, A. (1995). Emergence of multipotent hemopoietic cells in the yolk sac and paraaortic splanchnopleura in mouse embryos, beginning at 8.5 days postcoitus. *Proceedings of the National Academy of Sciences of the United States of America*, 92(3), 773–777. <https://doi.org/10.1073/pnas.92.3.773>
- Godoy, P., Hewitt, N. J., Albrecht, U., Andersen, M. E., Ansari, N., Bhattacharya, S., Bode, J. G., Bolleyn, J., Borner, C., Böttger, J., Braeuning, A., Budinsky, R. A., Burkhardt, B., Cameron, N. R., Camussi, G., Cho, C.-S., Choi, Y.-J., Craig Rowlands, J., Dahmen, U., ... Hengstler, J. G. (2013). Recent advances in 2D and 3D in vitro systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME. *Archives of Toxicology*, 87(8), 1315–1530. <https://doi.org/10.1007/s00204-013-1078-5>
- Goldie, L. C., Lucitti, J. L., Dickinson, M. E., & Hirschi, K. K. (2008). Cell signaling directing the formation and function of hemogenic endothelium during murine embryogenesis. *Blood*, 112(8), 3194–3204. <https://doi.org/10.1182/blood-2008-02-139055>
- Gomes, A. C., Hara, T., Lim, V. Y., Herndler-Brandstetter, D., Nevius, E., Sugiyama, T., Taniuchi, S., Schlenner, S., Richie, E., Rodewald, H.-R. R., Flavell, R. A., Nagasawa, T., Ikuta, K., Pereira, J. P., Cordeiro Gomes, A., Hara, T., Lim, V. Y., Herndler-Brandstetter, D., Nevius, E., ... Pereira, J. P. (2016). Hematopoietic Stem Cell Niches Produce Lineage-Instructive Signals to Control Multipotent Progenitor Differentiation. *Immunity*, 45(6), 1219–1231. <https://doi.org/10.1016/j.immuni.2016.11.004>
- Gomez-Perdiguero, E., Klapproth, K., Schulz, C., Busch, K., Azzoni, E., Crozet, L., Garner,

- H., Trouillet, C., de Bruijn, M. F. T. R., Geissmann, F., & Rodewald, H.-R. (2015). Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors. *Nature*, 518(7540), 547–551. <https://doi.org/10.1038/nature13989>
- Gregory, C., & Eaves, A. (1978). Three stages of erythropoietic progenitor cell differentiation distinguished by a number of physical and biologic properties. *Blood*, 51(3), 527–537. <https://doi.org/10.1182/blood.V51.3.527.527>
- Grossmann, M., Metcalf, D., Merryfull, J., Beg, A., Baltimore, D., & Gerondakis, S. (1999). The combined absence of the transcription factors Rel and RelA leads to multiple hemopoietic cell defects. *Proceedings of the National Academy of Sciences*, 96(21), 11848–11853. <https://doi.org/10.1073/pnas.96.21.11848>
- Guyot, C., Lepreux, S., Combe, C., Doudnikoff, E., Bioulac-Sage, P., Balabaud, C., & Desmoulière, A. (2006). Hepatic fibrosis and cirrhosis: The (myo)fibroblastic cell subpopulations involved. *International Journal of Biochemistry and Cell Biology*, 38(2), 135–151. <https://doi.org/10.1016/j.biocel.2005.08.021>
- Haas, J. D., Ravens, S., Düber, S., Sandroch, I., Oberdörfer, L., Kashani, E., Chennupati, V., Föhse, L., Naumann, R., Weiss, S., Krueger, A., Förster, R., & Prinz, I. (2012). Development of Interleukin-17-Producing $\gamma\delta$ T Cells Is Restricted to a Functional Embryonic Wave. *Immunity*, 37(1), 48–59. <https://doi.org/10.1016/j.immuni.2012.06.003>
- Haas, S., Trumpp, A., & Milsom, M. D. (2018). Causes and Consequences of Hematopoietic Stem Cell Heterogeneity. *Cell Stem Cell*, 22(5), 627–638. <https://doi.org/10.1016/j.stem.2018.04.003>
- Hanspal, M., & Hanspal, J. S. (1994). The association of erythroblasts with macrophages promotes erythroid proliferation and maturation: a 30-kD heparin-binding protein is involved in this contact. *Blood*, 84(10), 3494–3504. <http://www.ncbi.nlm.nih.gov/pubmed/7949103>
- Hanspal, M., Smockova, Y., & Uong, Q. (1998). Molecular identification and functional characterization of a novel protein that mediates the attachment of erythroblasts to macrophages. *Blood*, 92(8), 2940–2950. <http://www.ncbi.nlm.nih.gov/pubmed/9763581>
- Hata, M., Nanno, M., Doi, H., Satomi, S., Sakata, T., Suzuki, R., & Itoh, T. (1993). Establishment of a hepatocytic epithelial cell line from the murine fetal liver capable of promoting hemopoietic cell proliferation. *Journal of Cellular Physiology*, 154(2), 381–392. <https://doi.org/10.1002/jcp.1041540222>
- Hattangadi, S. M., Wong, P., Zhang, L., Flygare, J., & Lodish, H. F. (2011). From stem cell to red cell: Regulation of erythropoiesis at multiple levels by multiple proteins, RNAs, and chromatin modifications. In *Blood* (Vol. 118, Issue 24, pp. 6258–6268). <https://doi.org/10.1182/blood-2011-07-356006>
- Hayashi, Y., Sezaki, M., & Takizawa, H. (2019). Development of the hematopoietic system: Role of inflammatory factors. *Wiley Interdisciplinary Reviews: Developmental Biology*, 8(4), 1–17. <https://doi.org/10.1002/wdev.341>
- Hepatic stellate cell nomenclature. (1996). *Hepatology (Baltimore, Md.)*, 23(1), 193. <http://www.ncbi.nlm.nih.gov/pubmed/8550043>
- Herzenberg, L. A. (2015). Layered evolution in the immune system: a view from history. *Annals of the New York Academy of Sciences*, 1362(1), 1–5. <https://doi.org/10.1111/nyas.12795>
- Herzenberg, L. A., & Herzenberg, L. A. (1989). Toward a layered immune system. *Cell*, 59(6), 953–954. [https://doi.org/10.1016/0092-8674\(89\)90748-4](https://doi.org/10.1016/0092-8674(89)90748-4)
- Hirsch, E., Iglesias, A., Potocnik, A. J., Hartmann, U., & Fässler, R. (1996). Impaired migration but not differentiation of haematopoietic stem cells in the absence of $\beta 1$ integrins. *Nature*, 380(6570), 171–175. <https://doi.org/10.1038/380171a0>
- His, W. (1900). Lecithoblast und Angioblast der Wirbelthiere. *Wiss Math Phys*, 22:171.
- Hoeffel, G., Chen, J., Lavin, Y., Low, D., Almeida, F. F., See, P., Beaudin, A. E., Lum, J., Low, I., Forsberg, E. C., Poidinger, M., Zolezzi, F., Larbi, A., Ng, L. G., Chan, J. K. Y., Greter, M., Becher, B., Samokhvalov, I. M., Merad, M., & Ginhoux, F. (2015). C-Myb⁺ Erythro-Myeloid Progenitor-Derived Fetal Monocytes Give Rise to Adult Tissue-Resident Macrophages. *Immunity*, 42(4), 665–678. <https://doi.org/10.1016/j.immuni.2015.03.011>
- Holst, J., Watson, S., Lord, M. S., Eamegdool, S. S., Bax, D. V., Nivison-Smith, L. B.,

- Kondyurin, A., Ma, L., Oberhauser, A. F., Weiss, A. S., & Rasko, J. E. J. (2010). Substrate elasticity provides mechanical signals for the expansion of hemopoietic stem and progenitor cells. *Nature Biotechnology*, 28(10), 1123–1128. <https://doi.org/10.1038/nbt.1687>
- Houssaint, E. (1981). Differentiation of the mouse hepatic primordium. II. Extrinsic origin of the haemopoietic cell line. *Cell Differentiation*, 10(5), 243–252. [https://doi.org/10.1016/0045-6039\(81\)90007-5](https://doi.org/10.1016/0045-6039(81)90007-5)
- Hu, Y., & Smyth, G. K. (2009). ELDA: Extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. *Journal of Immunological Methods*, 347(1–2), 70–78. <https://doi.org/10.1016/j.jim.2009.06.008>
- Huang, H., & Auerbach, R. (1993). Identification and characterization of hematopoietic stem cells from the yolk sac of the early mouse embryo. *Proceedings of the National Academy of Sciences*, 90(21), 10110–10114. <https://doi.org/10.1073/pnas.90.21.10110>
- Huber, T. L., Kouskoff, V., Fehling, H. J., Palis, J., & Keller, G. (2004). Haemangioblast commitment is initiated in the primitive streak of the mouse embryo. *Nature*, 432(7017), 625–630. <https://doi.org/10.1038/nature03122>
- Ijpenberg, A., Pérez-Pomares, J. M., Guadix, J. A., Carmona, R., Portillo-Sánchez, V., Macías, D., Hohenstein, P., Miles, C. M., Hastie, N. D., & Muñoz-Chápuli, R. (2007). Wt1 and retinoic acid signaling are essential for stellate cell development and liver morphogenesis. *Developmental Biology*, 312(1), 157–170. <https://doi.org/10.1016/j.ydbio.2007.09.014>
- Ikuta, K., Kina, T., MacNeil, I., Uchida, N., Peault, B., Chien, Y. H., & Weissman, I. L. (1990). A developmental switch in thymic lymphocyte maturation potential occurs at the level of hematopoietic stem cells. *Cell*, 62(5), 863–874. [https://doi.org/10.1016/0092-8674\(90\)90262-D](https://doi.org/10.1016/0092-8674(90)90262-D)
- Iscove, N. N. (1977). THE ROLE OF ERYTHROPOIETIN IN REGULATION OF POPULATION SIZE AND CELL CYCLING OF EARLY AND LATE ERYTHROID PRECURSORS IN MOUSE BONE MARROW. *Cell Proliferation*, 10(4), 323–334. <https://doi.org/10.1111/j.1365-2184.1977.tb00300.x>
- Iscove, N. N., & Guilbert, L. J. (1978). Erythropoietin-Independence of Early Erythropoiesis and a Two-Regulator Model of Proliferative Control in the Hemopoietic System. In *In Vitro Aspects of Erythropoiesis* (pp. 3–7). Springer US. https://doi.org/10.1007/978-1-4612-6301-2_2
- Iscove, N. N., Sieber, F., & Winterhalter, K. H. (1974). Erythroid colony formation in cultures of mouse and human bone marrow: Analysis of the requirement for erythropoietin by gel filtration and affinity chromatography on agarose-concanavalin A. *Journal of Cellular Physiology*, 83(2), 309–320. <https://doi.org/10.1002/jcp.1040830218>
- Isern, J., Fraser, S. T., He, Z., & Baron, M. H. (2008). The fetal liver is a niche for maturation of primitive erythroid cells. *Proceedings of the National Academy of Sciences*, 105(18), 6662–6667. <https://doi.org/10.1073/pnas.0802032105>
- Isern, J., He, Z., Fraser, S. T., Nowotschin, S., Ferrer-Vaquer, A., Moore, R., Hadjantonakis, A. K., Schulz, V., Tuck, D., Gallagher, P. G., & Baron, M. H. (2012). Isern J, He Z, Fraser ST, et al. Single-lineage transcriptome analysis reveals key regulatory pathways in primitive erythroid progenitors in the mouse embryo. *Blood*. 2011;117(18):4924–4934. *Blood*, 120(24), 4903–4903. <https://doi.org/10.1182/blood-2012-10-464222>
- Jenkins, S. J., Ruckerl, D., Cook, P. C., Jones, L. H., Finkelman, F. D., van Rooijen, N., MacDonald, A. S., & Allen, J. E. (2011). Local Macrophage Proliferation, Rather than Recruitment from the Blood, Is a Signature of TH2 Inflammation. *Science*, 332(6035), 1284–1288. <https://doi.org/10.1126/science.1204351>
- Johnson, G. R., & Moore, M. A. S. (1975). Role of stem cell migration in initiation of mouse foetal liver haemopoiesis. *Nature*, 258(5537), 726–728. <https://doi.org/10.1038/258726a0>
- Jordan, C. T., Astle, C. M., Zawadzki, J., Mackarechtschian, K., Lemischka, I. R., & Harrison, D. E. (1995). Long-term repopulating abilities of enriched fetal liver stem cells measured by competitive repopulation. *Experimental Hematology*, 23(9), 1011–1015.

- <http://www.ncbi.nlm.nih.gov/pubmed/7635180>
- Jordan, C. T., McKearn, J. P., & Lemischka, I. R. (1990). Cellular and developmental properties of fetal hematopoietic stem cells. *Cell*, 61(6), 953–963. [https://doi.org/10.1016/0092-8674\(90\)90061-I](https://doi.org/10.1016/0092-8674(90)90061-I)
- Karamitros, D., Stoilova, B., Aboukhalil, Z., Hamey, F., Reinisch, A., Samitsch, M., Quek, L., Otto, G., Repapi, E., Doondeea, J., Usukhbayar, B., Calvo, J., Taylor, S., Goardon, N., Six, E., Pflumio, F., Porcher, C., Majeti, R., Göttgens, B., & Vyas, P. (2018). Single-cell analysis reveals the continuum of human lympho-myeloid progenitor cells. *Nature Immunology*, 19(1), 85–97. <https://doi.org/10.1038/s41590-017-0001-2>
- Kasaai, B., Caolo, V., Peacock, H. M., Lehoux, S., Gomez-Perdiguero, E., Luttun, A., & Jones, E. A. V. (2017). Erythro-myeloid progenitors can differentiate from endothelial cells and modulate embryonic vascular remodeling. *Scientific Reports*, 7(March), 25–28. <https://doi.org/10.1038/srep43817>
- Kaushansky, K. (1995). Thrombopoietin: the primary regulator of platelet production. *Blood*, 86(2), 419–431. <https://doi.org/10.1182/blood.V86.2.419.bloodjournal862419>
- Kellendonk, C., Opherk, C., Anlag, K., Schütz, G., & Tronche, F. (2000). Hepatocyte-specific expression of Cre recombinase. *Genesis*, 26(2), 151–153. [https://doi.org/10.1002/\(SICI\)1526-968X\(200002\)26:2<151::AID-GENE17>3.0.CO;2-E](https://doi.org/10.1002/(SICI)1526-968X(200002)26:2<151::AID-GENE17>3.0.CO;2-E)
- Khan, J. A., Mendelson, A., Kunisaki, Y., Birbrair, A., Kou, Y., Arnal-Estapé, A., Pinho, S., Ciero, P., Nakahara, F., Ma'ayan, A., Bergman, A., Merad, M., & Frenette, P. S. (2016). Fetal liver hematopoietic stem cell niches associate with portal vessels. *Science (New York, N.Y.)*, 351(6269), 176–180. <https://doi.org/10.1126/science.aad0084>
- Kiel, M. J., Yilmaz, Ö. H., Iwashita, T., Yilmaz, O. H., Terhorst, C., & Morrison, S. J. (2005). SLAM Family Receptors Distinguish Hematopoietic Stem and Progenitor Cells and Reveal Endothelial Niches for Stem Cells. *Cell*, 121(7), 1109–1121. <https://doi.org/10.1016/j.cell.2005.05.026>
- Kieusseian, A., de la Grange, P. B., Burlen-Defranoux, O., Godin, I., & Cumano, A. (2012). Immature hematopoietic stem cells undergo maturation in the fetal liver. *Development*, 139(19), 3521–3530. <https://doi.org/10.1242/dev.079210>
- Kikuchi, K., & Kondo, M. (2006). Developmental switch of mouse hematopoietic stem cells from fetal to adult type occurs in bone marrow after birth. *Proceedings of the National Academy of Sciences*, 103(47), 17852–17857. <https://doi.org/10.1073/pnas.0603368103>
- Kim, A. R., Ulirsch, J. C., Wilmes, S., Unal, E., Moraga, I., Karakukcu, M., Yuan, D., Kazerounian, S., Abdulhay, N. J., King, D. S., Gupta, N., Gabriel, S. B., Lander, E. S., Patisroglu, T., Ozcan, A., Ozdemir, M. A., Garcia, K. C., Piehler, J., Gazda, H. T., ... Sankaran, V. G. (2017). Functional Selectivity in Cytokine Signaling Revealed Through a Pathogenic EPO Mutation. *Cell*, 168(6), 1053–1064.e15. <https://doi.org/10.1016/j.cell.2017.02.026>
- Kim, I., He, S., Yilmaz, O. H., Kiel, M. J., & Morrison, S. J. (2006). Enhanced purification of fetal liver hematopoietic stem cells using SLAM family receptors. *Blood*, 108(2), 737–744. <https://doi.org/10.1182/blood-2005-10-4135>
- Kim, I., Yilmaz, O. H., & Morrison, S. J. (2005). CD144 (VE-cadherin) is transiently expressed by fetal liver hematopoietic stem cells. *Blood*, 106(3), 903–905. <https://doi.org/10.1182/blood-2004-12-4960>
- Kina, T., Ikuta, K., Takayama, E., Wada, K., Majumdar, A. Sen, Weissman, I. L., & Katsura, Y. (2000). The monoclonal antibody TER-119 recognizes a molecule associated with glycophorin A and specifically marks the late stages of murine erythroid lineage. *British Journal of Haematology*, 109(2), 280–287. <https://doi.org/10.1046/j.1365-2141.2000.02037.x>
- Kingsley, P. D., Malik, J., Emerson, R. L., Bushnell, T. P., McGrath, K. E., Bloedorn, L. A., Bulger, M., & Palis, J. (2006). “Maturation” globin switching in primary primitive erythroid cells. *Blood*, 107(4), 1665–1672. <https://doi.org/10.1182/blood-2005-08-3097>
- Kingsley, P. D., Malik, J., Fantauzzo, K. A., & Palis, J. (2004). Yolk sac-derived primitive erythroblasts enucleate during mammalian embryogenesis. *Blood*, 104(1), 19–25. <https://doi.org/10.1182/blood-2003-12-4162>

- Kiserud, T. (2005). Physiology of the fetal circulation. *Seminars in Fetal and Neonatal Medicine*, 10(6), 493–503. <https://doi.org/10.1016/j.siny.2005.08.007>
- Kissa, K., & Herbomel, P. (2010). Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *Nature*, 464(7285), 112–115. <https://doi.org/10.1038/nature08761>
- Kondo, M., Weissman, I. L., & Akashi, K. (1997). Identification of Clonogenic Common Lymphoid Progenitors in Mouse Bone Marrow. *Cell*, 91(5), 661–672. [https://doi.org/10.1016/S0092-8674\(00\)80453-5](https://doi.org/10.1016/S0092-8674(00)80453-5)
- Koury, M., & Bondurant, M. (1990a). Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells. *Science*, 248(4953), 378–381. <https://doi.org/10.1126/science.2326648>
- Koury, M., & Bondurant, M. (1990b). Control of red cell production: the roles of programmed cell death (apoptosis) and erythropoietin. *Transfusion*, 30(8), 673–674. <https://doi.org/10.1046/j.1537-2995.1990.30891020321.x>
- Koury, M. J., & Bondurant, M. C. (1988). Maintenance by erythropoietin of viability and maturation of murine erythroid precursor cells. *Journal of Cellular Physiology*, 137(1), 65–74. <https://doi.org/10.1002/jcp.1041370108>
- Kozhemyakina, E., Ionescu, A., & Lassar, A. B. (2014). GATA6 Is a Crucial Regulator of Shh in the Limb Bud. *PLoS Genetics*, 10(1), e1004072. <https://doi.org/10.1371/journal.pgen.1004072>
- Kristiansen, T. A., Jaensson Gyllenbäck, E., Zriwil, A., Björklund, T., Daniel, J. A., Sitnicka, E., Soneji, S., Bryder, D., & Yuan, J. (2016). Cellular Barcoding Links B-1a B Cell Potential to a Fetal Hematopoietic Stem Cell State at the Single-Cell Level. *Immunity*, 45(2), 346–357. <https://doi.org/10.1016/j.immuni.2016.07.014>
- Ku, H., Yonemura, Y., Kaushansky, K., & Ogawa, M. (1996). Thrombopoietin, the ligand for the Mpl receptor, synergizes with steel factor and other early acting cytokines in supporting proliferation of primitive hematopoietic progenitors of mice. *Blood*, 87(11), 4544–4551. <http://www.ncbi.nlm.nih.gov/pubmed/8639822>
- Kubota, H., Yao, H., & Reid, L. M. (2007). Identification and Characterization of Vitamin A-Storing Cells in Fetal Liver: Implications for Functional Importance of Hepatic Stellate Cells in Liver Development and Hematopoiesis. *Stem Cells*, 25(9), 2339–2349. <https://doi.org/10.1634/stemcells.2006-0316>
- Kumar, S., & Geiger, H. (2017). HSC Niche Biology and HSC Expansion Ex Vivo. *Trends in Molecular Medicine*, 23(9), 799–819. <https://doi.org/10.1016/j.molmed.2017.07.003>
- Kunisaki, Y., Bruns, I., Scheierrmann, C., Ahmed, J., Pinho, S., Zhang, D., Mizoguchi, T., Wei, Q., Lucas, D., Ito, K., Mar, J. C., Bergman, A., & Frenette, P. S. (2013). Arteriolar niches maintain haematopoietic stem cell quiescence. *Nature*, 502(7473), 637–643. <https://doi.org/10.1038/nature12612>
- Kusumbe, A. P., Ramasamy, S. K., Itkin, T., Mäe, M. A., Langen, U. H., Betsholtz, C., Lapidot, T., & Adams, R. H. (2016). Age-dependent modulation of vascular niches for haematopoietic stem cells. *Nature*, 532(7599), 380–384. <https://doi.org/10.1038/nature17638>
- Lai, C.-Y., Yamazaki, S., Okabe, M., Suzuki, S., Maeyama, Y., Iimura, Y., Onodera, M., Kakuta, S., Iwakura, Y., Nojima, M., Otsu, M., & Nakauchi, H. (2014). Stage-Specific Roles for Cxcr4 Signaling in Murine Hematopoietic Stem/Progenitor Cells in the Process of Bone Marrow Repopulation. *STEM CELLS*, 32(7), 1929–1942. <https://doi.org/10.1002/stem.1670>
- Lander, A. D., Kimble, J., Clevers, H., Fuchs, E., Montarras, D., Buckingham, M., Calof, A. L., Trumpp, A., & Oskarsson, T. (2012). What does the concept of the stem cell niche really mean today? *BMC Biology*, 10(1), 19. <https://doi.org/10.1186/1741-7007-10-19>
- Lassila, O., Eskola, J., Toivanen, P., Martin, C., & Dieterlen-Lievre, F. (1978). The origin of lymphoid stem cells studied in chick yolk sac–embryo chimaeras. *Nature*, 272(5651), 353–354. <https://doi.org/10.1038/272353a0>
- Laurenti, E., & Göttgens, B. (2018). From haematopoietic stem cells to complex differentiation landscapes. *Nature*, 553(7689), 418–426. <https://doi.org/10.1038/nature25022>
- Lee, S. H., Crocker, P. R., Westaby, S., Key, N., Mason, D. Y., Gordon, S., & Weatherall, D.

- J. (1988). Isolation and immunocytochemical characterization of human bone marrow stromal macrophages in hemopoietic clusters. *The Journal of Experimental Medicine*, 168(3), 1193–1198. <https://doi.org/10.1084/jem.168.3.1193>
- Lee, Y., Leslie, J., Yang, Y., & Ding, L. (2021). Hepatic stellate and endothelial cells maintain hematopoietic stem cells in the developing liver. *Journal of Experimental Medicine*, 218(3). <https://doi.org/10.1084/jem.20200882>
- Levine, J. H., Simonds, E. F., Bendall, S. C., Davis, K. L., Amir, E. A. D., Tadmor, M. D., Litvin, O., Fienberg, H. G., Jager, A., Zunder, E. R., Finck, R., Gedman, A. L., Radtke, I., Downing, J. R., Pe'er, D., & Nolan, G. P. (2015). Data-Driven Phenotypic Dissection of AML Reveals Progenitor-like Cells that Correlate with Prognosis. *Cell*, 162(1), 184–197. <https://doi.org/10.1016/j.cell.2015.05.047>
- Li, J., Hale, J., Bhagia, P., Xue, F., Chen, L., Jaffray, J., Yan, H., Lane, J., Gallagher, P. G., Mohandas, N., Liu, J., & An, X. (2014). Isolation and transcriptome analyses of human erythroid progenitors: BFU-E and CFU-E. *Blood*, 124(24), 3636–3645. <https://doi.org/10.1182/blood-2014-07-588806>
- Li, J., Ning, G., & Duncan, S. A. (2000). Mammalian hepatocyte differentiation requires the transcription factor HNF-4alpha. *Genes & Development*, 14(4), 464–474. <https://doi.org/10.1128/MMBR.66.1.39-63.2002>
- Li, L., Lee, J. Y., Gross, J., Song, S.-H., Dean, A., & Love, P. E. (2010). A requirement for Lim domain binding protein 1 in erythropoiesis. *The Journal of Experimental Medicine*, 207(12), 2543–2550. <https://doi.org/10.1084/jem.20100504>
- Liang, R., & Ghaffari, S. (2016). Advances in understanding the mechanisms of erythropoiesis in homeostasis and disease. *British Journal of Haematology*, 174(5), 661–673. <https://doi.org/10.1111/bjh.14194>
- Liao, Y., Smyth, G. K., & Shi, W. (2014). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*, 30(7), 923–930. <https://doi.org/10.1093/bioinformatics/btt656>
- Lin, C. S., Lim, S. K., D'Agati, V., & Costantini, F. (1996). Differential effects of an erythropoietin receptor gene disruption on primitive and definitive erythropoiesis. *Genes & Development*, 10(2), 154–164. <https://doi.org/10.1101/gad.10.2.154>
- Liu, J., Huang, X., Werner, M., Broering, R., Yang, D., & Lu, M. (2017). Advanced Method for Isolation of Mouse Hepatocytes, Liver Sinusoidal Endothelial Cells, and Kupffer Cells. In H. Guo & A. Cuconati (Eds.), *Hepatitis B Virus: Methods and Protocols* (pp. 249–258). Springer New York. https://doi.org/10.1007/978-1-4939-6700-1_21
- Liu, J., Zhang, J., Ginzburg, Y., Li, H., Xue, F., Franceschi, L. De, Chasis, J. A., Mohandas, N., & An, X. (2015). Quantitative analysis of murine terminal erythroid differentiation in vivo: novel method to study normal and disordered erythropoiesis. *Blood*, 121(8), 43–50. <https://doi.org/10.1182/blood-2012-09-456079>
- Lok, S., Kaushansky, K., Holly, R. D., Kuijper, J. L., Lofton-Day, C. E., Oort, P. J., Grant, F. J., Heipel, M. D., Burkhead, S. K., Kramer, J. M., Bell, L. A., Sprecher, C. A., Blumberg, H., Johnson, R., Prunkard, D., Ching, A. F. T., Mathewes, S. L., Bailey, M. C., Forstrom, J. W., ... Foster, D. C. (1994). Cloning and expression of murine thrombopoietin cDNA and stimulation of platelet production in vivo. *Nature*, 369(6481), 565–568. <https://doi.org/10.1038/369565a0>
- Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, 15(12), 550. <https://doi.org/10.1186/s13059-014-0550-8>
- Lua, I., & Asahina, K. (2016). The Role of Mesothelial Cells in Liver Development, Injury, and Regeneration. *Gut and Liver*, 10(2), 166. <https://doi.org/10.5009/gnl15226>
- Lyman, S. D., James, L., Bos, T. Vanden, de Vries, P., Brasel, K., Gliniak, B., Hollingsworth, L. T., Picha, K. S., McKenna, H. J., Splett, R. R., Fletcher, F. A., Maraskovsky, E., Farrah, T., Foxworthe, D., Williams, D. E., & Beckmann, M. P. (1993). Molecular cloning of a ligand for the flt3flk-2 tyrosine kinase receptor: A proliferative factor for primitive hematopoietic cells. *Cell*, 75(6), 1157–1167. [https://doi.org/10.1016/0092-8674\(93\)90325-K](https://doi.org/10.1016/0092-8674(93)90325-K)

- Makita, T. (2001). A developmental transition in definitive erythropoiesis: erythropoietin expression is sequentially regulated by retinoic acid receptors and HNF4. *Genes & Development*, 15(7), 889–901. <https://doi.org/10.1101/gad.871601>
- Manesia, J. K., Xu, Z., Broekaert, D., Boon, R., van Vliet, A., Eelen, G., Vanwelden, T., Stegen, S., Van Gastel, N., Pascual-Montano, A., Maria-Fendt, S., Carmeliet, G., Carmeliet, P., Khurana, S., & Verfaillie, C. M. (2015). Highly proliferative primitive fetal liver hematopoietic stem cells are fueled by oxidative metabolic pathways. *Stem Cell Research*, 15(3), 715–721. <https://doi.org/10.1016/j.scr.2015.11.001>
- Margagliotti, S., Clotman, F., Pierreux, C. E., Beaudry, J. B., Jacquemin, P., Rousseau, G. G., & Lemaigre, F. P. (2007). The Onecut transcription factors HNF-6/OC-1 and OC-2 regulate early liver expansion by controlling hepatoblast migration. *Developmental Biology*, 311(2), 579–589. <https://doi.org/10.1016/j.ydbio.2007.09.013>
- Mascarenhas, M. I., Parker, A., Dzierzak, E., & Ottersbach, K. (2009). Identification of novel regulators of hematopoietic stem cell development through refinement of stem cell localization and expression profiling. *Blood*, 114(21), 4645–4653. <https://doi.org/10.1182/blood-2009-06-230037>
- Matsubara, A., Iwama, A., Yamazaki, S., Furuta, C., Hirasawa, R., Morita, Y., Osawa, M., Motohashi, T., Eto, K., Ema, H., Kitamura, T., Vestweber, D., & Nakauchi, H. (2005). Endomucin, a CD34-like sialomucin, marks hematopoietic stem cells throughout development. *Journal of Experimental Medicine*, 202(11), 1483–1492. <https://doi.org/10.1084/jem.20051325>
- Matsumoto, K., Yoshitomi, H., Rossant, J., & Zaret, K. S. (2001). Liver organogenesis promoted by endothelial cells prior to vascular function. *Science*, 294(5542), 559–563. <https://doi.org/10.1126/science.1063889>
- Maximow, A. A. (1924). RELATION OF BLOOD CELLS TO CONNECTIVE TISSUES AND ENDOTHELIUM. *Physiological Reviews*, 4(4), 533–563. <https://doi.org/10.1152/physrev.1924.4.4.533>
- McDermott, D. H., Gao, J.-L., Liu, Q., Siwicki, M., Martens, C., Jacobs, P., Velez, D., Yim, E., Bryke, C. R., Hsu, N., Dai, Z., Marquesen, M. M., Stregevsy, E., Kwatema, N., Theobald, N., Long Priel, D. A., Pittaluga, S., Raffeld, M. A., Calvo, K. R., ... Murphy, P. M. (2015). Chromothriptic Cure of WHIM Syndrome. *Cell*, 160(4), 686–699. <https://doi.org/10.1016/j.cell.2015.01.014>
- McDevitt, M. A., Shivdasani, R. A., Fujiwara, Y., Yang, H., & Orkin, S. H. (1997). A “knockdown” mutation created by cis-element gene targeting reveals the dependence of erythroid cell maturation on the level of transcription factor GATA-1. *Proceedings of the National Academy of Sciences*, 94(13), 6781–6785. <https://doi.org/10.1073/pnas.94.13.6781>
- McGrath, K. E., Catherman, S. C., & Palis, J. (2017). Delineating stages of erythropoiesis using imaging flow cytometry. *Methods*, 112, 68–74. <https://doi.org/10.1016/j.ymeth.2016.08.012>
- McGrath, K. E., Frame, J. M., Fegan, K. H., Bowen, J. R., Conway, S. J., Catherman, S. C., Kingsley, P. D., Koniski, A. D., & Palis, J. (2015). Distinct Sources of Hematopoietic Progenitors Emerge before HSCs and Provide Functional Blood Cells in the Mammalian Embryo. *Cell Reports*, 11(12), 1892–1904. <https://doi.org/10.1016/j.celrep.2015.05.036>
- McGrath, K. E., Frame, J. M., Fromm, G. J., Koniski, A. D., Kingsley, P. D., Little, J., Bulger, M., & Palis, J. (2011). A transient definitive erythroid lineage with unique regulation of the β -globin locus in the mammalian embryo. *Blood*, 117(17), 4600–4608. <https://doi.org/10.1182/blood-2010-12-325357>
- McGrath, K. E., & Palis, J. (2008). Ontogeny of Erythropoiesis in the Mammalian Embryo. *Current Topics in Developmental Biology*, 82(07), 1–22. [https://doi.org/10.1016/S0070-2153\(07\)00001-4](https://doi.org/10.1016/S0070-2153(07)00001-4)
- Medvinsky, A., Dzierzak, E., Ridgeway, T., & Hill, M. (1996). Definitive Hematopoiesis Is Autonomously Initiated by the AGM Region. *Cell*, 86(6), 897–906. [https://doi.org/10.1016/S0092-8674\(00\)80165-8](https://doi.org/10.1016/S0092-8674(00)80165-8)
- Melchers, F. (1979). Murine embryonic B lymphocyte development in the placenta. *Nature*,

- 277(5693), 219–221. <https://doi.org/10.1038/277219a0>
- Méndez-Ferrer, S., Michurina, T. V., Ferraro, F., Mazloom, A. R., MacArthur, B. D., Lira, S. A., Scadden, D. T., Ma'ayan, A., Enikolopov, G. N., Frenette, P. S., Mag'Ayan, A., Enikolopov, G. N., Frenette, P. S., Ma'ayan, A., Enikolopov, G. N., & Frenette, P. S. (2010). Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature*, 466(7308), 829–834. <https://doi.org/10.1038/nature09262>
- Merad, M., Manz, M. G., Karsunky, H., Wagers, A., Peters, W., Charo, I., Weissman, I. L., Cyster, J. G., & Engleman, E. G. (2002). Langerhans cells renew in the skin throughout life under steady-state conditions. *Nature Immunology*, 3(12), 1135–1141. <https://doi.org/10.1038/ni852>
- Metcalf, D. (1999). Stem Cells, Pre-Progenitor Cells and Lineage-Committed Cells: Are Our Dogmas Correct? *Annals of the New York Academy of Sciences*, 872(1 HEMATOPOIETIC), 289–304. <https://doi.org/10.1111/j.1749-6632.1999.tb08473.x>
- Migliaccio, G., Migliaccio, A. R., Petti, S., Mavilio, F., Russo, G., Lazzaro, D., Testa, U., Marinucci, M., & Peschle, C. (1986). Human embryonic hemopoiesis. Kinetics of progenitors and precursors underlying the yolk sac-liver transition. *Journal of Clinical Investigation*, 78(1), 51–60. <https://doi.org/10.1172/JCI112572>
- Mikkola, H. K. A., Fujiwara, Y., Schlaeger, T. M., Traver, D., & Orkin, S. H. (2003a). Expression of CD41 marks the initiation of definitive hematopoiesis in the mouse embryo. *Blood*, 101(2), 508–516. <https://doi.org/10.1182/blood-2002-06-1699>
- Mikkola, H. K. A., Klintman, J., Yang, H., Hock, H., Schlaeger, T. M., Fujiwara, Y., & Orkin, S. H. (2003b). Haematopoietic stem cells retain long-term repopulating activity and multipotency in the absence of stem-cell leukaemia SCL/tal-1 gene. *Nature*, 421(6922), 547–551. <https://doi.org/10.1038/nature01345>
- Miller, C. L., Rebel, V. I., Helgason, C. D., Lansdorp, D. P. M., & Eaves, C. J. (1997). Impaired steel factor responsiveness differentially affects the detection and long-term maintenance of fetal liver hematopoietic stem cells in vivo. *Blood*, 89(4), 1214–1223. <https://doi.org/10.1182/blood.v89.4.1214>
- Mizoguchi, T., Pinho, S., Ahmed, J., Kunisaki, Y., Hanoun, M., Mendelson, A., Ono, N., Kronenberg, H. M., & Frenette, P. S. (2014). Osterix marks distinct waves of primitive and definitive stromal progenitors during bone marrow development. *Developmental Cell*, 29(3), 340–349. <https://doi.org/10.1016/j.devcel.2014.03.013>
- Mohandas, N., & Prenant, M. (1978). Three-dimensional model of bone marrow. *Blood*, 51(4), 633–643. <http://www.ncbi.nlm.nih.gov/pubmed/630113>
- Montecino-Rodriguez, E., & Dorshkind, K. (2012). B-1 B Cell Development in the Fetus and Adult. *Immunity*, 36(1), 13–21. <https://doi.org/10.1016/j.immuni.2011.11.017>
- Moore, K. A., Pytowski, B., Witte, L., Hicklin, D., & Lemischka, I. R. (1997). Hematopoietic activity of a stromal cell transmembrane protein containing epidermal growth factor-like repeat motifs. *Proceedings of the National Academy of Sciences*, 94(8), 4011–4016. <https://doi.org/10.1073/pnas.94.8.4011>
- Moore, M. A. S. (2009). The Ontogeny of the Hematopoietic System. In *Essentials of Stem Cell Biology* (Second Edi, Issue 8). Elsevier Inc. <https://doi.org/10.1016/B978-0-12-374729-7.00023-8>
- Moore, M. A. S., & Metcalf, D. (1970). Ontogeny of the haemopoietic system: yolk sac origin of in vivo and in vitro colony forming cells in the developing mouse embryo. *British Journal of Haematology*, 18(3), 279–296. <https://doi.org/10.1111/j.1365-2141.1970.tb01443.x>
- Moore, M. A. S., & Owen, J. J. T. (1967). Chromosome Marker Studies in the Irradiated Chick Embryo. *Nature*, 215(5105), 1081–1082. <https://doi.org/10.1038/2151081a0>
- Moriguchi, T., & Yamamoto, M. (2014). A regulatory network governing Gata1 and Gata2 gene transcription orchestrates erythroid lineage differentiation. *International Journal of Hematology*, 100(5), 417–424. <https://doi.org/10.1007/s12185-014-1568-0>
- Morrison, S. J., Hemmati, H. D., Wandycz, A. M., & Weissman, I. L. (1995). The purification and characterization of fetal liver hematopoietic stem cells. *Proceedings of the National Academy of Sciences*, 92(22), 10302–10306. <https://doi.org/10.1073/pnas.92.22.10302>
- Mucenski, M. L., McLain, K., Kier, A. B., Swerdlow, S. H., Schreiner, C. M., Miller, T. A.,

- Pietryga, D. W., Scott, W. J., & Potter, S. S. (1991). A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. *Cell*, 65(4), 677–689. [https://doi.org/10.1016/0092-8674\(91\)90099-K](https://doi.org/10.1016/0092-8674(91)90099-K)
- Müller, A. M., Medvinsky, A., Strouboulis, J., Grosveld, F., & Dzierzakt, E. (1994). Development of hematopoietic stem cell activity in the mouse embryo. *Immunity*, 1(4), 291–301. [https://doi.org/10.1016/1074-7613\(94\)90081-7](https://doi.org/10.1016/1074-7613(94)90081-7)
- Munugalavadla, V., & Kapur, R. (2005). Role of c-Kit and erythropoietin receptor in erythropoiesis. *Critical Reviews in Oncology/Hematology*, 54(1), 63–75. <https://doi.org/10.1016/j.critrevonc.2004.11.005>
- Murray, P. D. F. (1932). The Development in Vitro of the Blood of the Early Chick Embryo. *Proceedings of the Royal Society of London. Series B, Containing Papers of a Biological Character*, 111(773), 497–521.
- Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S. I., Kitamura, Y., Yoshida, N., Kikutani, H., & Kishimoto, T. (1996). Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. In *Nature* (Vol. 382, Issue 6592, pp. 635–638). <https://doi.org/10.1038/382635a0>
- Nakamura-Ishizu, A., Takizawa, H., & Suda, T. (2014). The analysis, roles and regulation of quiescence in hematopoietic stem cells. *Development*, 141(24), 4656–4666. <https://doi.org/10.1242/dev.106575>
- Nakashima, K., Zhou, X., Kunkel, G., Zhang, Z., Deng, J. M., Behringer, R. R., & de Crombrughe, B. (2002). The Novel Zinc Finger-Containing Transcription Factor Osterix Is Required for Osteoblast Differentiation and Bone Formation. *Cell*, 108(1), 17–29. [https://doi.org/10.1016/S0092-8674\(01\)00622-5](https://doi.org/10.1016/S0092-8674(01)00622-5)
- Nichogiannopoulou, A., Trevisan, M., Neben, S., Friedrich, C., & Georgopoulos, K. (1999). Defects in Hemopoietic Stem Cell Activity in Ikaros Mutant Mice. *The Journal of Experimental Medicine*, 190(9), 1201–1214. <https://doi.org/10.1084/jem.190.9.1201>
- Nierhoff, D., Ogawa, A., Oertel, M., Chen, Y.-Q., & Shafritz, D. A. (2005). Purification and characterization of mouse fetal liver epithelial cells with high *in vivo* repopulation capacity. *Hepatology*, 42(1), 130–139. <https://doi.org/10.1002/hep.20735>
- Nishina, H., Vaz, C., Billia, P., Nghiem, M., Sasaki, T., De la Pompa, J. L., Furlonger, K., Paige, C., Hui, C. C., Fischer, K. D., Kishimoto, H., Iwatsubo, T., Katada, T., Woodgett, J. R., & Penninger, J. M. (1999). Defective liver formation and liver cell apoptosis in mice lacking the stress signaling kinase SEK1/MKK4. *Development*, 126(3), 505–516.
- Nitou, M., Ishikawa, K., & Shiojiri, N. (2000). Immunohistochemical analysis of development of desmin-positive hepatic stellate cells in mouse liver. *Journal of Anatomy*, 197(4), 635–646. <https://doi.org/10.1046/j.1469-7580.2000.19740635.x>
- Nitou, M., Sugiyama, Y., Ishikawa, K., & Shiojiri, N. (2002). Purification of fetal mouse hepatoblasts by magnetic beads coated with monoclonal anti-E-cadherin antibodies and their *in vitro* culture. *Experimental Cell Research*, 279(2), 330–343. <https://doi.org/10.1006/excr.2002.5615>
- Nocka, K., Majumder, S., Chabot, B., Ray, P., Cervone, M., Bernstein, A., & Besmer, P. (1989). Expression of c-kit gene products in known cellular targets of W mutations in normal and W mutant mice--evidence for an impaired c-kit kinase in mutant mice. *Genes & Development*, 3(6), 816–826. <https://doi.org/10.1101/gad.3.6.816>
- Nonaka, H., Tanaka, M., Suzuki, K., & Miyajima, A. (2007). Development of murine hepatic sinusoidal endothelial cells characterized by the expression of hyaluronan receptors. *Developmental Dynamics*, 236(8), 2258–2267. <https://doi.org/10.1002/dvdy.21227>
- North, T. E., de Bruijn, M. F. T., Stacy, T., Talebian, L., Lind, E., Robin, C., Binder, M., Dzierzak, E., & Speck, N. A. (2002). Runx1 Expression Marks Long-Term Repopulating Hematopoietic Stem Cells in the Midgestation Mouse Embryo. *Immunity*, 16(5), 661–672. [https://doi.org/10.1016/S1074-7613\(02\)00296-0](https://doi.org/10.1016/S1074-7613(02)00296-0)
- Nuez, B., Michalovich, D., Bygrave, A., Ploemacher, R., & Grosveld, F. (1995). Defective haematopoiesis in fetal liver resulting from inactivation of the EKLF gene. *Nature*, 375(6529), 316–318. <https://doi.org/10.1038/375316a0>
- Oburoglu, L., Romano, M., Taylor, N., & Kinet, S. (2016). Metabolic regulation of

- hematopoietic stem cell commitment and erythroid differentiation. *Current Opinion in Hematology*, 23(3), 198–205. <https://doi.org/10.1097/MOH.0000000000000234>
- Ogawa, M., Nishikawa, S., Ikuta, K., Yamamura, F., Naito, M., Takahashi, K., & Nishikawa, S. (1988). B cell ontogeny in murine embryo studied by a culture system with the monolayer of a stromal cell clone, ST2: B cell progenitor develops first in the embryonal body rather than in the yolk sac. *The EMBO Journal*, 7(5), 1337–1343. <https://doi.org/10.1002/j.1460-2075.1988.tb02949.x>
- Ohneda, O., Fennie, C., Zheng, Z., Donahue, C., La, H., Villacorta, R., Cairns, B., & Lasky, L. A. (1998). Hematopoietic Stem Cell Maintenance and Differentiation Are Supported by Embryonic Aorta-Gonad-Mesonephros Region-Derived Endothelium. *Blood*, 92(3), 908–919. <https://doi.org/10.1182/blood.V92.3.908>
- Okuda, T., Van Deursen, J., Hiebert, S. W., Grosveld, G., & Downing, J. R. (1996). AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell*, 84(2), 321–330. [https://doi.org/10.1016/S0092-8674\(00\)80986-1](https://doi.org/10.1016/S0092-8674(00)80986-1)
- Omatsu, Y., Sugiyama, T., Kohara, H., Kondoh, G., Fujii, N., Kohno, K., & Nagasawa, T. (2010). The Essential Functions of Adipo-osteogenic Progenitors as the Hematopoietic Stem and Progenitor Cell Niche. *Immunity*, 33(3), 387–399. <https://doi.org/10.1016/j.immuni.2010.08.017>
- Onitsuka, I., Tanaka, M., & Miyajima, A. (2010). Characterization and Functional Analyses of Hepatic Mesothelial Cells in Mouse Liver Development. *Gastroenterology*, 138(4), 1525–1535.e6. <https://doi.org/10.1053/j.gastro.2009.12.059>
- Oostendorp, R. A. J., Medvinsky, A. J., Kusadasi, N., Nakayama, N., Harvey, K., Orelia, C., Ottersbach, K., Covey, T., Ploemacher, R. E., Saris, C., & Dzierzak, E. (2002). Embryonal subregion-derived stromal cell lines from novel temperature-sensitive SV40 T antigen transgenic mice support hematopoiesis. *Journal of Cell Science*, 115(Pt 10), 2099–2108. <http://www.ncbi.nlm.nih.gov/pubmed/11973351>
- Otsuka, H., Takito, J., Endo, Y., Yagi, H., Soeta, S., Yanagisawa, N., Nonaka, N., & Nakamura, M. (2016). The expression of embryonic globin mRNA in a severely anemic mouse model induced by treatment with nitrogen-containing bisphosphonate. *BMC Hematology*, 16(1), 4. <https://doi.org/10.1186/s12878-016-0041-0>
- Ottersbach, K., & Dzierzak, E. (2005). The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. *Developmental Cell*, 8(3), 377–387. <https://doi.org/10.1016/j.devcel.2005.02.001>
- Ozderdem, U., Grako, K. A., Dahlin-Huppe, K., Monosov, E., & Stallcup, W. B. (2001). NG2 proteoglycan is expressed exclusively by mural cells during vascular morphogenesis. *Developmental Dynamics*, 222(2), 218–227. <https://doi.org/10.1002/dvdy.1200>
- Palis, J. (2014). Primitive and definitive erythropoiesis in mammals. *Frontiers in Physiology*, 5(3), 1–10. <https://doi.org/10.3389/fphys.2014.00003>
- Palis, J. (2016). Hematopoietic stem cell-independent hematopoiesis: emergence of erythroid, megakaryocyte, and myeloid potential in the mammalian embryo. *FEBS Letters*, 590(22), 3965–3974. <https://doi.org/10.1002/1873-3468.12459>
- Palis, J., Chan, R. J., Koniski, A. D., Patel, R., Starr, M., & Yoder, M. C. (2001a). Spatial and temporal emergence of high proliferative potential hematopoietic precursors during murine embryogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, 98(8), 4528–4533. <https://doi.org/10.1073/pnas.071002398>
- Palis, J., Robertson, S., Kennedy, M., Wall, C., & Keller, G. (1999). Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. *Development*, 126(22), 5073–5084. <http://www.ncbi.nlm.nih.gov/pubmed/10529424>
- Palis, J., & Yoder, M. C. (2001b). Yolk-sac hematopoiesis: The first blood cells of mouse and man. *Experimental Hematology*, 29(8), 927–936. [https://doi.org/10.1016/S0301-472X\(01\)00669-5](https://doi.org/10.1016/S0301-472X(01)00669-5)
- Pappenheim, A. (1896). Ueber Entwicklung und Ausbildung der Erythroblasten. *Archiv Für Pathologische Anatomie Und Physiologie Und Für Klinische Medicin*, 145(3), 587–643. <https://doi.org/10.1007/BF01969901>

- Pardanaud, L., Luton, D., Prigent, M., Bourcheix, L. M., Catala, M., & Dieterlen-Lievre, F. (1996). Two distinct endothelial lineages in ontogeny, one of them related to hemopoiesis. *Development (Cambridge, England)*, 122(5), 1363–1371. <http://www.ncbi.nlm.nih.gov/pubmed/8625825>
- Parviz, F., Li, J., Kaestner, K. H., & Duncan, S. A. (2002). Generation of a conditionally null allele of hnf4 α . *Genesis*, 32(2), 130–133. <https://doi.org/10.1002/gene.10058>
- Passegué, E., Wagers, A. J., Giuriato, S., Anderson, W. C., & Weissman, I. L. (2005). Global analysis of proliferation and cell cycle gene expression in the regulation of hematopoietic stem and progenitor cell fates. *Journal of Experimental Medicine*, 202(11), 1599–1611. <https://doi.org/10.1084/jem.20050967>
- Perkins, A. C., Sharpe, A. H., & Orkin, S. H. (1995). Lethal β -thalassaemia in mice lacking the erythroid CACCC-transcription factor EKLF. *Nature*, 375(6529), 318–322. <https://doi.org/10.1038/375318a0>
- Perkins, A., Xu, X., Higgs, D. R., Patrinos, G. P., Arnaud, L., Bieker, J. J., & Philipsen, S. (2016). Krüppeling erythropoiesis: an unexpected broad spectrum of human red blood cell disorders due to KLF1 variants. *Blood*, 127(15), 1856–1862. <https://doi.org/10.1182/blood-2016-01-694331>
- Peschon, J. J., Morrissey, P. J., Grabstein, K. H., Ramsdell, F. J., Maraskovsky, E., Gliniak, B. C., Park, L. S., Ziegler, S. F., Williams, D. E., Ware, C. B., Meyer, J. D., & Davison, B. L. (1994). Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *The Journal of Experimental Medicine*, 180(5), 1955–1960. <https://doi.org/10.1084/jem.180.5.1955>
- Petit-Cocault, L., Volle-Challier, C., Fleury, M., Peault, B., & Souyri, M. (2007). Dual role of Mpl receptor during the establishment of definitive hematopoiesis. *Development*, 134(16), 3031–3040. <https://doi.org/10.1242/dev.001818>
- Pevny, L., Lin, C. S., D'Agati, V., Simon, M. C., Orkin, S. H., & Costantini, F. (1995). Development of hematopoietic cells lacking transcription factor GATA-1. *Development (Cambridge, England)*, 121(1), 163–172. <http://www.ncbi.nlm.nih.gov/pubmed/7867497>
- Pevny, L., Simon, M. C., Robertson, E., Klein, W. H., Tsai, S.-F., D'Agati, V., Orkin, S. H., & Costantini, F. (1991). Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature*, 349(6306), 257–260. <https://doi.org/10.1038/349257a0>
- Pietras, E. M., Reynaud, D., Kang, Y.-A., Carlin, D., Calero-Nieto, F. J., Leavitt, A. D., Stuart, J. M., Göttgens, B., & Passegué, E. (2015). Functionally Distinct Subsets of Lineage-Biased Multipotent Progenitors Control Blood Production in Normal and Regenerative Conditions. *Cell Stem Cell*, 17(1), 35–46. <https://doi.org/10.1016/j.stem.2015.05.003>
- Pinho, S., & Frenette, P. S. (2019). Haematopoietic stem cell activity and interactions with the niche. *Nature Reviews Molecular Cell Biology*, 20(5), 303–320. <https://doi.org/10.1038/s41580-019-0103-9>
- Pinho, S., Lacombe, J., Hanoun, M., Mizoguchi, T., Bruns, I., Kunisaki, Y., & Frenette, P. S. (2013). PDGFR α and CD51 mark human Nestin⁺ sphere-forming mesenchymal stem cells capable of hematopoietic progenitor cell expansion. *Journal of Experimental Medicine*, 210(7), 1351–1367. <https://doi.org/10.1084/jem.20122252>
- Popescu, D.-M., Botting, R. A., Stephenson, E., Green, K., Webb, S., Jardine, L., Calderbank, E. F., Polanski, K., Goh, I., Efremova, M., Acres, M., Maunder, D., Vegh, P., Gitton, Y., Park, J.-E., Vento-Tormo, R., Miao, Z., Dixon, D., Rowell, R., ... Haniffa, M. (2019). Decoding human fetal liver haematopoiesis. *Nature*, 574(7778), 365–371. <https://doi.org/10.1038/s41586-019-1652-y>
- Porter, F. D., Drago, J., Xu, Y., Cheema, S. S., Wassif, C., Huang, S. P., Lee, E., Grinberg, A., Massalas, J. S., Bodine, D., Alt, F., & Westphal, H. (1997). Lhx2, a LIM homeobox gene, is required for eye, forebrain, and definitive erythrocyte development. *Development (Cambridge, England)*, 124(15), 2935–2944. <http://www.ncbi.nlm.nih.gov/pubmed/9247336>
- Postic, C., Shiota, M., Niswender, K. D., Jetton, T. L., Chen, Y., Moates, J. M., Shelton, K. D., Lindner, J., Cherrington, A. D., & Magnuson, M. A. (1999). Dual Roles for Glucokinase in

- Glucose Homeostasis as Determined by Liver and Pancreatic β Cell-specific Gene Knock-outs Using Cre Recombinase. *Journal of Biological Chemistry*, 274(1), 305–315. <https://doi.org/10.1074/jbc.274.1.305>
- Prior, N., Hindley, C. J., Rost, F., Meléndez, E., Lau, W. W. Y., Göttgens, B., Rulands, S., Simons, B. D., & Huch, M. (2019). Lgr5⁺ stem and progenitor cells reside at the apex of a heterogeneous embryonic hepatoblast pool. *Development (Cambridge)*, 146(12), dev174557. <https://doi.org/10.1242/dev.174557>
- Ramond, C., Berthault, C., Burlen-Defranoux, O., De Sousa, A. P., Guy-Grand, D., Vieira, P., Pereira, P., & Cumano, A. (2014). Two waves of distinct hematopoietic progenitor cells colonize the fetal thymus. *Nature Immunology*, 15(1), 27–35. <https://doi.org/10.1038/ni.2782>
- Ramsfjell, V., Borge, O. J., Veiby, O. P., Cardier, J., Murphy, M. J., Lyman, S. D., Lok, S., & Jacobsen, S. E. (1996). Thrombopoietin, but not erythropoietin, directly stimulates multilineage growth of primitive murine bone marrow progenitor cells in synergy with early acting cytokines: distinct interactions with the ligands for c-kit and FLT3. *Blood*, 88(12), 4481–4492. <http://www.ncbi.nlm.nih.gov/pubmed/8977240>
- Ratajczak, M. Z., Perrotti, D., Melotti, P., Powzaniuk, M., Calabretta, B., Onodera, K., Kregenow, D. A., Machalinski, B., & Gewirtz, A. M. (1998). Myb and Ets proteins are candidate regulators of c-kit expression in human hematopoietic cells. *Blood*, 91(6), 1934–1946. https://doi.org/10.1182/blood.v91.6.1934.1934_1934_1946
- Reya, T., Morrison, S. J., Clarke, M. F., & Weissman, I. L. (2001). Stem cells, cancer, and cancer stem cells. *Nature*, 414(6859), 105–111. <https://doi.org/10.1038/35102167>
- Rhodes, K. E., Gekas, C., Wang, Y., Lux, C. T., Francis, C. S., Chan, D. N., Conway, S., Orkin, S. H., Yoder, M. C., & Mikkola, H. K. A. (2008a). The Emergence of Hematopoietic Stem Cells Is Initiated in the Placental Vasculature in the Absence of Circulation. *Cell Stem Cell*, 2(3), 252–263. <https://doi.org/10.1016/j.stem.2008.01.001>
- Rhodes, M. M., Kopsombut, P., Bondurant, M. C., Price, J. O., & Koury, M. J. (2008b). Adherence to macrophages in erythroblastic islands enhances erythroblast proliferation and increases erythrocyte production by a different mechanism than erythropoietin. *Blood*, 111(3), 1700–1708. <https://doi.org/10.1182/blood-2007-06-098178>
- Rich, I. N., & Kubanek, B. (1980). The ontogeny of erythropoiesis in the mouse detected by the erythroid colony-forming technique. *Journal of Embryology and Experimental Morphology*, 58(1), 143 LP – 155. <http://dev.biologists.org/content/58/1/143.abstract>
- Rieger, M. A., Hoppe, P. S., Smejkal, B. M., Eitelhuber, A. C., & Schroeder, T. (2009). Hematopoietic Cytokines Can Instruct Lineage Choice. *Science*, 325(5937), 217–218. <https://doi.org/10.1126/science.1171461>
- Robin, C., Bollerot, K., Mendes, S., Haak, E., Crisan, M., Cerisoli, F., Lauw, I., Kaimakis, P., Jorna, R., Vermeulen, M., Kayser, M., van der Linden, R., Imanirad, P., Verstegen, M., Nawaz-Yousaf, H., Papazian, N., Steegers, E., Cupedo, T., & Dzierzak, E. (2009). Human Placenta Is a Potent Hematopoietic Niche Containing Hematopoietic Stem and Progenitor Cells throughout Development. *Cell Stem Cell*, 5(4), 385–395. <https://doi.org/10.1016/j.stem.2009.08.020>
- Rodriguez-Fraticelli, A. E., Wolock, S. L., Weinreb, C. S., Panero, R., Patel, S. H., Jankovic, M., Sun, J., Calogero, R. A., Klein, A. M., & Camargo, F. D. (2018). Clonal analysis of lineage fate in native haematopoiesis. *Nature*, 553(7687), 212–216. <https://doi.org/10.1038/nature25168>
- Rogers, H., Wang, L., Yu, X., Alnaeeli, M., Cui, K., Zhao, K., Bieker, J. J., Prchal, J., Huang, S., Weksler, B., & Noguchi, C. T. (2012). T-cell Acute Leukemia 1 (TAL1) Regulation of Erythropoietin Receptor and Association with Excessive Erythrocytosis. *Journal of Biological Chemistry*, 287(44), 36720–36731. <https://doi.org/10.1074/jbc.M112.378398>
- Rossant, J., & Cross, J. C. (2001). Placental development: Lessons from mouse mutants. *Nature Reviews Genetics*, 2(7), 538–548. <https://doi.org/10.1038/35080570>
- Roy, V., & Verfaillie, C. M. (1999). Expression and function of cell adhesion molecules on fetal liver, cord blood and bone marrow hematopoietic progenitors: Implications for anatomical localization and developmental stage specific regulation of hematopoiesis. *Experimental*

- Hematology*, 27(2), 302–312. [https://doi.org/10.1016/S0301-472X\(98\)00031-9](https://doi.org/10.1016/S0301-472X(98)00031-9)
- Ruggeri, A., Labopin, M., Sormani, M. P., Sanz, G., Sanz, J., Volt, F., Michel, G., Locatelli, F., De Heredia, C. D., O'Brien, T., Arcese, W., Iori, A. P., Querol, S., Kogler, G., Lecchi, L., Pouthier, F., Garnier, F., Navarrete, C., Baudoux, E., ... Saccardi, R. (2014). Engraftment kinetics and graft failure after single umbilical cord blood transplantation using a myeloablative conditioning regimen. *Haematologica*, 99(9), 1509–1515. <https://doi.org/10.3324/haematol.2014.109280>
- Russel, E. S., Thompson, M. W., & McFarland, E. (1968). Analysis of effects of W and f genic substitutions on fetal mouse hematology. *Genetics*, 58(2), 259–270. <http://www.ncbi.nlm.nih.gov/pubmed/5691002>
- Sabin, & F.R. (1920). Studies on the origin of blood-vessels and of red blood-corpuscles as seen in the living blastoderm of chicks during the second day of incubation. *Contrib. Embryol Carneg Inst.*, 9, 214–262. <http://ci.nii.ac.jp/naid/10012241153/en/>
- Sacchetti, B., Funari, A., Michienzi, S., Di Cesare, S., Piersanti, S., Saggio, I., Tagliafico, E., Ferrari, S., Robey, P. G., Riminucci, M., & Bianco, P. (2007). Self-Renewing Osteoprogenitors in Bone Marrow Sinusoids Can Organize a Hematopoietic Microenvironment. *Cell*, 131(2), 324–336. <https://doi.org/10.1016/j.cell.2007.08.025>
- Saleque, S. (2002). The zinc-finger proto-oncogene Gfi-1b is essential for development of the erythroid and megakaryocytic lineages. *Genes & Development*, 16(3), 301–306. <https://doi.org/10.1101/gad.959102>
- Sánchez, M.-J., Holmes, A., Miles, C., & Dzierzak, E. (1996). Characterization of the First Definitive Hematopoietic Stem Cells in the AGM and Liver of the Mouse Embryo. *Immunity*, 5(6), 513–525. [https://doi.org/10.1016/S1074-7613\(00\)80267-8](https://doi.org/10.1016/S1074-7613(00)80267-8)
- Sanjuan-Pla, A., Macaulay, I. C., Jensen, C. T., Woll, P. S., Luis, T. C., Mead, A., Moore, S., Carella, C., Matsuoka, S., Jones, T. B., Chowdhury, O., Stenson, L., Lutteropp, M., Green, J. C. A., Facchini, R., Boukarabila, H., Grover, A., Gambardella, A., Thongjuea, S., ... Jacobsen, S. E. W. (2013). Platelet-biased stem cells reside at the apex of the haematopoietic stem-cell hierarchy. *Nature*, 502(7470), 232–236. <https://doi.org/10.1038/nature12495>
- Sasaki, K., & Sonoda, Y. (2000). Histometrical and Three-Dimensional Analyses of Liver Hematopoiesis in the Mouse Embryo. *Archives of Histology and Cytology*, 63(2), 137–146. <https://doi.org/10.1679/aohc.63.137>
- Sasaki, T., Mizuochi, C., Horio, Y., Nakao, K., Akashi, K., & Sugiyama, D. (2010). Regulation of hematopoietic cell clusters in the placental niche through SCF/Kit signaling in embryonic mouse. *Development*, 137(23), 3941–3952. <https://doi.org/10.1242/dev.051359>
- Sauvageau, G., Iscove, N. N., & Humphries, R. K. (2004). In vitro and in vivo expansion of hematopoietic stem cells. *Oncogene*, 23(43), 7223–7232. <https://doi.org/10.1038/sj.onc.1207942>
- Schacht, V., Dadras, S. S., Johnson, L. A., Jackson, D. G., Hong, Y.-K., & Detmar, M. (2005). Up-Regulation of the Lymphatic Marker Podoplanin, a Mucin-Type Transmembrane Glycoprotein, in Human Squamous Cell Carcinomas and Germ Cell Tumors. *The American Journal of Pathology*, 166(3), 913–921. [https://doi.org/10.1016/S0002-9440\(10\)62311-5](https://doi.org/10.1016/S0002-9440(10)62311-5)
- Schaefer, B. C., Schaefer, M. L., Kappler, J. W., Marrack, P., & Kedl, R. M. (2001). Observation of Antigen-Dependent CD8+ T-Cell/ Dendritic Cell Interactions in Vivo. *Cellular Immunology*, 214(2), 110–122. <https://doi.org/10.1006/cimm.2001.1895>
- Schmitt, C. E., Lizama, C. O., & Zovein, A. C. (2014). From transplantation to transgenics: Mouse models of developmental hematopoiesis. *Experimental Hematology*, 42(8), 707–716. <https://doi.org/10.1016/j.exphem.2014.06.008>
- Schofield, R. (1978). The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells*, 4(1–2), 7–25. <http://www.ncbi.nlm.nih.gov/pubmed/747780>
- Schulz, C., Gomez-Perdiguero, E., Chorro, L., Szabo-Rogers, H., Cagnard, N., Kierdorf, K., Prinz, M., Wu, B., Jacobsen, S. E. W., Pollard, J. W., Frampton, J., Liu, K. J., &

- Geissmann, F. (2012). A Lineage of Myeloid Cells Independent of Myb and Hematopoietic Stem Cells. *Science*, 336(6077), 86–90. <https://doi.org/10.1126/science.1219179>
- Schumann, K., Lämmermann, T., Bruckner, M., Legler, D. F., Polleux, J., Spatz, J. P., Schuler, G., Förster, R., Lutz, M. B., Sorokin, L., & Sixt, M. (2010). Immobilized Chemokine Fields and Soluble Chemokine Gradients Cooperatively Shape Migration Patterns of Dendritic Cells. *Immunity*, 32(5), 703–713. <https://doi.org/10.1016/j.immuni.2010.04.017>
- Schuster, J. A., Stupnikov, M. R., Ma, G., Liao, W., Lai, R., Ma, Y., & Aguila, J. R. (2012). Expansion of hematopoietic stem cells for transplantation: current perspectives. *Experimental Hematology & Oncology*, 1(1), 12. <https://doi.org/10.1186/2162-3619-1-12>
- Seki, M., & Shirasawa, H. (1965). Role of the reticular cells during maturation process of the erythroblast. III. The fate of phagocytized nucleus. *Pathology International*, 15(4), 387–405. <https://doi.org/10.1111/j.1440-1827.1965.tb01930.x>
- Severn, C. B. (1971). A morphological study of the development of the human liver. I. Development of the hepatic diverticulum. *American Journal of Anatomy*, 131(2), 133–158. <https://doi.org/10.1002/aja.1001310202>
- Shiojiri, N., & Sugiyama, Y. (2004). Immunolocalization of extracellular matrix components and integrins during mouse liver development. *Hepatology*, 40(2), 346–355. <https://doi.org/10.1002/hep.20303>
- Shivdasani, R. A., Mayer, E. L., & Orkin, S. H. (1995). Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. In *Nature* (Vol. 373, Issue 6513, pp. 432–434). <https://doi.org/10.1038/373432a0>
- Sigurdsson, V., Takei, H., Soboleva, S., Radulovic, V., Galeev, R., Siva, K., Leeb-Lundberg, L. M. F., Iida, T., Nittono, H., & Miharada, K. (2016). Bile Acids Protect Expanding Hematopoietic Stem Cells from Unfolded Protein Stress in Fetal Liver. *Cell Stem Cell*, 18(4), 522–532. <https://doi.org/10.1016/j.stem.2016.01.002>
- Silva, M., Grillet, D., Benito, A., Richard, C., Nunez, G., & Fernandez-Luna, J. (1996). Erythropoietin can promote erythroid progenitor survival by repressing apoptosis through Bcl-XL and Bcl-2. *Blood*, 88(5), 1576–1582. <https://doi.org/10.1182/blood.V88.5.1576.1576>
- Skutelsky, E., & Danon, D. (1972). On the expulsion of the erythroid nucleus and its phagocytosis. *The Anatomical Record*, 173(1), 123–126. <https://doi.org/10.1002/ar.1091730111>
- Soares-da-Silva, F., Peixoto, M., Cumano, A., & Pinto-do-Ó, P. (2020). Crosstalk Between the Hepatic and Hematopoietic Systems During Embryonic Development. *Frontiers in Cell and Developmental Biology*, 8(July), 1–20. <https://doi.org/10.3389/fcell.2020.00612>
- Stier, S., Ko, Y., Forkert, R., Lutz, C., Neuhaus, T., Grünewald, E., Cheng, T., Dombkowski, D., Calvi, L. M., Rittling, S. R., & Scadden, D. T. (2005). Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. *The Journal of Experimental Medicine*, 201(11), 1781–1791. <https://doi.org/10.1084/jem.20041992>
- Sudo, T., Ito, M., Ogawa, Y., Iizuka, M., Kodama, H., Kunisada, T., Hayashi, S., Ogawa, M., Sakai, K., & Nishikawa, S. (1989). Interleukin 7 production and function in stromal cell-dependent B cell development. *The Journal of Experimental Medicine*, 170(1), 333–338. <https://doi.org/10.1084/jem.170.1.333>
- Sugiyama, D., Kulkeaw, K., & Mizuochi, C. (2013). TGF-beta-1 up-regulates extra-cellular matrix production in mouse hepatoblasts. *Mechanisms of Development*, 130(2–3), 195–206. <https://doi.org/10.1016/j.mod.2012.09.003>
- Sugiyama, D., Kulkeaw, K., Mizuochi, C., Horio, Y., & Okayama, S. (2011). Hepatoblasts comprise a niche for fetal liver erythropoiesis through cytokine production. *Biochemical and Biophysical Research Communications*, 410(2), 301–306. <https://doi.org/10.1016/j.bbrc.2011.05.137>
- Sugiyama, T., Kohara, H., Noda, M., & Nagasawa, T. (2006). Maintenance of the Hematopoietic Stem Cell Pool by CXCL12-CXCR4 Chemokine Signaling in Bone Marrow Stromal Cell Niches. *Immunity*, 25(6), 977–988. <https://doi.org/10.1016/j.immuni.2006.10.016>

- Sugiyama, Y., Koike, T., & Shiojiri, N. (2010a). Developmental Changes of Cell Adhesion Molecule Expression in the Fetal Mouse Liver. *The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology*, 293(10), 1698–1710. <https://doi.org/10.1002/ar.21204>
- Sugiyama, Y., Takabe, Y., Nakakura, T., Tanaka, S., Koike, T., & Shiojiri, N. (2010b). Sinusoid development and morphogenesis may be stimulated by VEGF-Flk-1 signaling during fetal mouse liver development. *Developmental Dynamics*, 239(2), 386–397. <https://doi.org/10.1002/dvdy.22162>
- Sun, J., Ramos, A., Chapman, B., Johnnidis, J. B., Le, L., Ho, Y. J., Klein, A., Hofmann, O., & Camargo, F. D. (2014). Clonal dynamics of native haematopoiesis. *Nature*, 514(7522), 322–327. <https://doi.org/10.1038/nature13824>
- Suzuki, A., Zheng, Y. W., Kondo, R., Kusakabe, M., Takada, Y., Fukao, K., Nakauchi, H., & Taniguchi, H. (2000). Flow-cytometric separation and enrichment of hepatic progenitor cells in the developing mouse liver. *Hepatology*, 32(6), 1230–1239. <https://doi.org/10.1053/jhep.2000.20349>
- Suzuki, N., Obara, N., Pan, X., Watanabe, M., Jishage, K.-I., Minegishi, N., & Yamamoto, M. (2011). Specific Contribution of the Erythropoietin Gene 3' Enhancer to Hepatic Erythropoiesis after Late Embryonic Stages. *Molecular and Cellular Biology*, 31(18), 3896–3905. <https://doi.org/10.1128/MCB.05463-11>
- Swartley, O. M., Foley, J. F., Livingston, D. P., Cullen, J. M., & Elmore, S. A. (2016). Histology Atlas of the Developing Mouse Hepatobiliary Hemolymphatic Vascular System with Emphasis on Embryonic Days 11.5–18.5 and Early Postnatal Development. *Toxicologic Pathology*, 44(5), 705–725. <https://doi.org/10.1177/0192623316630836>
- Tajer, P., Pike-Overzet, K., Arias, S., Havenga, M., & Staal, F. (2019). Ex Vivo Expansion of Hematopoietic Stem Cells for Therapeutic Purposes: Lessons from Development and the Niche. *Cells*, 8(2), 169. <https://doi.org/10.3390/cells8020169>
- Takabe, Y., Yagi, S., Koike, T., & Shiojiri, N. (2012). Immunomagnetic exclusion of E-cadherin-positive hepatoblasts in fetal mouse liver cell cultures impairs morphogenesis and gene expression of sinusoidal endothelial cells. *Journal of Anatomy*, 221(3), 229–239. <https://doi.org/10.1111/j.1469-7580.2012.01532.x>
- Takahashi, K., Yamamura, F., & Naito, M. (1989). Differentiation, Maturation, and Proliferation of Macrophages in the Mouse Yolk Sac: A Light-Microscopic, Enzyme-Cytochemical, Immunohistochemical, and Ultrastructural Study. *Journal of Leukocyte Biology*, 45(2), 87–96. <https://doi.org/10.1002/jlb.45.2.87>
- Takebe, T., Enomura, M., Yoshizawa, E., Kimura, M., Koike, H., Ueno, Y., Matsuzaki, T., Yamazaki, T., Toyohara, T., Osafune, K., Nakauchi, H., Yoshikawa, H. Y., & Taniguchi, H. (2015). Vascularized and Complex Organ Buds from Diverse Tissues via Mesenchymal Cell-Driven Condensation. *Cell Stem Cell*, 16(5), 556–565. <https://doi.org/10.1016/j.stem.2015.03.004>
- Tallack, M. R., Whittington, T., Shan Yuen, W., Wainwright, E. N., Keys, J. R., Gardiner, B. B., Nourbakhsh, E., Cloonan, N., Grimmond, S. M., Bailey, T. L., & Perkins, A. C. (2010). A global role for KLF1 in erythropoiesis revealed by ChIP-seq in primary erythroid cells. *Genome Research*, 20(8), 1052–1063. <https://doi.org/10.1101/gr.106575.110>
- Tan, J. K. H., & Watanabe, T. (2017a). Stromal Cell Subsets Directing Neonatal Spleen Regeneration. *Scientific Reports*, 7, 1–11. <https://doi.org/10.1038/srep40401>
- Tan, K. S., Kulkeaw, K., Nakanishi, Y., & Sugiyama, D. (2017b). Expression of cytokine and extracellular matrix mRNAs in fetal hepatic stellate cells. *Genes to Cells*, 22(9), 836–844. <https://doi.org/10.1111/gtc.12517>
- Tanaka, M., Okabe, M., Suzuki, K., Kamiya, Y., Tsukahara, Y., Saito, S., & Miyajima, A. (2009). Mouse hepatoblasts at distinct developmental stages are characterized by expression of EpCAM and DLK1: Drastic change of EpCAM expression during liver development. *Mechanisms of Development*, 126(8–9), 665–676. <https://doi.org/10.1016/j.mod.2009.06.939>
- Tanaka, Y., Kurosawa, S., Tajima, K., Tanaka, T., Ito, R., Inoue, Y., Okinaka, K., Inamoto, Y., Fuji, S., Kim, S. W., Tanosaki, R., Yamashita, T., & Fukuda, T. (2016). Analysis of non-

- relapse mortality and causes of death over 15 years following allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplantation*, 51(4), 553–559. <https://doi.org/10.1038/bmt.2015.330>
- Tanimizu, N., Nishikawa, M., Saito, H., Tsujimura, T., & Miyajima, A. (2003). Isolation of hepatoblasts based on the expression of Dlk/Pref-1. *Journal of Cell Science*, 116(9), 1775–1786. <https://doi.org/10.1242/jcs.00388>
- Tanimizu, N., Saito, H., Mostov, K., & Miyajima, A. (2004). Long-term culture of hepatic progenitors derived from mouse Dlk+ hepatoblasts. *Journal of Cell Science*, 117(26), 6425–6434. <https://doi.org/10.1242/jcs.01572>
- Taoudi, S., Gonneau, C., Moore, K., Sheridan, J. M., Blackburn, C. C., Taylor, E., & Medvinsky, A. (2008). Extensive hematopoietic stem cell generation in the AGM region via maturation of VE-Cadherin+CD45+ pre-definitive HSCs. *Cell Stem Cell*, 3(1), 99–108. <https://doi.org/10.1016/j.stem.2008.06.004>
- Taoudi, S., & Medvinsky, A. (2007). Functional identification of the hematopoietic stem cell niche in the ventral domain of the embryonic dorsal aorta. *Proceedings of the National Academy of Sciences of the United States of America*, 104(22), 9399–9403. <https://doi.org/10.1073/pnas.0700984104>
- Taoudi, S., Morrison, A. M., Inoue, H., Gribi, R., Ure, J., & Medvinsky, A. (2005). Progressive divergence of definitive haematopoietic stem cells from the endothelial compartment does not depend on contact with the foetal liver. *Development*, 132(18), 4179–4191. <https://doi.org/10.1242/dev.01974>
- Thomas, M. L., & Lefrançois, L. (1988). Differential expression of the leucocyte-common antigen family. *Immunology Today*, 9(10), 320–326. [https://doi.org/10.1016/0167-5699\(88\)91326-6](https://doi.org/10.1016/0167-5699(88)91326-6)
- Till, J. E., & McCulloch, E. A. (1961). A Direct Measurement of the Radiation Sensitivity of Normal Mouse Bone Marrow Cells. *Radiation Research*, 14(2), 213. <https://doi.org/10.2307/3570892>
- Till, J. E., McCulloch, E. A., & Siminovitch, L. (1964). A STOCHASTIC MODEL OF STEM CELL PROLIFERATION, BASED ON THE GROWTH OF SPLEEN COLONY-FORMING CELLS. *Proceedings of the National Academy of Sciences*, 51(1), 29–36. <https://doi.org/10.1073/pnas.51.1.29>
- Tober, J., Koniski, A. D., McGrath, K. E., Vemishetti, R., Emerson, R. L. R., de Mesy-Bentley, K. K. L. L., Waugh, R. E., & Palis, J. (2007). The megakaryocyte lineage originates from hemangioblast precursors and is an integral component both of primitive and of definitive hematopoiesis. *Blood*, 109(4), 1433–1441. <https://doi.org/10.1182/blood-2006-06-031898>
- Tober, J., McGrath, K. E., & Palis, J. (2008). Primitive erythropoiesis and megakaryopoiesis in the yolk sac are independent of c-myb. *Blood*, 111(5), 2636–2639. <https://doi.org/10.1182/blood-2007-11-124685>
- Tremblay, K. D., & Zaret, K. S. (2005). Distinct populations of endoderm cells converge to generate the embryonic liver bud and ventral foregut tissues. *Developmental Biology*, 280(1), 87–99. <https://doi.org/10.1016/j.ydbio.2005.01.003>
- Tsai, F.-Y., Keller, G., Kuo, F. C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F. W., & Orkin, S. H. (1994). An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature*, 371(6494), 221–226. <https://doi.org/10.1038/371221a0>
- Tsai, S., Emerson, S. G., Sieff, C. A., & Nathan, D. G. (1986). Isolation of a human stromal cell strain secreting hemopoietic growth factors. *Journal of Cellular Physiology*, 127(1), 137–145. <https://doi.org/10.1002/jcp.1041270117>
- Tsuneto, M., Tokoyoda, K., Kajikhina, E., Hauser, A. E., Hara, T., Tani-ichi, S., Ikuta, K., & Melchers, F. (2013). B-Cell Progenitors and Precursors Change Their Microenvironment in Fetal Liver During Early Development. *STEM CELLS*, 31(12), 2800–2812. <https://doi.org/10.1002/stem.1421>
- Udan, R. S., Culver, J. C., & Dickinson, M. E. (2013). Understanding vascular development. *Wiley Interdisciplinary Reviews: Developmental Biology*, 2(3), 327–346. <https://doi.org/10.1002/wdev.91>

- Vegiopoulos, A., Garcia, P., Emambokus, N., & Frampton, J. (2006). Coordination of erythropoiesis by the transcription factor c-Myb. *Blood*, 107(12), 4703–4710. <https://doi.org/10.1182/blood-2005-07-2968>
- Velten, L., Haas, S. F., Raffel, S., Blaszkiewicz, S., Islam, S., Hennig, B. P., Hirche, C., Lutz, C., Buss, E. C., Nowak, D., Boch, T., Hofmann, W. K., Ho, A. D., Huber, W., Trumpp, A., Essers, M. A. G., & Steinmetz, L. M. (2017). Human haematopoietic stem cell lineage commitment is a continuous process. *Nature Cell Biology*, 19(4), 271–281. <https://doi.org/10.1038/ncb3493>
- Wake, K. (1971). “Sternzellen” in the liver: Perisinusoidal cells with special reference to storage of vitamin A. *American Journal of Anatomy*, 132(4), 429–461. <https://doi.org/10.1002/aja.1001320404>
- Walasek, M. A., van Os, R., & de Haan, G. (2012). Hematopoietic stem cell expansion: challenges and opportunities. *Annals of the New York Academy of Sciences*, 1266(1), 138–150. <https://doi.org/10.1111/j.1749-6632.2012.06549.x>
- Wang, H. U., Chen, Z. F., & Anderson, D. J. (1998). Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell*, 93(5), 741–753. [https://doi.org/10.1016/S0092-8674\(00\)81436-1](https://doi.org/10.1016/S0092-8674(00)81436-1)
- Wang, Q., Stacy, T., Binder, M., Marin-Padilla, M., Sharpe, A. H., & Speck, N. A. (1996a). Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. *Proceedings of the National Academy of Sciences*, 93(8), 3444–3449. <https://doi.org/10.1073/pnas.93.8.3444>
- Wang, Q., Stacy, T., Miller, J. D., Lewis, A. F., Gu, T.-L., Huang, X., Bushweller, J. H., Bories, J.-C., Alt, F. W., Ryan, G., Liu, P. P., Wynshaw-Boris, A., Binder, M., Marin-Padilla, M., Sharpe, A. H., & Speck, N. A. (1996b). The CBF β Subunit Is Essential for CBF α 2 (AML1) Function In Vivo. *Cell*, 87(4), 697–708. [https://doi.org/10.1016/S0092-8674\(00\)81389-6](https://doi.org/10.1016/S0092-8674(00)81389-6)
- Warren, A. J., Colledge, W. H., Carlton, M. B. L., Evans, M. J., Smith, A. J. H., & Rabbitts, T. H. (1994). The Oncogenic Cysteine-rich LIM domain protein Rbtl2 is essential for erythroid development. *Cell*, 78(1), 45–57. [https://doi.org/10.1016/0092-8674\(94\)90571-1](https://doi.org/10.1016/0092-8674(94)90571-1)
- Watanabe, T., Nakagawa, K., Ohata, S., Kitagawa, D., Nishitai, G., Seo, J., Tanemura, S., Shimizu, N., Kishimoto, H., Wada, T., Aoki, J., Arai, H., Iwatsubo, T., Mochita, M., Watanabe, T., Satake, M., Ito, Y., Matsuyama, T., Mak, T. W., ... Katada, T. (2002). SEK1/MKK4-Mediated SAPK/JNK Signaling Participates in Embryonic Hepatoblast Proliferation via a Pathway Different from NF- κ B-Induced Anti-Apoptosis. *Developmental Biology*, 250(2), 332–347. <https://doi.org/10.1006/dbio.2002.0781>
- Wei, Q., & Frenette, P. S. (2018). Niches for Hematopoietic Stem Cells and Their Progeny. *Immunity*, 48(4), 632–648. <https://doi.org/10.1016/j.immuni.2018.03.024>
- Weiss, M. J., Keller, G., & Orkin, S. H. (1994). Novel insights into erythroid development revealed through in vitro differentiation of GATA-1 embryonic stem cells. *Genes & Development*, 8(10), 1184–1197. <https://doi.org/10.1101/gad.8.10.1184>
- Widness, J. A. (2008). Pathophysiology of Anemia During the Neonatal Period, Including Anemia of Prematurity. *NeoReviews*, 9(11), e520–e525. <https://doi.org/10.1542/neo.9-11-e520>
- Wilkinson, A. C., Igarashi, K. J., & Nakauchi, H. (2020). Haematopoietic stem cell self-renewal in vivo and ex vivo. *Nature Reviews Genetics*, 1–14. <https://doi.org/10.1038/s41576-020-0241-0>
- Wilkinson, A. C., Ishida, R., Kikuchi, M., Sudo, K., Morita, M., Crisostomo, R. V., Yamamoto, R., Loh, K. M., Nakamura, Y., Watanabe, M., Nakauchi, H., & Yamazaki, S. (2019). Long-term ex vivo haematopoietic-stem-cell expansion allows nonconditioned transplantation. *Nature*, 571(7763), 117–121. <https://doi.org/10.1038/s41586-019-1244-x>
- Wilson, J. W., Groat, C. S., & Leduc, E. H. (1963). HISTOGENESIS OF THE LIVER. *Annals of the New York Academy of Sciences*, 111(1), 8–24. <https://doi.org/10.1111/j.1749-6632.1963.tb36945.x>
- Wineman, J., Moore, K., Lemischka, I. R., & Müller-Sieburg, C. (1996). Functional heterogeneity of the hematopoietic microenvironment: rare stromal elements maintain

- long-term repopulating stem cells. *Blood*, 87(10), 4082–4090. <http://www.ncbi.nlm.nih.gov/pubmed/8639765>
- Wohrer, S., Knapp, D. J. H. F., Copley, M. R., Benz, C., Kent, D. G., Rowe, K., Babovic, S., Mader, H., Oostendorp, R. A. J., & Eaves, C. J. (2014). Distinct Stromal Cell Factor Combinations Can Separately Control Hematopoietic Stem Cell Survival, Proliferation, and Self-Renewal. *Cell Reports*, 7(6), 1956–1967. <https://doi.org/10.1016/j.celrep.2014.05.014>
- Wolber, F. M., Leonard, E., Michael, S., Orschell-Traycoff, C. M., Yoder, M. C., & Srouf, E. F. (2002). Roles of spleen and liver in development of the murine hematopoietic system. *Experimental Hematology*, 30(9), 1010–1019. [https://doi.org/10.1016/S0301-472X\(02\)00881-0](https://doi.org/10.1016/S0301-472X(02)00881-0)
- Wong, J. B., Hewitt, S. L., Heltemes-Harris, L. M., Mandal, M., Johnson, K., Rajewsky, K., Koralov, S. B., Clark, M. R., Farrar, M. A., & Skok, J. A. (2019). B-1a cells acquire their unique characteristics by bypassing the pre-BCR selection stage. *Nature Communications*, 10(1), 4768. <https://doi.org/10.1038/s41467-019-12824-z>
- Wong, P. P. M. C., Chung, S.-W. S., Reicheld, S. M. S., & Chui, D. D. H. K. (1986). Hemoglobin switching during murine embryonic development: evidence for two populations of embryonic erythropoietic progenitor cells. *Blood*, 67(3), 716–721. <https://doi.org/10.1182/blood.V67.3.716.716>
- Wu, H., Klingmüller, U., Besmer, P., & Lodish, H. F. (1995a). Interaction of the erythropoietin and stem-cell-factor receptors. *Nature*, 377(6546), 242–246. <https://doi.org/10.1038/377242a0>
- Wu, H., Liu, X., Jaenisch, R., & Lodish, H. F. (1995b). Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. *Cell*, 83(1), 59–67. [https://doi.org/10.1016/0092-8674\(95\)90234-1](https://doi.org/10.1016/0092-8674(95)90234-1)
- Xu, M., Matsuoka, S., Yang, F.-C., Ebihara, Y., Manabe, A., Tanaka, R., Eguchi, M., Asano, S., Nakahata, T., & Tsuji, K. (2001). Evidence for the presence of murine primitive megakaryocytopoiesis in the early yolk sac. *Blood*, 97(7), 2016–2022. <https://doi.org/10.1182/blood.V97.7.2016>
- Xu, M., Tsuji, K., Ueda, T., Mukoyama, Y., Hara, T., Yang, F.-C., Ebihara, Y., Matsuoka, S., Manabe, A., Kikuchi, A., Ito, M., Miyajima, A., & Nakahata, T. (1998). Stimulation of Mouse and Human Primitive Hematopoiesis by Murine Embryonic Aorta-Gonad-Mesonephros-Derived Stromal Cell Lines. *Blood*, 92(6), 2032–2040. <https://doi.org/10.1182/blood.V92.6.2032>
- Yamamoto, R., Morita, Y., Ooehara, J., Hamanaka, S., Onodera, M., Rudolph, K. L., Ema, H., & Nakauchi, H. (2013). Clonal Analysis Unveils Self-Renewing Lineage-Restricted Progenitors Generated Directly from Hematopoietic Stem Cells. *Cell*, 154(5), 1112–1126. <https://doi.org/10.1016/j.cell.2013.08.007>
- Yang, L., Li, L. C., Lamaoqiezhong, Wang, X., Wang, W. H., Wang, Y. C., & Xu, C. R. (2019). The contributions of mesoderm-derived cells in liver development. *Seminars in Cell and Developmental Biology*, 92(March), 63–76. <https://doi.org/10.1016/j.semcdb.2018.09.003>
- Yang, L., Wang, W. H., Qiu, W. L., Guo, Z., Bi, E., & Xu, C. R. (2017). A single-cell transcriptomic analysis reveals precise pathways and regulatory mechanisms underlying hepatoblast differentiation. *Hepatology*, 66(5), 1387–1401. <https://doi.org/10.1002/hep.29353>
- Yoder, M. C., Hiatt, K., Dutt, P., Mukherjee, P., Bodine, D. M., & Orlic, D. (1997). Characterization of Definitive Lymphohematopoietic Stem Cells in the Day 9 Murine Yolk Sac. *Immunity*, 7(3), 335–344. [https://doi.org/10.1016/S1074-7613\(00\)80355-6](https://doi.org/10.1016/S1074-7613(00)80355-6)
- Yoder, M. C., Papaioannou, V. E., Breitfeld, P. P., & Williams, D. A. (1994). Murine yolk sac endoderm- and mesoderm-derived cell lines support in vitro growth and differentiation of hematopoietic cells. *Blood*, 83(9), 2436–2443. <http://www.ncbi.nlm.nih.gov/pubmed/8167333>
- Yoshida, H., Kawamoto, H., Santee, S. M., Hashi, H., Honda, K., Nishikawa, S., Ware, C. F., Katsura, Y., & Nishikawa, S.-I. (2001). Expression of $\alpha 4 \beta 7$ Integrin Defines a Distinct

- Pathway of Lymphoid Progenitors Committed to T Cells, Fetal Intestinal Lymphotoxin Producer, NK, and Dendritic Cells. *The Journal of Immunology*, 167(5), 2511–2521. <https://doi.org/10.4049/jimmunol.167.5.2511>
- Yu, V. W. C., & Scadden, D. T. (2016). Hematopoietic Stem Cell and Its Bone Marrow Niche. *Current Topics in Developmental Biology*, 118, 21–44. <https://doi.org/10.1016/bs.ctdb.2016.01.009>
- Zanjani, E. D., Ascensao, J. L., McGlave, P. B., Banisadre, M., & Ash, R. C. (1981). Studies on the Liver to Kidney Switch of Erythropoietin Production. *Journal of Clinical Investigation*, 67(4), 1183–1188. <https://doi.org/10.1172/JCI110133>
- Zhang, C. C., Kaba, M., Ge, G., Xie, K., Tong, W., Hug, C., & Lodish, H. F. (2006). Angiopoietin-like proteins stimulate ex vivo expansion of hematopoietic stem cells. *Nature Medicine*, 12(2), 240–245. <https://doi.org/10.1038/nm1342>
- Zhang, C. C., & Lodish, H. F. (2004). Insulin-like growth factor 2 expressed in a novel fetal liver cell population is a growth factor for hematopoietic stem cells. *Growth (Lakeland)*, 103(7), 2513–2521. <https://doi.org/10.1182/blood-2003-08-2955>. Supported
- Zheng, J., Huynh, H., Umikawa, M., Silvany, R., & Zhang, C. C. (2011). Angiopoietin-like protein 3 supports the activity of hematopoietic stem cells in the bone marrow niche. *Blood*, 117(2), 470–479. <https://doi.org/10.1182/blood-2010-06-291716>
- Zheng, J., Umikawa, M., Cui, C., Li, J., Chen, X., Zhang, C. C., Huynh, H., Kang, X., Silvany, R., Wan, X., Ye, J., Cantó, A. P., Chen, S.-H., Wang, H.-Y., Ward, E. S., & Zhang, C. C. (2012). Inhibitory receptors bind ANGPTLs and support blood stem cells and leukaemia development. *Nature*, 485(7400), 656–660. <https://doi.org/10.1038/nature11095>
- Zhou, B. O., Yue, R., Murphy, M. M., Peyer, J. G., & Morrison, S. J. (2014). Leptin-receptor-expressing mesenchymal stromal cells represent the main source of bone formed by adult bone marrow. *Cell Stem Cell*, 15(2), 154–168. <https://doi.org/10.1016/j.stem.2014.06.008>
- Zon, L. I., Youssoufian, H., Mather, C., Lodish, H. F., & Orkin, S. H. (1991). Activation of the erythropoietin receptor promoter by transcription factor GATA-1. *Proceedings of the National Academy of Sciences*, 88(23), 10638–10641. <https://doi.org/10.1073/pnas.88.23.10638>
- Zovein, A. C., Hofmann, J. J., Lynch, M., French, W. J., Turlo, K. A., Yang, Y., Becker, M. S., Zanetta, L., Dejana, E., Gasson, J. C., Tallquist, M. D., & Iruela-Arispe, M. L. (2008). Fate Tracing Reveals the Endothelial Origin of Hematopoietic Stem Cells. *Cell Stem Cell*, 3(6), 625–636. <https://doi.org/10.1016/j.stem.2008.09.018>



Crosstalk Between the Hepatic and Hematopoietic Systems During Embryonic Development

Francisca Soares-da-Silva^{1,2,3,4,5,6†}, Márcia Peixoto^{1,2,3,4,5,6†}, Ana Cumano^{4,5,6} and Perpetua Pinto-do-Ó^{1,2,3*}

¹ Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal, ² Instituto Nacional de Engenharia Biomédica, Universidade do Porto, Porto, Portugal, ³ Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal, ⁴ Lymphocytes and Immunity Unit, Immunology Department, Pasteur Institute, Paris, France, ⁵ INSERM U1223, Paris, France, ⁶ Université Paris Diderot, Sorbonne Paris Cité, Paris, France

OPEN ACCESS

Edited by:

Joana Paiva Miranda,
University of Lisbon, Portugal

Reviewed by:

Kenichi Miharada,
Lund University, Sweden
Sandra Pinho,
University of Illinois at Chicago,
United States

*Correspondence:

Perpetua Pinto-do-Ó
perpetua@ineb.up.pt

†These authors have contributed
equally to this work

Specialty section:

This article was submitted to
Stem Cell Research,
a section of the journal
Frontiers in Cell and Developmental
Biology

Received: 21 April 2020

Accepted: 19 June 2020

Published: 22 July 2020

Citation:

Soares-da-Silva F, Peixoto M,
Cumano A and Pinto-do-Ó P (2020)
Crosstalk Between the Hepatic
and Hematopoietic Systems During
Embryonic Development.
Front. Cell Dev. Biol. 8:612.
doi: 10.3389/fcell.2020.00612

Hematopoietic stem cells (HSCs) generated during embryonic development are able to maintain hematopoiesis for the lifetime, producing all mature blood lineages. HSC transplantation is a widely used cell therapy intervention in the treatment of hematologic, autoimmune and genetic disorders. Its use, however, is hampered by the inability to expand HSCs *ex vivo*, urging for a better understanding of the mechanisms regulating their physiological expansion. In the adult, HSCs reside in the bone marrow, in specific microenvironments that support stem cell maintenance and differentiation. Conversely, while developing, HSCs are transiently present in the fetal liver, the major hematopoietic site in the embryo, where they expand. Deeper insights on the dynamics of fetal liver composition along development, and on how these different cell types impact hematopoiesis, are needed. Both, the hematopoietic and hepatic fetal systems have been extensively studied, albeit independently. This review aims to explore their concurrent establishment and evaluate to what degree they may cross modulate their respective development. As insights on the molecular networks that govern physiological HSC expansion accumulate, it is foreseeable that strategies to enhance HSC proliferation will be improved.

Keywords: hematopoietic stem cells, fetal liver, fetal liver microenvironment, fetal hematopoiesis, hematopoietic stem cell expansion, hematopoietic stem cell niche, self-renewal, cytokine signaling

INTRODUCTION

In the adult organism, hematopoietic stem cells (HSCs) constitute a rare and largely quiescent cell population residing in the bone marrow (BM) (Cheshier et al., 1999). The current dogma states that HSCs self-renew to maintain their pool throughout life and reenter cell cycle in response to stress (Wilson et al., 2008). The balance between self-renewal and differentiation in adult BM has been extensively studied, with the identification of different cellular niches and molecular cues as important elements in HSC maintenance and differentiation – reviewed in Crane et al. (2017) and Pinho and Frenette (2019).

During ontogeny, HSCs undergo a high proliferative stage, expanding in the fetal liver (FL), one of the anatomical locations of embryonic hematopoiesis (Ema and Nakauchi, 2000). Therefore, it has long been assumed that the hepatic microenvironment may drive the proliferation of HSCs while sustaining their primary “stemness” hallmark (functional capacity to reconstitute the hematopoietic compartment of irradiated recipients). So far, however, limited information is available on an HSC supportive environment in the FL and the mechanisms conveying these functional properties remain elusive, hindering effective translation into clinical applications.

A thorough dissection of the architecture and cellular organization of the liver is critical to elucidate the nature of the hematopoietic FL niche and disclose the elements (soluble and/or cell-bound signals, cell-cell contact, cell-matrix interactions, physical properties, etc.) contributing for the regulation of HSCs. This review aims to discuss the role of the FL stroma (encompassing all non-hematopoietic FL cells) and explore the interplay of the two fetal systems – hepatic and hematopoietic – in mouse (or otherwise stated) and how they mutually influence their development.

THE EMERGENCE OF THE HEMATOPOIETIC SYSTEM DURING EMBRYOGENESIS

The adult hematopoietic system relies on a robust process whereby HSCs divide and differentiate generating all mature blood lineages. In physiological conditions, this process takes place in the BM in both humans and mice. Even though adult hematopoiesis occurs in the BM, this is merely the end-site of an otherwise thrilling journey through different anatomic locations (Figure 1).

The first hematopoietic cells emerge in the yolk sac (YS) in extra-embryonic structures named blood islands at around embryonic day (E) 7.5 in mice and 2–3 weeks post-conception (wpc) in humans (Bloom and Bartelmez, 1940; Palis et al., 1999). Primitive erythroid progenitors (EryP) generate primitive erythrocytes, large nucleated cells that express embryonic globins (Kingsley et al., 2006), which are found in circulation after the onset of cardiac contractions at E8.25 (~3 wpc in humans) (Ji et al., 2003; Tavian et al., 1999) and oxygenate the developing embryo. Myeloid progenitors such as macrophage colony-forming cells or megakaryocyte colony-forming cells are also represented during early stages, concomitantly with EryP (Palis et al., 1999), suggesting that primitive hematopoiesis is limited to these three lineages.

Around 24 h later, at E8.5 (4–5 wpc in humans), a second wave of hematopoiesis initiates, with erythro-myeloid progenitors (EMPs) (Migliaccio et al., 1986; Bertrand et al., 2005b) emerging in the vascular plexus of the YS, in a process denominated endothelial to hematopoietic transition (EHT) (Frame et al., 2016; Kasaai et al., 2017). EMPs proliferate and differentiate in the YS into erythroid and myeloid cells but can also be identified in circulation and in the developing liver at later stages

(E10.5) via surface expression of c-Kit, CD16/32 and low levels of CD45 (McGrath et al., 2015). These progenitors generate the first definitive erythrocytes, megakaryocytes, macrophages and other myeloid lineages such as neutrophils, granulocytes and mast cells, but lack HSC activity (Palis et al., 1999; McGrath et al., 2015). Although EMPs are a transient population at early stages of embryonic development, they generate different tissue-resident macrophages that, depending on the organ, can persist throughout adulthood (Gomez-Perdiguero et al., 2015), mast cells that are maintained until birth (Gentek et al., 2018), and are the major producers of erythrocytes throughout embryonic life (Soares-da-Silva et al., 2020, Preprint).

A third wave of hematopoiesis occurs between E9.5–E11 in mice (~4 wpc in humans), with HSCs emergence in the aorta-gonads-mesonephros (AGM) region (Cumano et al., 1996; Medvinsky et al., 1996; Tavian et al., 1996) through EHT (Bertrand et al., 2010; Kissa and Herbomel, 2010). After generation, immature HSCs (imHSCs) undergo a maturation process as they migrate to the FL (Taoudi et al., 2008; Kieusseian et al., 2012) where they proliferate [expanding in numbers by >30-fold (Ema and Nakauchi, 2000)] and differentiate into all blood lineages: erythroid, myeloid and lymphoid. HSCs can be defined by their ability to provide long-term multilineage hematopoietic reconstitution (LTR) when transplanted to lethally irradiated mice (Morrison et al., 1995) and further repopulate secondary recipients. These cells can be found within the Lin[−]CD45⁺Sca1⁺c-Kit⁺ (LSK) compartment and be further divided according to their reconstitution ability in long-term (LSK CD150⁺CD48[−] LT-HSC) or short-term (LSK CD150[−]CD48[−] ST-HSC) reconstituting cells (Kim et al., 2006). Downstream progenitors of HSCs such as multipotent progenitors (MPPs), lympho-myeloid-primed progenitors (LMPPs), common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs) can also be found in FL and are responsible for the seeding of other hematopoietic organs such as the thymus (Ramond et al., 2014). Although adult and embryonic HSCs have similar lineage potentials, some lymphoid lineages are only produced during embryonic development, namely dendritic epidermal T cells (Ikuta et al., 1990), lymphoid tissue-inducer cells (Eberl et al., 2004), and a subset of IL-17-producer $\gamma\delta$ T cells (Haas et al., 2012). Embryonic hematopoiesis also takes place in the placenta, starting at E10.5–E11 (~6 wpc in humans) and declining at around E15.5 (Gekas et al., 2005; Ottersbach and Dzierzak, 2005; Robin et al., 2009). HSCs and other progenitors are also found in the fetal spleen after E15.5 (Christensen et al., 2004), even though without evidence for significant expansion and mostly differentiating into the macrophage lineage (Bertrand et al., 2006). At around E16.5 (~10 wpc in humans) HSCs migrate to the BM, where they are maintained through adulthood (Charbord et al., 1996; Christensen et al., 2004). In the adult, BM HSCs are largely quiescent (Cheshier et al., 1999) and only divide to maintain the stem cell pool, while the replenishment of blood lineages appears to be guaranteed by downstream MPPs (Sun et al., 2014; Busch et al., 2015; Pei et al., 2017; Rodriguez-Fraticelli et al., 2018).

Thus, embryonic hematopoiesis is characterized by an overlap in time and space of three waves with distinct anatomical origins

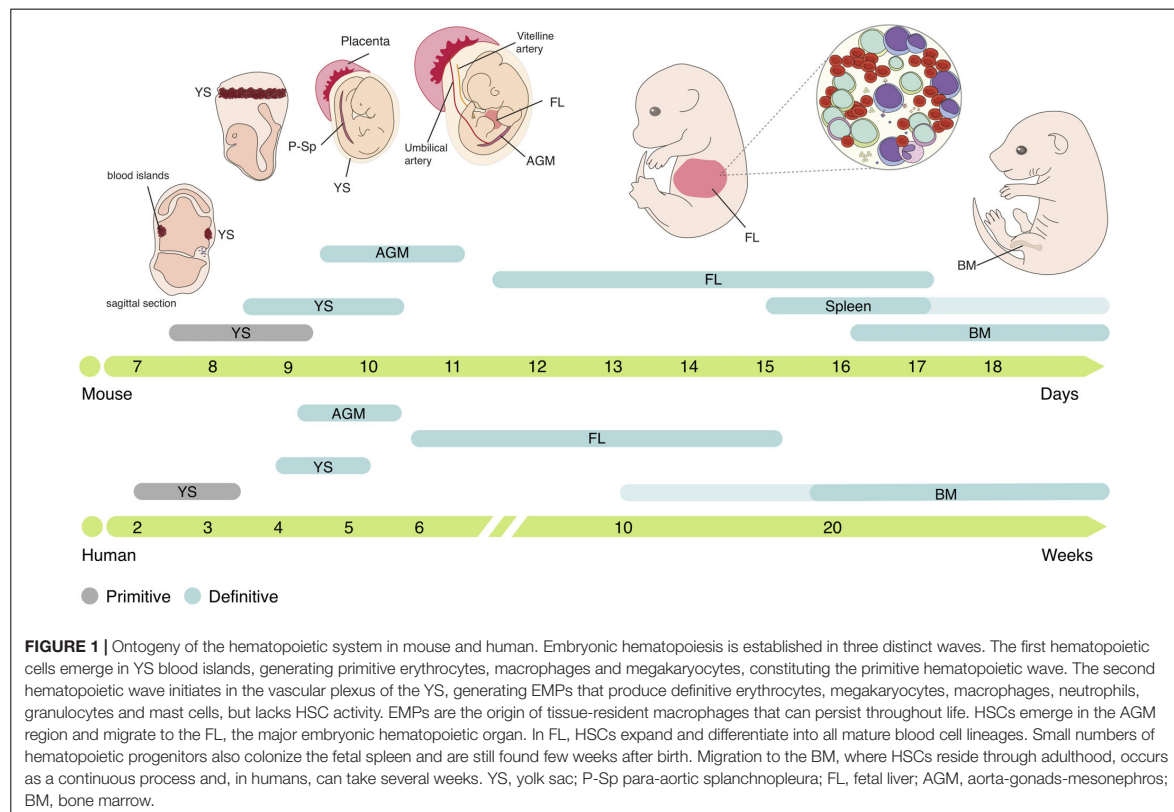


FIGURE 1 | Ontogeny of the hematopoietic system in mouse and human. Embryonic hematopoiesis is established in three distinct waves. The first hematopoietic cells emerge in YS blood islands, generating primitive erythrocytes, macrophages and megakaryocytes, constituting the primitive hematopoietic wave. The second hematopoietic wave initiates in the vascular plexus of the YS, generating EMPs that produce definitive erythrocytes, megakaryocytes, macrophages, neutrophils, granulocytes and mast cells, but lacks HSC activity. EMPs are the origin of tissue-resident macrophages that can persist throughout life. HSCs emerge in the AGM region and migrate to the FL, the major embryonic hematopoietic organ. In FL, HSCs expand and differentiate into all mature blood cell lineages. Small numbers of hematopoietic progenitors also colonize the fetal spleen and are still found few weeks after birth. Migration to the BM, where HSCs reside through adulthood, occurs as a continuous process and, in humans, can take several weeks. YS, yolk sac; P-Sp para-aortic splanchnopleura; FL, fetal liver; AGM, aorta-gonads-mesonephros; BM, bone marrow.

and lineage potential. All waves converge to the FL, the major hematopoietic organ during embryogenesis.

THE COLONIZATION OF THE FL BY THE HEMATOPOIETIC SYSTEM

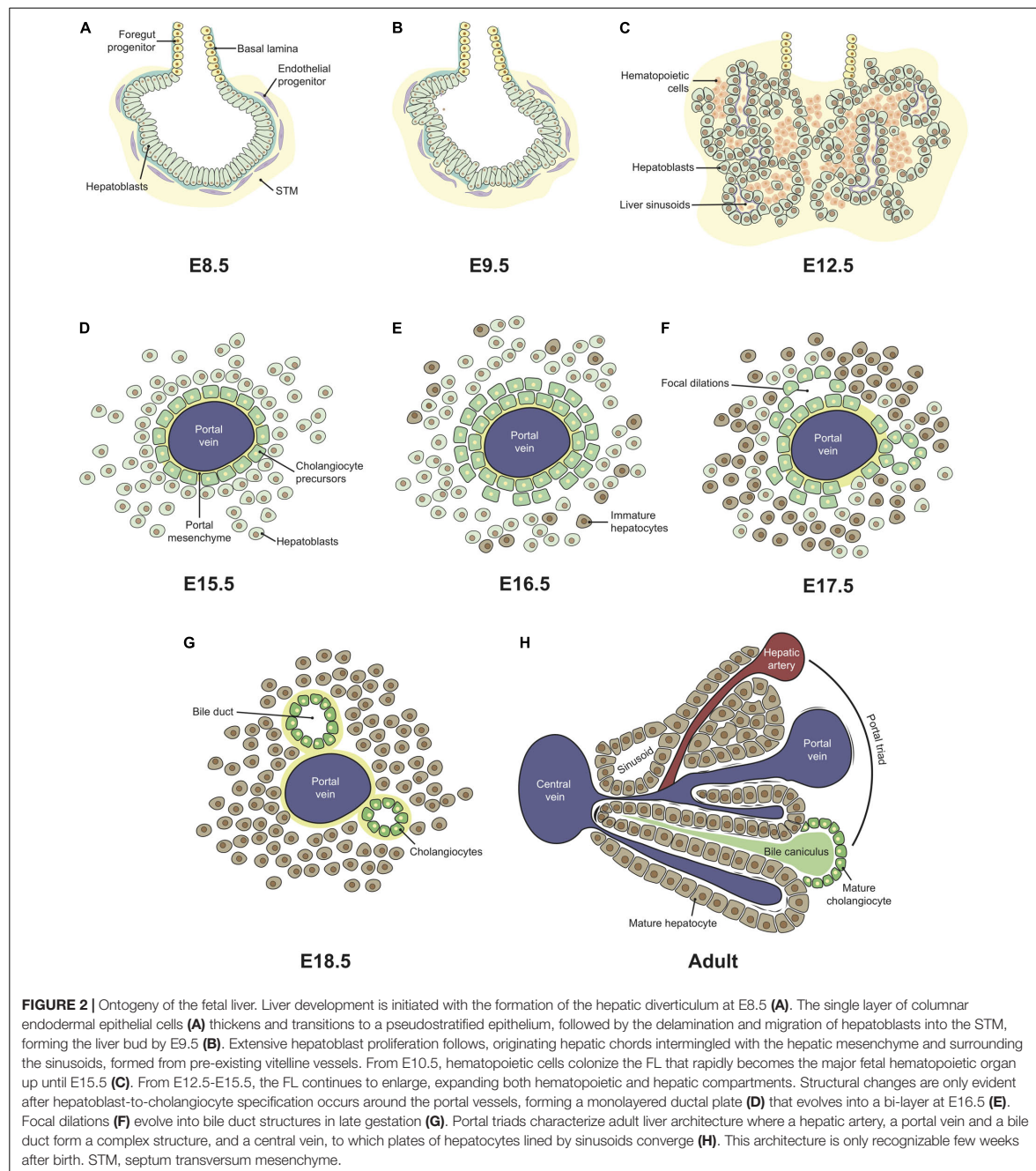
Which Cells Are Present When Hematopoietic Progenitors Arrive?

As the embryo gastrulates and folds, endoderm envelops the YS ultimately forming a hollow structure, the primitive gut tube, subsequently patterned into foregut, midgut and hindgut regions. The foregut, located in the anterior endoderm, adjacent to the developing heart, generates the liver, alongside with the lungs, thyroid, and pancreas (Tremblay and Zaret, 2005). Embryonic liver development starts at around E8.5-E9 (~4 wpc in humans) with the formation of the hepatic diverticulum, an extension of the ventral foregut epithelium that invades the septum transverse mesenchyme (STM) and forms a liver bud, by E9.5 (Severn, 1971; Wilson et al., 2006). The liver bud originates from a single-sheet of columnar endodermal epithelium with a gut morphology (Figure 2A), which then transitions to pseudostratified epithelial hepatoblasts (Figure 2B; Bort et al., 2006). At this stage, these cells are separated from the STM by a basement membrane

rich in laminin and composed of other extracellular matrix (ECM) molecules, such as nidogen, type IV collagen, fibronectin, and heparan sulfate proteoglycan (Shiojiri and Sugiyama, 2004). A process of extensive hepatoblast proliferation follows, during which the cells outgrow the liver bud, disrupting the basement membrane, into the STM (Figure 2C; Douarin, 1975). The other constituents of the organ, sinusoidal endothelial cells (SECs), mesothelial, sub-mesothelial and hepatic stellate cells have a mesoderm origin (reviewed by Yang et al., 2019), as described below. Angioblasts or endothelial progenitor cells are found delimiting the basement membrane (Figure 2A), resembling a loose “necklace” of cells, and were shown to promote liver organogenesis. In *Flk-1^{-/-}* mutant embryos, which lack endothelial cells, hepatic specification occurs, but proliferation and migration into the STM are impaired (Matsumoto et al., 2001). At E10.5–E11.0 (~5–6 wpc in humans), hematopoietic cells colonize the FL that rapidly becomes the major fetal hematopoietic organ (Johnson and Moore, 1975; Migliaccio et al., 1986; Palis et al., 2001).

Hepatoblasts

Hepatoblasts are bipotent immature FL cells that differentiate into hepatocytes – the hepatic parenchyma main constituent – or cholangiocytes – the biliary epithelial cells. At the onset



of liver development, bipotent hepatoblasts express the liver-specific transcription factors (TFs) hematopoietically-expressed homeobox protein (HHEX) (Bogue et al., 2000), prospero homeobox protein 1 (PROX1) (Dudas et al., 2004), and co-express the hepatocytes' markers alpha-fetoprotein (AFP),

albumin (ALB) (Cascio and Zaret, 1991), cytokeratin 18 (CK18) (Tanimizu et al., 2003), hepatocyte nuclear factor 4 α (HNF4 α) (Li et al., 2000) and cholangiocytes' markers such as cytokeratin 19 (CK19) (Tanimizu et al., 2004). Other typical hepatoblast markers are listed in Table 1.

TABLE 1 | Fetal liver non-hematopoietic compartment: cell types and associated markers.

Cell type	Gene expression	Markers used for isolation
Hepatoblasts	<i>Hhex, Prox1, Alb, Afp, Ck8, Ck18, Ck19, Met, Hnf6, Oc2, Hnf4a, Ttr, Foxm1, Foxa2, Tbx3, Dlk1, Lgr5, Epcam, Cdh1, Trgb1, Itga6, Liv2, Prom1, Anpep</i>	DLK1 ⁺ (Tanimizu et al., 2003; Tan et al., 2017) E-Cadherin ⁺ (Nitou et al., 2002; Nierhoff et al., 2005) EpCAM ⁺ DLK1 ⁺ (Tanaka et al., 2009) Liv2 ⁺ CD31 [−] CD45 [−] Lgr5-eGFP ⁺ (Prior et al., 2018) Ter119 [−] CD45 [−] c-Kit [−] CD49f ⁺ CD29 ⁺ (Suzuki et al., 2000) VCAM1 ⁺ ALCAM ⁺ DLK1 ⁺ (Tsuneto et al., 2013) Ter119 [−] CD45 [−] CD51 ⁺ VCAM1 ⁺ PDGFRα [−] (Brouard et al., 2017)
Hepatocytes	<i>Alb, Afp, Ttr, G6p, ApoA1, Apoh Por, Cps1</i>	–
Cholangiocytes	<i>Sox9, Hnf6, Oc2, Spp1, Ck19, Epcam, Krt7</i>	EpCAM ⁺ (Yang et al., 2017)
Endothelial cells	<i>Flk1, Flt1, Ve-cadherin, Pecam1, Mcam, Tek, Tie, Lyve1, Kdr</i>	CD45 [−] Ter119 [−] CD31 ⁺ (Khan et al., 2016) DLK1 [−] CD45 [−] Ter119 [−] CD31 ⁺ LYVE-1 ⁺ and DLK1 [−] CD45 [−] Ter119 [−] CD31 ⁺ LYVE-1 [−] (Tan et al., 2017)
Mesothelial cells	<i>Cytokeratin, Cd200, Gpm6a, Alcam, Gp38, Wt1, Podxl, Msln</i>	Flk1 [−] PODXL ^{high} (Onitsuka et al., 2010)
Sub-mesothelial cells	<i>Alcam, Desmin, Nestin, p75NTR, Pdgfra, Wt1</i>	–
Hepatic stellate cells	<i>Vimentin, Acta2, Desmin, p75NTR, Foxf1, Lhx2, Hlx</i>	DLK1 [−] Ter119 [−] CD45 [−] CD31 [−] LYVE-1 [−] p75NTR ⁺ (Tan et al., 2017)
Pericytes	<i>NG2, Nestin, Vimentin, Acta2, Pdgfra, Pdgfrb, Dlk1, Itgav, Endoglin, Vcam, Mcam, Nr2f2</i>	Ter119 [−] CD45 [−] CD31 [−] Nestin ⁺ NG2 ⁺ (Khan et al., 2016) CD45 [−] CD56 [−] CD34 [−] CD146 ⁺ (Gerlach et al., 2012)

¹ Study performed in humans.

Different markers have been used to isolate hepatoblasts, namely delta like non-canonical Notch ligand [DLK1 or preadipocyte factor 1 (Pref-1)] (Tanimizu et al., 2003), epithelial cadherin (E-cadherin) or CD324 (Nitou et al., 2002), epithelial cell adhesion molecule (EpCAM) or CD326 (Tanaka et al., 2009), and leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) (Prior et al., 2018). DLK1 is strongly expressed by hepatoblasts in the E10.5 liver bud and continues to be highly expressed until around E16.5, being significantly downregulated thereafter and absent in mature hepatocytes and cholangiocytes (Tanimizu et al., 2003; Tanaka et al., 2009). E-Cadherin, present at the onset of liver outgrowth, is downregulated by the time hepatoblasts migrate to the STM, disrupting the epithelial sheet, although it can be used as a hepatoblast-specific marker after E12.5 (Nitou et al., 2000; Margagliotti et al., 2007). Transient EpCAM expression labels newly formed hepatoblasts but is significantly reduced after E12, while expression after E16 specifically labels bile duct cells (Tanaka et al., 2009). Recently, combining multicolor clonal genetic lineage tracing, organoid cultures and analysis of single-cell RNA sequencing, LGR5 was shown to mark a subpopulation of *bonafide* bipotential hepatoblasts at E9.5–E10 as the origin of the hepatoblast pool (Prior et al., 2018).

Endothelial Cells

The main blood vessels in the adult liver are the portal and central veins and the hepatic artery. Up until birth, the hepatic artery is absent and embryonic circulation is sustained by a transient afferent vascular system, the extraembryonic umbilical and vitelline veins (Collardeau-Frachon and Scoazec, 2008). The portal vein arises early in the liver development, between

E10.5–E12.5 in mouse (4–6 wpc in human) (Collardeau-Frachon and Scoazec, 2008; Swartley et al., 2016). The hepatic sinusoids are the first vessels to appear, by E10–E10.5, originating from the pre-existing vitelline vessels. The latter sprouts throughout the STM, by angiogenesis, receiving signals from the surrounding mesenchyme (Figure 2C; Swartley et al., 2016). Hepatoblasts were also identified as a positive stimulator of sinusoid morphogenesis and maturation (Takabe et al., 2012). Stabilin 2 (STAB-2) and lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) (commonly used as a marker of lymphatics) – hyaluronan receptors – start to be expressed in SECs at E9.5 and E10.5, respectively, and continue to be expressed thereafter (Nonaka et al., 2007; Takabe et al., 2012). Of note, lymphatic vessels were only reported after birth (Swartley et al., 2016). At E9.5, endothelial cells located around the liver diverticulum (Figure 2A) express both CD31/PECAM-1 and Flk-1 (Sugiyama et al., 2010b). CD31 and Flk1 expression in SECs is strong in the early stages of liver development, but is downregulated with time. In adult livers, endothelial cells of portal and hepatic veins strongly express CD31, while it is absent or weakly detected in SECs (Sugiyama et al., 2010b; Takabe et al., 2012). Primitive SECs also strongly express Flk-1, contrarily to endothelial cells of portal and hepatic veins (Sugiyama et al., 2010b). During embryonic liver development, portal vessels express the arterial markers Ephrin-B2 and Neuropilin-1, but not the venous marker EphB4. This expression profile is inverted at the end of gestation, with the transition into a venular phenotype (Wang et al., 1998; Khan et al., 2016). Liver endothelial cells constitute a heterogeneous cellular compartment and different markers should be used for their identification according to vascular location and developmental stage.

Mesothelial and Sub-Mesothelial Cells

Mesothelial cells (MCs) compose a single epithelial layer (mesothelium) lining the liver parenchyma on the surface of lobes. From E12.5, MCs are characterized by the expression of cytokeratin, CD200, glycoprotein M6A (GPM6A), podoplanin (PDPN/Gp38), podocalyxin-like protein 1 (PODXL), and mesothelin (MSLN) (Lua and Asahina, 2016). PODXL is highly expressed in immature MCs, being downregulated during development, while MSLN is upregulated. MCs proliferate during liver development and remain quiescent after birth. Wilm's tumor-1 (WT1) is mainly expressed by MCs (Onitsuka et al., 2010). WT1^{-/-} embryos show incomplete lobulation compared to control littermates at E13.5, reduced numbers of Flk1⁻PODXL^{high} MCs, DLK1⁺ hepatoblasts, and total FL cells, suggesting that hepatic development was impaired due to defective MCs (Ijpenberg et al., 2007; Onitsuka et al., 2010). This is supported by the observation that fetal MCs express growth factors (PTN, MDK, and HGF) involved in hepatic development (Onitsuka et al., 2010).

Underneath the MC sheet lays a population of cells expressing Desmin, Nerve growth factor receptor (NGFR/p75NTR) and platelet-derived growth factor receptor α (PDGFR α /CD140a), associated with type IV collagen of the basal lamina, commonly referred as "sub-mesothelial cells" (sub-MC) or capsular fibroblasts. The expression of activated leukocyte cell adhesion molecule (ALCAM/CD166) and WT1 was also observed in MC and sub-MC around E11–E14 and, before that, in the STM by E9–E10 (Asahina et al., 2011; Lua and Asahina, 2016).

Hepatic Stellate Cells and/or Pericytes

Although the terms hepatic stellate cells and pericytes have been used by many authors as synonyms, it is not consensual they represent the same population. In adult liver, there is a population of perisinusoidal cells residing in the space of Disse between hepatocytes and SECs, that stores vitamin D lipids (Wake, 1971), and is a major player in liver fibrogenesis (Guyot et al., 2006). MesP1-expressing mesoderm has been considered its earliest ancestry, as it gives rise to the STM – the origin of the liver mesothelium and mesenchymal cells. Migration inward of MC and sub-MC from the liver surface is assumed to give rise to hepatic stellate cells and perivascular mesenchymal cells (Asahina, 2012). Hepatic stellate cells express Desmin, p75NTR, but not the MC markers ALCAM, WT1, and Gp38 (Asahina et al., 2010).

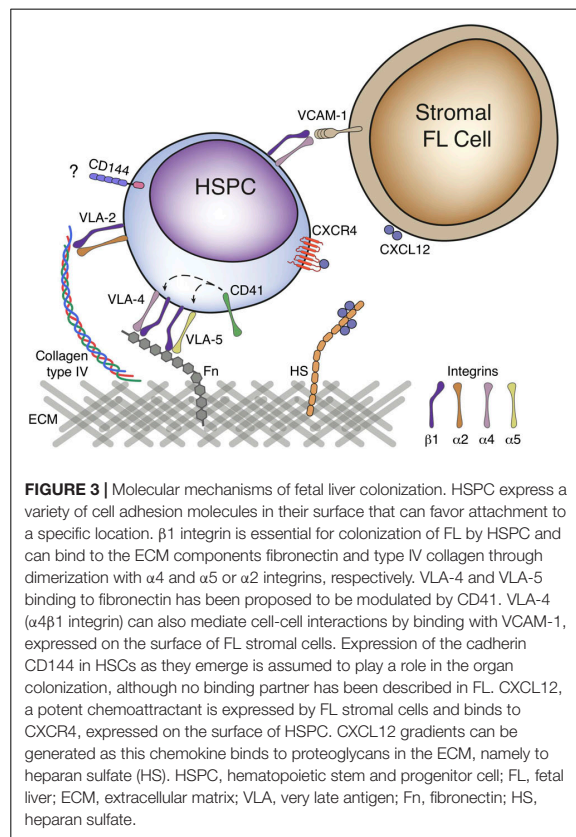
Gerlach et al. (2012) isolated CD146⁺CD45⁻CD56⁻CD34⁻ cells from fetal and adult human livers and identified them as pericytes, a distinct population from hepatic stellate cells. They showed that these cells express NG2 and vimentin, but not GFAP *in situ*, and are found around periportal but not pericentral blood vessels neither within the space of Disse. These cells exhibit high osteogenic and myogenic, but low adipogenic or chondrogenic differentiation potential, in *in vitro* differentiation assays. In mice, a population characterized by the expression of Nestin and NG2 was identified as periportal pericytes, which expresses mesenchymal markers and shows trilineage mesenchymal capacity *in vitro* (Khan et al., 2016).

Law of Attraction: What Brings Hematopoietic Progenitors to the Developing Liver?

Hematopoietic stem cells emerge from the dorsal aorta directly into circulation and can, therefore, be found in different locations (Cumano et al., 1996; Medvinsky et al., 1996). These cells can travel through the umbilical arteries to the placenta and return to the embryo through the umbilical veins. The umbilical veins drain directly into the liver by fusing with the intrahepatic vascular plexus of the vitelline veins, forming the hepatic sinus. Cells traveling in the right umbilical vein can bypass the liver directly to the vena cava through the *ductus venosus*, a structure only present during fetal development, and can be directed into other embryonic regions through the heart. The liver is also irrigated by the vitelline veins, which transport blood from the YS, and eventually mature to become the portal vein. Another route for newly formed HSCs is to travel from the dorsal aorta through the subcardinal vein to the liver or inferior cardinal vein to the heart. From the heart, circulating cells can reach the developing lungs or upper half of the body (Kiserud, 2005). However, it is in the developing FL that HSCs establish and remain until they migrate to the BM. Transplantation studies show that HSCs can also be found in the placenta (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). The placenta of a mouse model lacking a functional circulatory system was shown to still harbor hematopoietic activity, suggesting that the placenta could generate *de novo* hematopoietic cells with multilineage potential (Rhodes et al., 2008), however, direct evidence for HSC emergence from the placenta is yet to attain.

Hematopoietic stem or progenitor cells (HSPCs) but also EMPs, colonize the FL after liver bud formation at E10.5 (Johnson and Moore, 1975; Palis et al., 2001). Distinct mechanisms of FL colonization have been proposed, mostly relying on cell adhesion-mediated processes and/or chemoattraction (cytokines, chemokine signaling, and growth factors) (Figure 3; Hayashi et al., 2019).

Several cell adhesion molecules have been identified in embryonic HSPCs, including integrins, selectins, cadherins, and others. Hematopoietic progenitors express vascular-endothelial cadherin (VE-Cadherin/CD144) as they emerge from the YS (in the case of EMPs) or AGM (in the case of HSCs) that is downregulated thereafter and undetectable in BM HSCs (Fraser et al., 2002; Taoudi et al., 2005). FL CD34⁺ progenitors express higher levels of the integrins β 1 (CD29), α 2 (CD49b), and α 5 (CD49e), similar levels of integrins α 4 (CD49d) and α 6 (CD49f), E- and P-selectins (CD62E and CD62P, respectively) and CD11b and CD11c molecules, but lower levels of integrin β 2 (CD18), CD11a and CD44 than their adult BM equivalent (Roy and Verfaillie, 1999). Seminal studies showed that HSPCs lacking β 1 integrin were unable to colonize the FL but were still present in the circulation and capable of generating all blood lineages, suggesting a role for integrin-mediated cell adhesion in FL colonization (Hirsch et al., 1996). Integrin receptors result from the dimerization of α and β subunits and analysis of the expression of the α chain partner of β 1 integrin in LSK progenitors revealed integrins α 4 and α 6 as the most



predominant (Sugiyama et al., 2013). FL CD34⁺ progenitors bind the ECM component fibronectin through the integrin receptors $\alpha 4\beta 1$ and $\alpha 5\beta 1$ (also known as VLA-4 and VLA-5) and this binding has been proposed to be modulated by the integrin $\alpha 2\beta 1$ (GPIIb or CD41), also expressed in these cells (Figure 3; Roy and Verfaillie, 1999; Emambokus and Frampton, 2003). Other ECM components have been tested for adhesion of FL CD34⁺ progenitors such as type I and type IV collagen and laminin, however, only type IV collagen promotes adherence at levels equivalent to that of fibronectin, through $\alpha 2\beta 1$ integrin (VLA-2) (Roy and Verfaillie, 1999). In the FL, hepatoblasts (defined as DLK1⁺ cells) are the major producers of ECM components, namely vitronectin and fibronectin (Sugiyama et al., 2013). Embryos lacking hepatoblasts can still form the liver bud but die between E10.5–E12.5 (Nishina et al., 1999). These FL show decreased expression of vitronectin and fibronectin that may play an important role in FL colonization by HSCs and YS EMPs, although this role has not been specifically assessed (Sugiyama et al., 2013). Integrins can also mediate cell-cell interactions. Cellular bound counterparts of VLA-4 include the vascular cell adhesion molecule-1 (VCAM-1/CD106), expressed by FL hepatoblasts (Sugiyama et al., 2010a).

Cytokine and chemokine signaling can also stand at the basis of FL colonization. The stromal cell-derived factor-1 (SDF-1), commonly known as CXCL12, acts through binding to its receptor CXCR4 present in HSPCs and has been extensively studied in the adult BM – reviewed in Yu and Scadden (2016); and Wei and Frenette (2018). CXCL12 expression is stabilized at the cell surface or in the surrounding ECM through proteoglycans binding, allowing the creation of chemokine gradients essential for cell migration (Schumann et al., 2010). In the FL, CXCL12 is expressed by DLK1⁺ hepatoblasts (Chou and Lodish, 2010) and Nestin⁺NG2⁺ pericytes (Khan et al., 2016). The role of CXCL12 in FL colonization was analyzed using CXCL12^{-/-} embryos. At early stages (E12.5–E14.5), the number of HSCs was similar in the FL of mutant animals and controls. By E16.5 FL HSCs were reduced by more than twofold and an abnormally high number was found in circulation. These observations indicate that CXCL12 is an important factor for retaining HSCs in FL, but not for its initial colonization (Ara et al., 2003).

Owing to the particular architecture of fetal circulation, FL is in an anatomically privileged location. Even if HSCs are traveling directly within the embryo through the subcardinal vein, or the umbilical veins after passing in the placenta, the FL is the first intraembryonic organ they encounter. Whether a passive retainment of circulating cells (e.g., through $\beta 1$ -integrin) occurs, or specific signals directly promote chemoattraction of HSPCs to FL is still unclear.

How Do the Hepatic and Hematopoietic Cell Types Organize During Development?

The structure of the FL changes dramatically during embryonic development. Crawford et al. (2010) extensively characterized the mouse developing hepatobiliary system from E9.5 to E18.5 creating a histology atlas. At initial stages (E10.5–E12.5), the liver is mainly constituted by a vascular plexus and migrating hepatoblasts that later form hepatic chords. At E11.5, the hepatic sinusoids are wide, which may favor the access and establishment of the newly generated hematopoietic progenitors. From E12.5 onward, the organization of the cells in the FL changes as the frequency of hematopoietic cells increases. At E13.5, the most frequent FL population are nucleated erythrocytes that, at early stages, are located throughout the liver parenchyma, in between the hepatic chords, but after E14.5 more mature enucleated erythroid cells are found within the vessels (Ayres-Silva et al., 2011). At this stage, megakaryocytes and erythroblastic islands, which consist of a central macrophage surrounded by erythroid cells, are also distinguishable in the liver parenchyma. These macrophages are responsible for the phagocytosis of the expelled nuclei during erythroid maturation (Bessis et al., 1978). Megakaryocytes are essential to thrombosis and hemostasis and may be determinant in an organ that is mostly constituted by erythroid cells. Moreover, the developing liver seems to provide a unique microenvironment for the expansion of megakaryocyte progenitors (Brouard et al., 2017).

From E13.5 to late gestation, no dramatic changes occur in the histology of the FL. Other hematopoietic cells such as B cell progenitors identified by Pax5 expression, can be found interspersed in the tissue by E12.5 and also forming perivascular aggregates by E18.5. Granulocytes are scattered throughout the tissue from E16.5 onward, concentrating/converging around central veins and in the periphery by E17.5, correlating with the presence of mesenchymal cells and suggesting a crosstalk between these distinct cell types (Ayres-Silva et al., 2011). By this time-point, as hematopoietic cells exit the organ and migrate to the BM, hepatoblasts and hepatocytes regain contact (Crawford et al., 2010).

Disclosure of HSCs distribution within the FL has been hindered by the multi-marker assessment required, i.e., Lin⁻c-Kit⁺Sca1⁺CD150⁺CD48⁻, to phenotypically identify these cells. Hematopoietic progenitors (defined by c-Kit expression) are found in close association with DLK1⁺ hepatoblasts at E14.5 (Sugiyama et al., 2013). Nevertheless, c-Kit⁺ cells could mostly represent erythroid progenitors as they are the most frequent population at this stage (Soares-da-Silva et al., 2020, Preprint). Other approaches include the use of transgenic Ly6a-GFP (labeling Sca1⁺ cells) mice, that together with Runx1 localized HSPCs at E11.5 in close contact with endothelial cells (Tamplin et al., 2015). HSCs, profiled as CD150⁺CD48⁻Lin⁻, have been found in close association with Nestin⁺NG2⁺ pericytes surrounding the portal vessels (Khan et al., 2016). Although this characterization more closely identifies a potential HSC, FL studies using mouse models that directly label HSCs are still missing. Generation of a mouse with a single-color reporter driven by endogenous *Hoxb5* (*Hoxb5*-tri-mCherry), which expression in the BM is limited to LT-HSCs and *in situ* imaging evidenced the close proximity of LT-HSCs with VE-Cadherin⁺ cells (Chen et al., 2016). Recently, another HSC-specific reporter line has been described, yet also only analyzed in the adult bone (Christodoulou et al., 2020).

THE INTERPLAY BETWEEN THE DEVELOPING HEPATIC-HEMATOPOIETIC TISSUES

How Does the FL Environment Modulate Hematopoiesis? A Role in Maturation, Expansion and Differentiation of HSCs?

Emerging imHSCs lack long-term reconstitution activity in conventional or Rag2^{-/-} immunocompromised mice but can reconstitute NK-deficient Rag2^{γc}^{-/-} animals (Cumano et al., 2001; Bertrand et al., 2005a). After co-culture with the OP9 BM stromal cell line in the presence of thrombopoietin (TPO) or with E10.5 FL rudiments, CD31⁺c-Kit⁺CD45⁻ imHSCs acquire an adult HSC phenotype (LSK CD150⁺CD48⁻) and develop LTR activity in Rag2^{-/-} or conventional mice as they upregulate MHC class I molecules (Kieusseian et al., 2012). These experiments suggest that the FL provides the signals necessary for the maturation of newly formed HSCs. FL stroma also supports the differentiation of committed hematopoietic

progenitors towards distinct lineages. Interleukine 7 (IL-7) promotes the survival and proliferation of lymphoid progenitors and controls the determination of the B cell lineage (Sudo et al., 1989; Peschon et al., 1994). In FL, IL-7 is produced by VCAM1⁺ALCAM⁺DLK1⁺ hepatoblasts (Tsuneto et al., 2013) and controls the number of lymphoid progenitors that develop into the B-cell lineage by stabilizing the B-cell transcriptional signature (Berthault et al., 2017). For instance, erythropoietin (EPO) is produced by DLK1⁺ hepatoblasts and is required for proliferation and terminal differentiation of erythroid progenitors (Sugiyama et al., 2011). Also, TPO expressing Ter119⁻CD45⁻CD51⁺VCAM1⁺PDGFRα⁻ FL hepatoblasts support the production of megakaryocytes from adult BM HSCs in a contact-dependent manner (Brouard et al., 2017). TPO is the main regulator of megakaryocyte differentiation and platelet production (Kaushansky, 1995; Eaton and de Sauvage, 1997) but has also been shown to promote survival and proliferation of BM HSPCs *in vitro* (Borge et al., 1996; Ku et al., 1996), the proliferation of fetal hematopoietic progenitors *in vivo* (Alexander et al., 1996) or expansion of BM HSCs following transplantation (Fox et al., 2002). Lack of TPO signaling causes decreased HSC function and numbers (Kimura et al., 1998; Solar et al., 1998), a consequence from the exit of a quiescent state (Nakamura et al., 2007; Qian et al., 2007), possibly leading to a premature exhaustion of the stem cell pool. The survival and proliferation effects of TPO are enhanced when used in combination with other early cytokines, namely FMS-like tyrosine kinase 3 ligand (FLT3L) and c-Kit ligand [KITL, also known as stem cell factor (SCF) or steel factor (SF)] both in murine and human adult BM cells (Ramsfjell et al., 1996; Borge et al., 1997). The highest levels of TPO in the adult are found in the liver (Lok et al., 1994). Systemic TPO produced by hepatocytes, but not by hematopoietic, osteoblast or BM mesenchymal stromal cells is required for BM HSC maintenance (Decker et al., 2018). It can be detected in FL as early as E10.5, having a strong impact on HSC expansion and survival in this organ (Petit-Cocault et al., 2007). Indeed, several cytokines/chemokines/growth factors are important for HSC proliferation, maintenance and survival, namely KITL, FLT3L, insulin growth factor (IGF), angiopoietin-3, angiopoietin-like 2, Wnt family growth factors, Ephrin2a, CSF1, EPO, CXCL12, and IL-6 – reviewed in Sauvageau et al. (2004). In FL, some of these cytokines are expressed by hepatoblasts or other stromal cells, such as stellate cells or pericytes (see Table 2 and Figure 4; Charbord and Moore, 2005; Chou and Lodish, 2010; Khan et al., 2016; Tan et al., 2017).

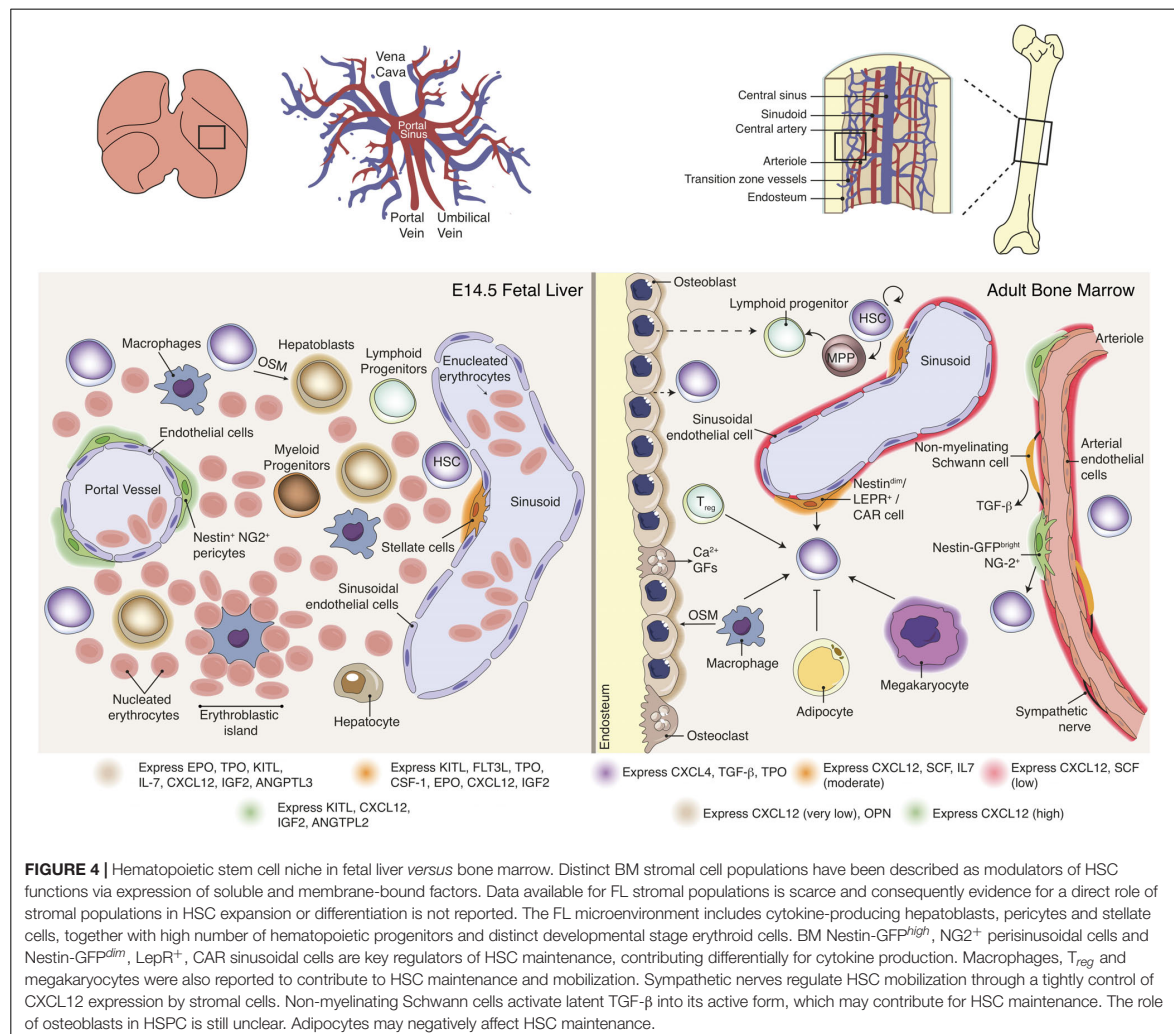
The hypothesis that stem cells are regulated by their environment has been proposed by Schofield (1978) and postulates that stem cell properties are maintained by the surrounding cells designated “niche.” Stromal regulation of hematopoiesis has been proposed by many and early studies of co-culture of hematopoietic progenitors with either FL fibroblast or epithelial-stromal cell lines showed erythroid and myeloid support (Tsai et al., 1986; Hata et al., 1993). More than 200 FL stromal cell lines have been developed and tested for maintenance or expansion of HSCs, however, only a few were able to maintain their repopulating activity

TABLE 2 | Cytokine signaling in fetal liver.

FL Supportive Stroma		Hematopoietic progenitors	
Pathway involved	Expressing cell	Receptor	Effect
KITL	DLK1 ⁺ hepatoblasts (Chou and Lodish, 2010; Sugiyama et al., 2011) Nestin ⁺ cells (Khan et al., 2016) Stellate cells (Tan et al., 2017)	c-Kit (CD117) (Yarden et al., 1987)	BM HSC survival and self-renewal (Barker, 1994; Miller et al., 1997)
ANGPTL2	Nestin ⁺ cells (Khan et al., 2016)	PirB (Zheng et al., 2012)	BM HSC proliferation (Zhang et al., 2006)
ANGPTL3	DLK1 ⁺ hepatoblasts (Chou and Lodish, 2010)	PirB (Zheng et al., 2012)	BM HSC maintenance (Zheng et al., 2011) BM HSC proliferation (Zhang et al., 2006)
FLT3L	Stellate cells (Tan et al., 2017)	Flt3 (CD135) (Matthews et al., 1991)	FL HSPC proliferation (Lyman et al., 1993)
TPO	DLK1 ⁺ hepatoblasts (Chou and Lodish, 2010; Sugiyama et al., 2011; Brouard et al., 2017) Stellate cells (Tan et al., 2017)	MPL (TPO-R, CD110) (Vigon et al., 1992)	FL HSC survival and proliferation (Petit-Cocault et al., 2007) BM HSC quiescence (Nakamura et al., 2007; Qian et al., 2007)
CSF1	Stellate cells (Tan et al., 2017)	Csf-1 Receptor (Guilbert and Stanley, 1980)	Commitment to macrophage lineage (Rieger et al., 2009)
EPO	DLK1 ⁺ hepatoblasts (Sugiyama et al., 2011) Stellate cells (Tan et al., 2017)	EPO receptor (Sawyer et al., 1987)	Proliferation and differentiation of FL erythroid progenitors (Lin et al., 1996)
CXCL12	DLK1 ⁺ hepatoblasts (Chou and Lodish, 2010) Nestin ⁺ cells (Khan et al., 2016) Stellate cells (Kubota et al., 2007)	CXCR4 (Bleul et al., 1996)	FL and FBM B-cell lymphopoiesis and FBM myelopoiesis (Nagasawa et al., 1996) BM HSC engraftment post-transplantation (Lai et al., 2014; McDermott et al., 2015) FL HSC retention (Ara et al., 2003) BM HSC retention (Sugiyama et al., 2006)
IL-7	VCAM1 ⁺ ALCAM ⁺ DLK1 ⁺ hepatoblasts (Tsuneto et al., 2013)	IL-7R α (Park et al., 1990)	Lymphocyte expansion (Peschon et al., 1994)
IGF2	DLK1 ⁺ hepatoblasts (Chou and Lodish, 2010) Nestin ⁺ cells (Khan et al., 2016) Stellate cells (Tan et al., 2017)	IGF1-R (Rubin et al., 1983) IGF2-R (Morgan et al., 1987) Insulin receptor (House and Weidemann, 1970)	FL and BM HSC proliferation (Zhang and Lodish, 2004)

over a 3-week co-culture period (Wineman et al., 1996). The functional heterogeneity observed was not due to distinct cytokine production as all cell lines expressed similar cytokine profiles, including *Flt3l*, *Kitl*, *Tpo*, *Igf1*, *Il6*, *Il11*, leukemia inhibitory factor (*Lif*), granulocyte-colony stimulating factor (G-CSF/*Csf3*), granulocyte/macrophage-colony stimulating factor (GM-CSF/*Csf2*), and transforming growth factor, beta 1 (*Tgfb1*). Importantly, the successful stromal cell lines have in common the expression of *Dlk1*, and overexpression of this factor is sufficient to enable hematopoietic support (Moore et al., 1997). Although these cell lines only maintain HSC potential and do not promote HSC expansion, some FL cells have been described to do so. Recent studies have addressed the role of specific FL populations *in vivo* or *in vitro*. It is the case of DLK1⁺ hepatoblasts that are able to expand LT-HSC around 20-fold after a 3-week co-culture period (Chou et al., 2013). This expansion seems to be contact-dependent as DLK1⁺ conditioned medium only allows expansion of ST-HSC and cells cultured in transwell inserts did not show the same expansion levels. Although promising, it is worth mentioning that these cultures were supplemented

with KITL, TPO, and FLT3L and, therefore, it remains unclear which mechanisms underlie HSCs expansion in DLK1⁺ cell co-cultures. Moreover, DLK1 knockdown in human hepatoblasts results in decreased hematopoietic support *in vitro* (Gerlach et al., 2019). The role of hepatoblasts *in vivo* has been difficult to assess as transgenic mice with hepatoblast deficiencies die between E10.5 and E12.5 and studies of liver development do not usually investigate the hematopoietic compartment (Nishina et al., 1999). DLK1 has been used to identify hepatoblasts but it is also expressed by the majority of Nestin⁺ cells surrounding the portal vessels, a cell type that has been implicated as part of the FL niche (Tanimizu et al., 2003; Chou and Lodish, 2010; Chou et al., 2013; Khan et al., 2016). HSCs numbers are modestly reduced when Nestin⁺NG2⁺ pericytes are selectively eliminated and the remaining HSCs are less proliferative (Khan et al., 2016). So far, this is the only study that addresses the role of a specific cell type in HSC expansion and maintenance *in vivo*. Nestin⁺ cells are the major producers of *Cxcl12* at E14.5 when compared to Nestin⁻ cells, therefore, the reduction in FL HSCs could result from a defect in HSC expansion together with a displacement



of cells into circulation. Moreover, Nestin⁺ cells seem to be a transient population, important for HSC localization around the portal vessels during FL hematopoiesis but are no longer present postnatally, a stage at which HSCs migrate and reside in the BM. These cells have also been described in adult BM and ablation of Nestin⁺NG2⁺ pericytes alters HSCs localization away from arterioles (Kunisaki et al., 2013). In the rat, fetal hepatic stellate cells were shown to express VCAM-1 and to secrete *Cxcl12* and hepatocyte growth factor (*Hgf*), revealing a potential role for the hematopoietic and hepatic development (Kubota et al., 2007). Accordingly, mouse FL hepatic stellate cells (defined as p75NTR⁺) express a range of hematopoietic cytokines, *Csf1*, *Igf2*, *Tpo*, *Kitl*, *Epo*, *Igf1*, *Il11*, *Flt3l* and Oncostatin M (*Osm*, involved in hepatic maturation) and were therefore proposed as potential niche components (Tan et al., 2017).

To date, researchers have undertaken a cell type-directed approach, however, it is conceivable that different FL populations play distinct roles in the maintenance and expansion of HSCs and act through cellular networks. Only an unsupervised analysis of the FL constituents as a whole will shed light on the part each cell type takes in the support of hematopoiesis.

The vascular labyrinthine of the placenta (where embryonic circulation meets maternal circulation) has also been proposed as a niche for HSC expansion (Gekas et al., 2005; Ottersbach and Dzierzak, 2005; Robin et al., 2009). Human placenta-derived stromal cell lines with pericyte characteristics were shown to support *in vitro* maintenance of cord blood (CB) hematopoietic progenitors and hematopoietic cells were found in close contact with pericytes/perivascular cells in the placenta by immunostaining (Robin et al., 2009). Taken together with what

has already been described for the FL (Khan et al., 2016), pericyte-like cells are likely to play a role on supporting hematopoiesis.

Can the Adult Liver Be a Hematopoietic Site?

Extramedullary hematopoiesis (EMH) is a process in which HSPCs leave their microenvironment in the BM and establish in distinct anatomical locations wherein they continue to produce mature blood cells. Although it is a physiologic process during embryonic development (YS, AGM, FL, and fetal spleen), in the adult it only occurs in pathological settings of BM failure, myelostimulation, tissue inflammation, or abnormal cytokine production (Johns and Christopher, 2012). EMH can occur sporadically in lymph nodes, spinal cord, kidneys, gastrointestinal tract, and lung (Chiu et al., 2015). It is interesting, however, that the predominant sites of EMH are shared between the embryo and the adult: the spleen and the liver. In fact, splenic or liver hematopoiesis can still be observed postnatally in many mammals but disappears before adulthood. Hematopoietic foci in the adult liver can be found within sinusoids and in close association with macrophages (Barberá-Guillem et al., 1989). BM HSPCs co-cultured with liver sinusoid endothelial cells (LSECs) were maintained for more than 6 weeks in cytokine supplemented media, demonstrating a putative niche role of endothelial cells in the adult liver (Cardier and Barberá-Guillem, 1997). An adherent layer of liver cells has been suggested to support megakaryopoiesis by the production of TPO and B lymphopoiesis by the production of *Il7* and *Flt3l* (Cardier and Dempsey, 1998; Wittig et al., 2010). Although endothelial cells have been reported to express TPO, this cytokine is mostly produced by the liver parenchyma (hepatocytes) (Nomura et al., 1997). In the adult, EPO is produced by the kidney, although hepatocytes can also support hepatic erythropoiesis in physiological or pathological conditions (Ploemacher and van Soest, 1977; Semenza et al., 1991; Eckardt et al., 1994; Weidemann and Johnson, 2009). In conclusion, under physiologic conditions, the liver harbors low numbers of HSPCs and supports extramedullary hematopoiesis (Taniguchi et al., 1996; Watanabe et al., 1996). Thus, it is conceivable that adult liver may keep some of its embryonic niche properties.

Is the Development of the FL and Hematopoietic Cells Symbiotic?

In mid-gestation, embryonic liver functions as a “bag” accommodating the expanding hematopoietic system before BM development. During the temporal window in which the organ is essentially a hematopoietic tissue – from E12.5 to E16.5 (~6–18 wpc in humans) – and the ratio of non-hematopoietic cells/total liver cells is very low, the organ’s architecture is far less complex than that of its adult counterpart. In the adult, hepatocytes are the main parenchymal cell type, organized in cords interspaced by an intricate vascular and biliary system (Figure 2H). Alongside with the massive migration of hematopoietic cells to the BM at E16.5 (Christensen et al., 2004), the liver tissue starts to mature – hepatocytes and cholangiocytes differentiate from hepatoblasts and cellular adhesion increases,

creating tight hepatic parenchyma with dense hepatocyte cords (Crawford et al., 2010). Transcriptomic and proteomic analysis throughout FL development identified E15.5 as the time of onset of metabolic, detoxification and immune programs (Guo et al., 2009).

Hepatoblasts differentiate into hepatocytes starting at ~E13.5 in mice and around 14 wpc in humans (Haruna et al., 1996; Yang et al., 2017). Single-cell transcriptomic studies along development (E10.5–E17.5) of hepatoblasts/hepatocytes/cholangiocytes (sorted based on the expression of DLK-1 and EpCAM) suggest that hepatoblast-to-hepatocyte lineage specification is the default process. Cholangiocyte specification occurs as early as E11.5 and is completed by E14.5 (Yang et al., 2017). Hepatoblasts fate decision is modulated by a gradient of Activin/TGF- β signaling, controlled by Onecut (OC) TFs (OC-1/HNF-6 and OC-2) (Clotman, 2005). Cholangiocyte-primed hepatoblasts appear in low numbers at E13.5 around the portal vein and portal sinus, forming a single-layered ductal plate at E15.5, that evolves to a double-layer by E16.5 (Figures 2D,E), characterized by CK19 and CK8 cytokeratins and β 2 integrin (CD18) (Van Eyken et al., 1988; Tanimizu et al., 2009). At this stage, CK19 expression becomes specific to ductal plate cells (Van Eyken et al., 1988). Focal dilations between the two ductal plate cell layers give rise to the bile ducts (Figures 2F,G), whereas the remaining tissue progressively regresses (Clotman et al., 2002). This ductal plate remodeling involves tubulogenesis (Antoniou et al., 2009) and apoptosis (Terada and Nakanuma, 1995). Around birth, the portal mesenchyme encircles the cells of the ductal plate (Swartley et al., 2016). Hepatoblasts located away from the portal vein will gradually differentiate into hepatocytes and by E17 start to exhibit a characteristic polarized epithelial morphology disposed in hepatic cords alongside the bile canaliculi (Zorn, 2008). Because cholangiocyte differentiation occurs along the hilum-to-periphery axis, different maturation states can be observed at a given developmental time (Yang et al., 2017). Three-dimensional reconstructions of serial cross-sections/whole-mount immunostained FL and carbon ink injection have been used to disclose the morphogenesis of intrahepatic bile ducts (Vestentoft et al., 2011; Takashima et al., 2015; Tanimizu et al., 2016).

Kinoshita et al. (1999) showed that hematopoietic cells expand when cultured in a monolayer of primary fetal hepatic cells (in presence of hematopoietic cytokines) and that the addition of OSM suppresses *in vitro* hematopoiesis, by inducing the maturation of the hepatic cells. Since hematopoietic cells produce OSM, its expansion increases the local OSM concentration, consequently promoting hepatic development (Kamiya et al., 1999). It was hypothesized that a metabolically active liver no longer supports hematopoiesis (Miyajima et al., 2000). However, it is still not clear whether the displacement of the hematopoietic cells out of the FL facilitates liver maturation or if the changes in the microenvironment no longer support hematopoiesis.

Impaired hematopoiesis in *c-Myb* mutant (Mucenski et al., 1991) or *Ubc*^{-/-} mice (Ryu et al., 2012) and abnormal erythropoiesis in *Rb*-deficient mice (Lee et al., 1992) also results in impaired liver growth. However, the

early embryonic lethality (at around E15) has hindered the analysis of the impact of the hematopoietic compartment in liver development.

The vasculature remodeling at the end of gestation was also correlated with the rapid loss of HSCs in the postnatal liver (Khan et al., 2016). After birth, the portal vein no longer receives blood from the vitelline vein, collecting the blood from the gut, draining it to the central vein and the hepatic artery arises. With the ligation of the umbilical inlet, the portal vessels acquire a vein phenotype and lose the periportal pericytes (Khan et al., 2016).

HEMATOPOIETIC EXIT FROM FL AND ESTABLISHMENT IN THE BM

What Makes the Hematopoietic System Move? How Is the BM Niche Established?

The BM is the ultimate destination of the hematopoietic system journey in the embryo, being the production hub for blood cells throughout life. LSK cell homing and colonization of mouse BM was reported at E15.5–E16.5, coinciding with the fetal bone marrow (FBM) vascularization (Gekas et al., 2005; Coşkun et al., 2014; Cao et al., 2019), although LT-HSC activity cannot be detected before E17.5 (Christensen et al., 2004). In mice, HSCs migrating out of the FL also seed the spleen, starting at E15.5, with HSC activity still being detected a few weeks after birth (Wolber et al., 2002; Christensen et al., 2004; Bertrand et al., 2006). In humans, although early colonization of fetal long bones was reported at ~10 wpc (Charbord et al., 1996), the hematopoietic shift from the FL to the FBM occurs later, around 20 wpc (Figure 1).

Coşkun et al. (2014) showed that hematopoietic progenitor cells reside in the vascularized regions of fetal long bones, stage at which they are still proliferative. Previous work showed that in the BM, HSCs are cycling during the first 3 weeks after birth and become quiescent thereafter (Bowie et al., 2006). The shift to a quiescence state seems to be dependent on the cellular composition of the microenvironment (Coşkun et al., 2014). LSK cells isolated from *Osx*^{-/-} FBM [that lack osteolineage cells (Nakashima et al., 2002) and some stromal populations (Mizoguchi et al., 2014)] form multi-lineage colonies *in vitro*, but fail to repopulate transplanted recipients (Mizoguchi et al., 2014). LSK cells exhibited dysregulated cell cycle progression and defective homing ability, suggesting that osteolineage and/or mesenchymal cells are necessary to establish and sustain BM LT-HSCs (Coşkun et al., 2014). Additionally, E15.5 CD105⁺Thy1⁻ mesenchymal progenitors transplanted under the kidney capsule give rise to donor-derived chondrocytes and can create an ectopic niche with the recruitment of host-derived marrow and HSCs, evidencing the importance of endochondral ossification for HSC niche formation (Chan et al., 2009).

Osteopontin (OPN), also known as secreted phosphoprotein 1 (SPP1) has been suggested to be an important niche factor in BM. Its concentration in stromal cells is inversely correlated

with HSC proliferation in the adult BM (Stier et al., 2005). The OPN dominant form thrombin-cleaved osteopontin (trOPN) is highly expressed in FBM (at the trabecular bone surface), but neglectable-to-none in the early-mid gestation FL (Cao et al., 2019). trOPN receptor $\alpha 4 \beta 1$ integrin is upregulated in fetal compared to adult HSCs. The differential concentration of the divalent metal cations, Ca²⁺, Mg²⁺, and Mn²⁺ between FL and FBM, being highly prevalent in the latter, was assumed to activate $\alpha 4 \beta 1$ in HSCs, possibly hindering their expansion in the BM (Cao et al., 2019).

CXCL12 expression in the vicinity of vascular endothelial cells in FBM supports the hematopoietic colonization of the organ (Nagasawa et al., 1996; Ara et al., 2003). HSCs isolated from CXCL12^{-/-} embryos failed to colonize the BM in long-term repopulation assays whereas their migration ability could be rescued by enforced expression of CXCL12 under the control of vascular-specific Tie-2 regulatory sequences (Ara et al., 2003). Moreover, the CXCL12-mediated migration of HSPCs *in vitro* seems to be enhanced by the presence of KITL, indicating an additive effect, only found in fetal HSCs (Christensen et al., 2004). This suggests an important role of KITL for HSC seeding and homing during embryonic development.

CXCL12-GFP knock-in mice allowed the identification of CXCL12-abundant reticular (CAR) cells, a population with high expression of CXCL12, distributed near SECs and at a lower extent near the endosteum (Sugiyama et al., 2006). These cells show *in vitro* potential to differentiate into adipocytic and osteoblastic lineages (Omatsu et al., 2010). Specific markers have been identified for mesenchymal cells in BM. Leptin receptor (Lepr) is highly enriched in *Scf*-GFP-expressing perivascular stromal cells (Ding et al., 2012) and Lepr⁺ cells were shown to largely overlap with CAR cells (Zhou et al., 2014). A rare population of Nestin⁺ cells that contains all of the fibroblastic CFU (CFU-F) activity within the mouse BM, capable of generating mesenspheres (mesenchymal spheres) *in vitro*, multipotent and with self-renewal potential was identified as a mesenchymal stem cell (MSC) population (Méndez-Ferrer et al., 2010). Later, two different Nestin-GFP populations were discriminated based on the fluorescence intensity by microscopy. Rare quiescent *Nestin*-GFP^{bright} cells, positive for the pericyte marker NG2 and α -smooth muscle actin, are enriched for CFU-F activity and express *Cxcl12* and *Kitl*. These cells are located alongside arterioles, close to dormant HSCs (Kunisaki et al., 2013), and associated with sympathetic nerves, that regulate their CXCL12 expression through the β_3 -adrenergic receptor (Méndez-Ferrer et al., 2010). *Nestin*-GFP^{dim} cells have a reticular shape, are mitotically active and line sinusoids, largely overlapping with Lepr⁺ cells (~80%) (Kunisaki et al., 2013). Selective ablation of mouse Nestin⁺ cells (Méndez-Ferrer et al., 2010) or CAR cells (Omatsu et al., 2010) significantly impacts the maintenance of HSCs. The structural differences of the blood vessels and perivascular populations seem to be associated with heterogeneity in HSC function. Besides, the selective deletion of *Cxcl12* from arteriolar NG2⁺ cells, but not sinusoidal Lepr⁺ cells, significantly reduced the HSCs compartment in the BM and a similar effect was observed by deletion of *Kitl* in Lepr⁺; but not NG2⁺ cells, evidencing the differential contribution of

perivascular populations in the cytokine production (Asada et al., 2017). BM microenvironment is illustrated in **Figure 4**.

Nestin⁺ cells can be prospectively isolated using PDGFR α and CD51 markers in the mouse and human fetal and adult BM (Pinho et al., 2013) and show MSC's properties and enrichment of *Cxcl12*, *Vcam1*, *Angpt1*, *Opn*, and *Scf* genes. Of note, in humans, these cells represent a small subset of CD146⁺ cells (Pinho et al., 2013), the latter harboring all the CFU-F activity in BM (Sacchetti et al., 2007). Co-culture of human PDGFR α ⁺CD51⁺ mesenspheres with human FBM CD34⁺ cells in a serum-free, but cytokine supplemented (TPO, SCF, FLT3L) culture media can expand MPPs that engraft immunodeficient mice (Pinho et al., 2013).

In the FL, Nestin-GFP⁺NG2⁺ cells, associated with portal vessels, form a niche promoting HSC expansion during the FL development that is no longer found after birth. Concomitantly, the phenotype of the portal vessel transits from Neuropilin-1⁺Ephrin-B2⁺ arterial to EphB4⁺ venular vessels (Khan et al., 2016). This role of Nestin⁺NG2⁺ cells in FL is opposite to that in BM, where Nestin⁺NG2⁺ arteriolar pericytes were proposed to maintain HSC quiescent (Kunisaki et al., 2013).

UNRESOLVED QUESTIONS – EX VIVO EXPANSION OF HSCs

Hematopoietic stem cells are the only cells of the hematopoietic compartment with the potential to replenish all mature blood cells and to divide without triggering differentiation programs, a process known as self-renewal. The mechanisms conveying these properties have been under investigation over the last 60 years, however, to date, they remain poorly understood. The concept that specific BM niche/microenvironment components regulate the fate of HSCs has been proposed by many authors. Cordeiro Gomes et al. (2016) analyzed not only HSCs but also different hematopoietic progenitors and found both HSCs and MPPs locate near or in contact with the same mesenchymal progenitor CAR-cells, expressing CXCL12 and SCF, fundamental to maintain the HSC pool and IL-7 that acts as a short-range signal for lymphoid differentiation. These observations suggest that both maintenance and multilineage differentiation are locally regulated by the same niche (Cordeiro Gomes et al., 2016). In the FL, HSCs expand considerably and differentiate, producing different mature lineages. If a given stromal population is also involved in the regulation of both processes, by the expression of various cytokines, or different cell populations contribute with distinct cytokines it still unresolved.

Hematopoietic stem cell transplantation is a widely used cell therapy intervention in the treatment of hematologic, autoimmune and genetic disorders. However, this therapy is still associated with high mortality rates, mainly due to infection, graft-versus-host disease (GVHD) and organ dysfunction, urging the need for improvement (Tanaka et al., 2016). The most common source of HSCs for transplantation is the BM or mobilized circulating HSPCs. However, matching of major histocompatibility complex antigens is needed to avoid GVHD (Schuster et al., 2012; Walasek et al., 2012). CB can be successfully

used as a source of partially mismatched HSCs, as it is readily available through CB banks and elicits low levels of GVHD. Low numbers of HSCs in CB and consequent absent or delayed reconstitution leads to post-transplantation infections, limiting the use of CB in adult patients (Ruggeri et al., 2014).

In vitro generation of HSCs would overcome some of the current clinical difficulties that transplantation faces, however, despite countless efforts to derive HSCs from pluripotent stem cell sources, generation of HSC *in vitro* has not yet been achieved – reviewed in Freire and Butler (2020). Another possibility to obtain higher numbers of HSCs would be to expand them *ex vivo* prior to transplantation. Distinct cytokine/growth factor cocktails have shown promising for expansion of HSCs, yet, limited success was reported in clinical studies due to a lack of LT-HSC expansion and rather proliferation of downstream progenitors together with undesirable stem cell differentiation – reviewed in Kumar and Geiger (2017) and Tajer et al. (2019). Cytokines currently used for *ex vivo* HSC expansion include KITL, TPO, IL-3, and FLT3L. *Kitl* and *Tpo* knockout mice show normal fetal development but reduced HSC numbers in the adult, suggesting that these cytokines are important for stem cell survival and proliferation in adulthood but might not be the drivers of HSC expansion during the embryonic period (Fujita et al., 1989; de Sauvage et al., 1996). There is growing evidence that the physical and mechanical properties of the microenvironment could impact on HSC decisions – reviewed in Kumar and Geiger (2017). A combination of cytokines with stroma derived ECM components – fibronectin and collagen – has shown encouraging results (Wohrer et al., 2014; Wilkinson et al., 2019). Moreover, culture of BM HSPCs in tropoelastin, the most elastic biomaterial known, induces a sixfold increase of LSK cells without supplementation with cytokines, suggesting that tropoelastin mediates a similar effect in survival and proliferation of LSK cells and its use could replace exogenous cytokines (Holst et al., 2010). Most cytokines studied have a role in HSC function in the BM, a site where HSC expansion does not occur in physiological conditions. The same cytokines have been found in FL stroma, but whether these are responsible for fetal HSC expansion is not known. Most likely, the FL expansion of newly generated HSCs results from intrinsic cellular properties together with a suitable microenvironment, physical cues included. Efforts have been made to replicate the embryonic microenvironment where HSCs expand, particularly by co-culture with FL hepatic or mesenchymal cells (Wineman et al., 1996; Chou and Lodish, 2010; Chou et al., 2013; Khan et al., 2016). Most strategies used so far focused on specific cell populations, either hepatoblasts or mesenchymal cells, but overlooked the possibility that distinct populations might need to interact. Only the co-culture of HSCs in a system where all the cytokine-expressing populations of the FL are present would reproduce the FL microenvironment. Such a system where hepatoblasts, endothelial and mesenchymal cells are cultured together in a 3D aggregate has already been devised, but no co-culture with hematopoietic cells has been attempted (Takebe et al., 2015). Liver cell culture models have been extensively developed for pharmacological and toxicological research or as a source for transplantation, to obtain an *in vitro* system that resembles a mature liver – reviewed in

Godoy et al. (2013), and for that reason are not suitable for recreating the FL niche environment.

Whereas the FL interactions between different cell types have been correlated with their contribution to the massive transient expansion of the hematopoietic system, other niche factors need to be addressed. Sigurdsson et al. (2016) reported the role of FL bile acids (BAs) as chemical chaperones, critical to sustain high protein production by expanding LT-HSCs without triggering endoplasmic reticulum (ER) stress. Inhibiting the biosynthesis of BAs *in vivo* resulted in reduced numbers of Lin[−], LSKs, and LSK CD48[−]CD150⁺ in the FL, with no apparent effect in the number of HSCs in the mother's BM (Sigurdsson et al., 2016). Comparison of FL and BM HSCs transcriptomes demonstrated that FL HSCs metabolism relies on oxygen-dependent pathways, which may be a requirement for extensive energy production during expansion. Contrary to BM HSCs, FL HSCs use oxidative phosphorylation (aside from glycolysis), have higher number of mitochondria and up regulate genes associated with antioxidant and DNA repair pathways, that are speculated to confer protection from reactive oxygen species-mediated (geno)toxicity (Manesia et al., 2015).

Exploring the complexity of HSC niches (cellular composition, cytokine and growth factors milieu, physical properties, oxygen availability, etc.) will improve our understanding of HSC self-renewal capacity (recently reviewed by Wilkinson et al., 2020), that is currently insufficient to devise efficient strategies for HSC expansion *ex vivo*.

CONCLUDING REMARKS

Distinct studies have identified and characterized FL stromal populations that may contribute with specific cues, enabling the HSC expansion in this organ. Despite the efforts, the role of

the FL microenvironment has not been directly shown. The possibility that HSCs proliferate exclusively due to intrinsic properties is however remote. Therefore, a model in which distinct populations cooperate is conceivable. An analysis that contemplates such complex multicellular networks has not been attempted. This review aimed to compile information on the cellular populations that could signal to HSCs during development, highlighting the advances and the unresolved questions in the field.

AUTHOR CONTRIBUTIONS

FS-d-S and MP: conceptualization and writing. AC: discussion and revision. PP-d-Ó: conceptualization and revision. All authors contributed to the article and approved the submitted version.

FUNDING

FS-d-S funded by FCT grants PD/BD/114128/2015 and POCI-0100145-FEDER-01638. MP funded by FCT grant SFRH/BD/143605/2019. AC financed by the Institut Pasteur, INSERM, ANR (grant Twothyme and EpiDev) and REVIVE Future Investment Program and Pasteur-Weizmann Foundation. PP-d-Ó financed by Portuguese funds through FCT/MCTES in the framework of the projects PTDC/MED-OUT/32656/2017, POCI-01-0145-FEDER-032656, and by – FCT – UIDB/04293/2020.

ACKNOWLEDGMENTS

We thank the members of PP-d-Ó and AC laboratories for critical discussions.

REFERENCES

- Alexander, W. S., Roberts, A. W., Nicola, N. A., Li, R., and Metcalf, D. (1996). Deficiencies in progenitor cells of multiple hematopoietic lineages and defective megakaryocytopoiesis in mice lacking the thrombopoietic receptor c-Mpl. *Blood* 87, 2162–2170.
- Antoniou, A., Raynaud, P., Cordi, S., Zong, Y., Tronche, F., Stanger, B. Z., et al. (2009). Intrahepatic bile ducts develop according to a new mode of tubulogenesis regulated by the transcription factor SOX9. *Gastroenterology* 136, 2325–2333. doi: 10.1053/j.gastro.2009.02.051
- Ara, T., Tokoyoda, K., Sugiyama, T., Egawa, T., Kawabata, K., and Nagasawa, T. (2003). Long-term hematopoietic stem cells require stromal cell-derived factor-1 for colonizing bone marrow during ontogeny. *Immunity* 19, 257–267. doi: 10.1016/S1074-7613(03)00201-2
- Asada, N., Kunisaki, Y., Pierce, H., Wang, Z., Fernandez, N. F., Birbrair, A., et al. (2017). Differential cytokine contributions of perivascular haematopoietic stem cell niches. *Nat. Cell Biol.* 19, 214–223. doi: 10.1038/ncb3475
- Asahina, K. (2012). Hepatic stellate cell progenitor cells. *J. Gastroenterol. Hepatol.* 27(Suppl. 2), 80–84. doi: 10.1111/j.1440-1746.2011.07001.x
- Asahina, K., Tsai, S. Y., Li, P., Ishii, M. Jr., and Henry, M. (2010). Mesenchymal origin of hepatic stellate cells, submesothelial cells, and perivascular mesenchymal cells during mouse liver development. *Hepatology* 49, 998–1011. doi: 10.1002/hep.22721.Mesenchymal
- Asahina, K., Zhou, B., Pu, W. T., and Tsukamoto, H. (2011). Septum transversum-derived mesothelium gives rise to hepatic stellate cells and perivascular mesenchymal cells in developing mouse liver. *Hepatology* 53, 983–995. doi: 10.1002/hep.24119
- Ayres-Silva, J. D. P., Manso, P. P. D. A., Madeira, M. R. D. C., Pelajo-Machado, M., and Lenzi, H. L. (2011). Sequential morphological characteristics of murine fetal liver hematopoietic microenvironment in Swiss Webster mice. *Cell Tissue Res.* 344, 455–469. doi: 10.1007/s00441-011-1170-1
- Barberá-Guillem, E., Ayala, R., and Vidal-Vanaclocha, F. (1989). Differential location of hemopoietic colonies within liver acini of postnatal and phenylhydrazine-treated adult mice. *Hepatology* 9, 29–36. doi: 10.1002/hep.1840090106
- Barker, J. E. (1994). SL/SLd hematopoietic progenitors are deficient in situ. *Exp. Hematol.* 22, 174–177.
- Berthault, C., Ramond, C., Buren-Defranoux, O., Soubigou, G., Chea, S., Golub, R., et al. (2017). Asynchronous lineage priming determines commitment to T cell and B cell lineages in fetal liver. *Nat. Immunol.* 18, 1139–1149. doi: 10.1038/ni.3820
- Bertrand, J., Chi, N. C., Santoso, B., Teng, S., Stainier, D. Y. R., and Traver, D. (2010). Haematopoietic stem cells derive directly from aortic endoderm during development. *Nature* 464, 108–111. doi: 10.1038/nature08738
- Bertrand, J., Desanti, G. E., Lo-Man, R., Leclerc, C., Cumano, A., and Golub, R. (2006). Fetal spleen stroma drives macrophage commitment. *Development* 133, 3619–3628. doi: 10.1242/dev.02510

- Bertrand, J., Giroux, S., Golub, R., Klaine, M., Jalil, A., Boucontet, L., et al. (2005a). Characterization of purified intraembryonic hematopoietic stem cells as a tool to define their site of origin. *Proc. Natl. Acad. Sci. U.S.A.* 102, 134–139. doi: 10.1073/pnas.0402270102
- Bertrand, J., Jalil, A., Klaine, M., Jung, S., Cumano, A., and Godin, I. (2005b). Three pathways to mature macrophages in the early mouse yolk sac. *Blood* 106, 3004–3011. doi: 10.1182/blood-2005-02-0461
- Bessis, M., Mize, C., and Prenant, M. (1978). Erythropoiesis: comparison of in vivo and in vitro amplification. *Blood Cells* 4, 155–174.
- Bleul, C. C., Farzan, M., Choe, H., Parolin, C., Clark-Lewis, I., Sodroski, J., et al. (1996). The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature* 382, 829–833. doi: 10.1038/382829a0
- Bloom, W., and Bartelmez, G. W. (1940). Hematopoiesis in young human embryos. *Am. J. Anat.* 67, 21–53. doi: 10.1002/aja.1000670103
- Bogue, C. W., Ganea, G. R., Sturm, E., Ianucci, R., and Jacobs, H. C. (2000). Hex expression suggests a role in the development and function of organs derived from foregut endoderm. *Dev. Dyn.* 219, 84–89.
- Borge, O., Ramsfjell, V., Cui, L., and Jacobsen, S. E. (1997). Ability of early acting cytokines to directly promote survival and suppress apoptosis of human primitive CD34+CD38- bone marrow cells with multilineage potential at the single-cell level: key role of thrombopoietin. *Blood* 90, 2282–2292.
- Borge, O., Ramsfjell, V., Veiby, O., Murphy, M., Lok, S., and Jacobsen, S. (1996). Thrombopoietin, but not erythropoietin promotes viability and inhibits apoptosis of multipotent murine hematopoietic progenitor cells in vitro. *Blood* 88, 2859–2870. doi: 10.1182/blood.V88.8.2859.bloodjournal88.82859
- Bort, R., Signore, M., Tremblay, K., Barbera, J. P. M., and Zaret, K. S. (2006). Hex homeobox gene controls the transition of the endoderm to a pseudostratified, cell emergent epithelium for liver bud development. *Dev. Biol.* 290, 44–56. doi: 10.1016/j.ydbio.2005.11.006
- Bowie, M. B., McKnight, K. D., Kent, D. G., McCaffrey, L., Hoodless, P. A., and Eaves, C. J. (2006). Hematopoietic stem cells proliferate until after birth and show a reversible phase-specific engraftment defect. *J. Clin. Invest.* 116, 2808–2816. doi: 10.1172/JCI28310
- Brouard, N., Jost, C., Matthias, N., Albrecht, C., Egard, S., Gandhi, P., et al. (2017). A unique microenvironment in the developing liver supports the expansion of megakaryocyte progenitors. *Blood Adv.* 1, 1854–1866. doi: 10.1182/bloodadvances.2016003541
- Busch, K., Klapproth, K., Barile, M., Flossdorf, M., Holland-Letz, T., Schlenger, S. M., et al. (2015). Fundamental properties of unperturbed haematopoiesis from stem cells in vivo. *Nature* 518, 542–546. doi: 10.1038/nature14242
- Cao, H., Cao, B., Heazlewood, C. K., Domingues, M., Sun, X., Debele, E., et al. (2019). Osteopontin is an important regulatory component of the fetal bone marrow hematopoietic stem cell niche. *Cells* 8:985. doi: 10.3390/cells8090985
- Cardier, J., and Barberá-Guillem, E. (1997). Extramedullary hematopoiesis in the adult mouse liver is associated with specific hepatic sinusoidal endothelial cells. *Hepatology* 26, 165–175. doi: 10.1002/hep.510260122
- Cardier, J., and Dempsey, J. (1998). Thrombopoietin and its receptor, c-mpl, are constitutively expressed by mouse liver endothelial cells: evidence of thrombopoietin as a growth factor for liver endothelial cells. *Blood* 91, 923–929. doi: 10.1182/blood.V91.3.923
- Cascio, S., and Zaret, K. S. (1991). Hepatocyte differentiation initiates during endodermal-mesenchymal interactions prior to liver formation. *Development* 113, 217–225.
- Chan, C. K. F., Chen, C.-C., Luppen, C. A., Kim, J.-B., DeBoer, A. T., Wei, K., et al. (2009). Endochondral ossification is required for haematopoietic stem-cell niche formation. *Nature* 457, 490–494. doi: 10.1038/nature07547
- Charbord, P., and Moore, K. A. (2005). Gene expression in stem cell-supporting stromal cell lines. *Ann. N. Y. Acad. Sci.* 1044, 159–167. doi: 10.1196/annals.1349.020
- Charbord, P., Tavian, M., Humeau, L., and Peault, B. (1996). Early ontogeny of the human marrow from long bones: an immunohistochemical study of hematopoiesis and its microenvironment. *Blood* 87, 4109–4119. doi: 10.1182/blood.V87.10.4109.bloodjournal87104109
- Chen, J. Y., Miyazaki, M., Wang, S. K., Yamazaki, S., Sinha, R., Kao, K. S., et al. (2016). Hoxb5 marks long-term haematopoietic stem cells and reveals a homogenous perivascular niche. *Nature* 530, 223–227. doi: 10.1038/nature16943
- Cheshier, S. H., Morrison, S. J., Liao, X., and Weissman, I. L. (1999). In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 96, 3120–3125. doi: 10.1073/pnas.96.6.3120
- Chiu, S.-C., Liu, H.-H., Chen, C.-L., Chen, P.-R., Liu, M.-C., Lin, S.-Z., et al. (2015). Extramedullary hematopoiesis (EMH) in laboratory animals: offering an insight into stem cell research. *Cell Transplant.* 24, 349–366. doi: 10.3727/096368915X686850
- Chou, S., Flygare, J., and Lodish, H. F. (2013). Fetal hepatic progenitors support long-term expansion of hematopoietic stem cells. *Exp. Hematol.* 41, 479–490. doi: 10.1016/j.exphem.2013.02.003
- Chou, S., and Lodish, H. F. (2010). Fetal liver hepatic progenitors are supportive stromal cells for hematopoietic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 107, 7799–7804. doi: 10.1073/pnas.1003586107
- Christensen, J. L., Wright, D. E., Wagers, A. J., and Weissman, I. L. (2004). Circulation and chemotaxis of fetal hematopoietic stem cells. *PLoS Biol.* 2:e020075. doi: 10.1371/journal.pbio.0020075
- Christodoulou, C., Spencer, J. A., Yeh, S.-C. A., Turcotte, R., Kokkalis, K. D., Panero, R., et al. (2020). Live-animal imaging of native haematopoietic stem and progenitor cells. *Nature* 578, 278–283. doi: 10.1038/s41586-020-1971-z
- Clotman, F. (2005). Control of liver cell fate decision by a gradient of TGF signaling modulated by Onecut transcription factors. *Genes Dev.* 19, 1849–1854. doi: 10.1101/gad.340305
- Clotman, F., Lannoy, V. J., Reber, M., Cereghini, S., Cassiman, D., Jacquemin, P., et al. (2002). The onecut transcription factor HNF6 is required for normal development of the biliary tract. *Development* 129, 1819–1828.
- Collardeau-Frachon, S., and Scoazec, J. Y. (2008). Vascular development and differentiation during human liver organogenesis. *Anat. Rec.* 291, 614–627. doi: 10.1002/ar.20679
- Cordeiro Gomes, A., Hara, T., Lim, V. Y., Herndler-Brandstetter, D., Nevius, E., Sugiyama, T., et al. (2016). Hematopoietic stem cell niches produce lineage-instructive signals to control multipotent progenitor differentiation. *Immunity* 45, 1219–1231. doi: 10.1016/j.immuni.2016.11.004
- Coşkun, S., Chao, H., Vasavada, H., Heydari, K., Gonzales, N., Zhou, X., et al. (2014). Development of the fetal bone marrow niche and regulation of HSC quiescence and homing ability by emerging osteolineage cells. *Cell Rep.* 9, 581–590. doi: 10.1016/j.celrep.2014.09.013
- Crane, G. M., Jeffery, E., and Morrison, S. J. (2017). Adult haematopoietic stem cell niches. *Nat. Rev. Immunol.* 17, 573–590. doi: 10.1038/nri.2017.53
- Crawford, L. W., Foley, J. F., and Elmore, S. A. (2010). Histology atlas of the developing mouse hepatobiliary system with emphasis on embryonic days 9.5–18.5. *Toxicol. Pathol.* 38, 872–906. doi: 10.1177/0192623310374329
- Cumano, A., Dieterlen-Lievre, F., and Godin, I. (1996). Lymphoid potential, probed before circulation in mouse, is restricted to caudal intraembryonic splanchnopleura. *Cell* 86, 907–916. doi: 10.1016/S0092-8674(00)80166-X
- Cumano, A., Ferraz, J. C., Klaine, M., Di Santo, J. P., and Godin, I. (2001). Intraembryonic, but Not Yolk Sac hematopoietic precursors, isolated before circulation, provide long-term multilineage reconstitution. *Immunity* 15, 477–485. doi: 10.1016/S1074-7613(01)00190-X
- de Sauvage, F. J., Carver-Moore, K., Luoh, S. M., Ryan, A., Dowd, M., Eaton, D. L., et al. (1996). Physiological regulation of early and late stages of megakaryocytopoiesis by thrombopoietin. *J. Exp. Med.* 183, 651–656. doi: 10.1084/jem.183.2.651
- Decker, M., Leslie, J., Liu, Q., and Ding, L. (2018). Hepatic thrombopoietin is required for bone marrow hematopoietic stem cell maintenance. *Science* 360, 106–110. doi: 10.1126/science.aap8861
- Ding, L., Saunders, T. L., Enikolopov, G., and Morrison, S. J. (2012). Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature* 481, 457–462. doi: 10.1038/nature10783
- Douarin, N. M. (1975). An experimental analysis of liver development. *Med. Biol.* 53, 427–455.
- Dudas, J., Papoutsis, M., Hecht, M., Elmaouhoub, A., Saile, B., Christ, B., et al. (2004). The homeobox transcription factor Prox1 is highly conserved in embryonic hepatoblasts and in adult and transformed hepatocytes, but is absent

- from bile duct epithelium. *Anat. Embryol.* 208, 359–366. doi: 10.1007/s00429-004-0403-4
- Eaton, D. L., and de Sauvage, F. J. (1997). Thrombopoietin: the primary regulator of megakaryocytopoiesis and thrombopoiesis. *Exp. Hematol.* 25, 1–7.
- Eberl, G., Marmon, S., Sunshine, M. J., Rennert, P. D., Choi, Y., and Littmann, D. R. (2004). An essential function for the nuclear receptor ROR γ t in the generation of fetal lymphoid tissue inducer cells. *Nat. Immunol.* 5, 64–73. doi: 10.1038/nl1022
- Eckardt, K. U., Pugh, C. W., Meier, M., Tan, C. C., Ratcliffe, P. J., and Kurtz, A. (1994). Production of erythropoietin by liver cells in vivo and in vitro. *Ann. N. Y. Acad. Sci.* 718, 50–60. doi: 10.1111/j.1749-6632.1994.tb55703.x
- Ema, H., and Nakauchi, H. (2000). Expansion of hematopoietic stem cells in the developing liver of a mouse embryo. *Blood* 95, 2284–2289.
- Emambokus, N. R., and Frampton, J. (2003). The glycoprotein IIb molecule is expressed on early murine hematopoietic progenitors and regulates their numbers in sites of hematopoiesis. *Immunity* 19, 33–45. doi: 10.1016/S1074-7613(03)00173-0
- Fox, N., Priestley, G., Papayannopoulou, T., and Kaushansky, K. (2002). Thrombopoietin expands hematopoietic stem cells after transplantation. *J. Clin. Invest.* 110, 389–394. doi: 10.1172/JCI15430
- Frame, J. M., Fegan, K. H., Conway, S. J., McGrath, K. E., and Palis, J. (2016). Definitive hematopoiesis in the yolk sac emerges from wnt-responsive hemogenic endothelium independently of circulation and arterial identity. *Stem Cells* 34, 431–444. doi: 10.1002/stem.2213
- Fraser, S. T., Ogawa, M., Yu, R. T., Nishikawa, S., Yoder, M. C., and Nishikawa, S. I. (2002). Definitive hematopoietic commitment within the embryonic vascular endothelial-cadherin+ population. *Exp. Hematol.* 30, 1070–1078. doi: 10.1016/S0301-472X(02)00887-1
- Freire, A. G., and Butler, J. M. (2020). Blood making: learning what to put into the dish. *F1000Research* 9:38. doi: 10.12688/f1000research.21245.1
- Fujita, J., Onoue, H., Ebi, Y., Nakayama, H., and Kanakura, Y. (1989). In vitro duplication and in vivo cure of mast-cell deficiency of Sl/Sld mutant mice by cloned 3T3 fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.* 86, 2888–2891. doi: 10.1073/pnas.86.8.2888
- Gekas, C., Dieterlen-Lièvre, F., Orkin, S. H., and Mikkola, H. K. A. (2005). The placenta is a niche for hematopoietic stem cells. *Dev. Cell* 8, 365–375. doi: 10.1016/j.devcel.2004.12.016
- Gentek, R., Ghigo, C., Hoeffel, G., Bulle, M. J., Msallam, R., Gautier, G., et al. (2018). Hemogenic endothelial fate mapping reveals dual developmental origin of mast cells. *Immunity* 48, 1160–1171. doi: 10.1016/j.immuni.2018.04.025
- Gerlach, J. C., Over, P., Turner, M. E., Thompson, R. L., Foka, H. G., Chen, W. C. W., et al. (2012). Perivascular mesenchymal progenitors in human fetal and adult liver. *Stem Cells Dev.* 21, 3258–3269. doi: 10.1089/scd.2012.0296
- Gerlach, J. C., Thompson, R. L., Gridelli, B., and Schmelzer, E. (2019). Effects of delta-like noncanonical Notch ligand 1 expression of human fetal liver hepatoblasts on hematopoietic progenitors. *Stem Cells Intern.* 2019:7916275. doi: 10.1155/2019/7916275
- Godoy, P., Hewitt, N. J., Albrecht, U., Andersen, M. E., Ansari, N., Bhattacharya, S., et al. (2013). Recent advances in 2D and 3D in vitro systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME. *Arch. Toxicol.* 87:1315. doi: 10.1007/s00204-013-1078-5
- Gomez-Perdiguer, E., Klapproth, K., Schulz, C., Busch, K., Azzoni, E., Crozet, L., et al. (2015). Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors. *Nature* 518, 547–551. doi: 10.1038/nature13989
- Guilbert, L. J., and Stanley, E. R. (1980). Specific interaction of murine colony-stimulating factor with mononuclear phagocytic cells. *J. Cell Biol.* 85, 153–159. doi: 10.1083/jcb.85.1.153
- Guo, Y., Zhang, X., Huang, J., Zeng, Y., Liu, W., Geng, C., et al. (2009). Relationships between hematopoiesis and hepatogenesis in the midtrimester fetal liver characterized by dynamic transcriptomic and proteomic profiles. *PLoS One* 4:e7641. doi: 10.1371/journal.pone.0007641
- Guyot, C., Lepreux, S., Combe, C., Doudnikoff, E., Bioulac-Sage, P., Balabaud, C., et al. (2006). Hepatic fibrosis and cirrhosis: the (myo)fibroblastic cell subpopulations involved. *Intern. J. Biochem. Cell Biol.* 38, 135–151. doi: 10.1016/j.biocel.2005.08.021
- Haas, J. D., Ravens, S., Düber, S., Sandrock, I., Oberdörfer, L., Kashani, E., et al. (2012). Development of interleukin-17-producing $\gamma\delta$ T cells is restricted to a functional embryonic wave. *Immunity* 37, 48–59. doi: 10.1016/j.immuni.2012.06.003
- Haruna, Y., Saito, K., Spaulding, S., Nalesnik, M. A., and Gerber, M. A. (1996). Identification of bipotential progenitor cells in human liver development. *Hepatology* 23, 476–481. doi: 10.1002/hep.510230312
- Hata, M., Nanno, M., Doi, H., Satomi, S., Sakata, T., Suzuki, R., et al. (1993). Establishment of a hepatocytic epithelial cell line from the murine fetal liver capable of promoting hemopoietic cell proliferation. *J. Cell. Physiol.* 154, 381–392. doi: 10.1002/jcp.1041540222
- Hayashi, Y., Sezaki, M., and Takizawa, H. (2019). Development of the hematopoietic system: Role of inflammatory factors. *Wil. Interdiscipl. Rev. Dev. Biol.* 8, 1–17. doi: 10.1002/wdev.341
- Hirsch, E., Iglesias, A., Potocnik, A. J., Hartmann, U., and Fässler, R. (1996). Impaired migration but not differentiation of haematopoietic stem cells in the absence of β 1 integrins. *Nature* 380, 171–175. doi: 10.1038/380171a0
- Holst, J., Watson, S., Lord, M. S., Eamegdool, S. S., Bax, D. V., Nivison-Smith, L. B., et al. (2010). Substrate elasticity provides mechanical signals for the expansion of hemopoietic stem and progenitor cells. *Nat. Biotechnol.* 28, 1123–1128. doi: 10.1038/nbt1687
- House, P. D. R., and Weidemann, M. J. (1970). Characterization of an [125I]-Insulin binding plasma membrane fraction from rat liver. *Biochem. Biophys. Res. Commun.* 41, 541–548. doi: 10.1016/0006-291X(70)90046-X
- Ijpenberg, A., Pérez-Pomares, J. M., Guadix, J. A., Carmona, R., Portillo-Sánchez, V., Macías, D., et al. (2007). Wt1 and retinoic acid signaling are essential for stellate cell development and liver morphogenesis. *Dev. Biol.* 312, 157–170. doi: 10.1016/j.ydbio.2007.09.014
- Ikuta, K., Kina, T., MacNeil, I., Uchida, N., Peault, B., Chien, Y., et al. (1990). A developmental switch in thymic lymphocyte maturation potential occurs at the level of hematopoietic stem cells. *Cell* 62, 863–874. doi: 10.1016/0092-8674(90)90262-D
- Ji, R. P., Phoon, C. K. L., Aristizábal, O., McGrath, K. E., Palis, J., and Turnbull, D. H. (2003). Onset of cardiac function during early mouse embryogenesis coincides with entry of primitive erythroblasts into the embryo proper. *Circ. Res.* 92, 133–135. doi: 10.1161/01.RES.0000056532.18710.C0
- Johns, J. L., and Christopher, M. M. (2012). Extramedullary hematopoiesis: a new look at the underlying stem cell niche, theories of development, and occurrence in animals. *Vet. Pathol.* 49, 508–523. doi: 10.1177/0300985811432344
- Johnson, G. R., and Moore, M. A. S. (1975). Role of stem cell migration in initiation of mouse foetal liver haemopoiesis. *Nature* 258, 726–728. doi: 10.1038/258726a0
- Kamiya, A., Kinoshita, T., Ito, Y., Matsui, T., Morikawa, Y., Senba, E., et al. (1999). Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer. *EMBO J.* 18, 2127–2136. doi: 10.1093/emboj/18.8.2127
- Kasaai, B., Caolo, V., Peacock, H. M., Lehoux, S., Gomez-Perdiguer, E., Luttun, A., et al. (2017). Erythro-myeloid progenitors can differentiate from endothelial cells and modulate embryonic vascular remodeling. *Sci. Rep.* 7, 25–28. doi: 10.1038/srep43817
- Kaushansky, K. (1995). Thrombopoietin: the primary regulator of platelet production. *Blood* 86, 419–431. doi: 10.1182/blood.V86.2.419.bloodjournal862419
- Khan, J., Mendelson, A., Kunisaki, Y., Birbrair, A., Kou, Y., Arnal-Estapé, A., et al. (2016). Fetal liver hematopoietic stem cell niches associate with portal vessels. *Science* 351, 176–180. doi: 10.1126/science.aad0084
- Kiessseian, A., de la Grange, P. B., Buren, Defranoux, O., Godin, I., and Cumano, A. (2012). Immature hematopoietic stem cells undergo maturation in the fetal liver. *Development* 139, 3521–3530. doi: 10.1242/dev.079210
- Kim, I., He, S., Yilmaz, O. H., Kiel, M. J., Morrison, S. J., and Yilmaz, H. (2006). Enhanced purification of fetal liver hematopoietic stem cells using SLAM family receptors. *Blood* 108, 737–744. doi: 10.1182/blood-2005-10-4135
- Kimura, S., Roberts, A. W., Metcalf, D., and Alexander, W. S. (1998). Hematopoietic stem cell deficiencies in mice lacking c-Mpl, the receptor for thrombopoietin. *Proc. Natl. Acad. Sci. U.S.A.* 95, 1195–1200. doi: 10.1073/pnas.95.3.1195
- Kingsley, P. D., Malik, J., Emerson, R. L., Bushnell, T. P., McGrath, K. E., Bloedorn, L. A., et al. (2006). “Maturation” globin switching in primary primitive erythroid cells. *Blood* 107, 1665–1672. doi: 10.1182/blood-2005-08-3097

- Kinoshita, T., Sekiguchi, T., Xu, M.-J., Ito, Y., Kamiya, A., Tsuji, K.-I., et al. (1999). Hepatic differentiation induced by oncostatin M attenuates fetal liver hematopoiesis. *Proc. Natl. Acad. Sci. U.S.A.* 96, 7265–7270. doi: 10.2177/jsci.21.supplement_215
- Kiserud, T. (2005). Physiology of the fetal circulation. *Semin. Fet. Neonat. Med.* 10, 493–503. doi: 10.1016/j.siny.2005.08.007
- Kissa, K., and Herbolom, P. (2010). Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *Nature* 464, 112–115. doi: 10.1038/nature08761
- Ku, H., Yonemura, Y., Kaushansky, K., and Ogawa, M. (1996). Thrombopoietin, the ligand for the Mpl receptor, synergizes with steel factor and other early acting cytokines in supporting proliferation of primitive hematopoietic progenitors of mice. *Blood* 87, 4544–4551.
- Kubota, H., Yao, H., and Reid, L. M. (2007). Identification and characterization of vitamin A-storing cells in fetal liver: implications for functional importance of hepatic stellate cells in liver development and hematopoiesis. *Stem Cells* 25, 2339–2349. doi: 10.1634/stemcells.2006-0316
- Kumar, S., and Geiger, H. (2017). HSC niche biology and hsc expansion *Ex Vivo*. *Trends Mol. Med.* 23, 799–819. doi: 10.1016/j.molmed.2017.07.003
- Kunisaki, Y., Bruns, I., Scheiermann, C., Ahmed, J., Pinho, S., Zhang, D., et al. (2013). Arteriolar niches maintain haematopoietic stem cell quiescence. *Nature* 502, 637–643. doi: 10.1038/nature12612
- Lai, C.-Y., Yamazaki, S., Okabe, M., Suzuki, S., Maeyama, Y., Iimura, Y., et al. (2014). Stage-specific roles for Cxcr4 signaling in murine hematopoietic stem/progenitor cells in the process of bone marrow repopulation. *Stem Cells* 32, 1929–1942. doi: 10.1002/stem.1670
- Lee, E. Y.-H. P., Chang, C.-Y., Hu, N., Wang, Y.-C. J., Lai, C.-C., Herrup, K., et al. (1992). Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. *Nature* 359, 288–294. doi: 10.1038/359288a0
- Li, J., Ning, G., and Duncan, S. A. (2000). Mammalian hepatocyte differentiation requires the transcription factor HNF-4 α . *Genes Dev.* 14, 464–474. doi: 10.1126/MMBR.66.1.39-63.2002
- Lin, C. S., Lim, S. K., D'Agati, V., and Costantini, F. (1996). Differential effects of an erythropoietin receptor gene disruption on primitive and definitive erythropoiesis. *Genes Dev.* 10, 154–164. doi: 10.1101/gad.10.2.154
- Lok, S., Kaushansky, K., Holly, R. D., Kuijper, J. L., Lofton-Day, C. E., Oort, P. J., et al. (1994). Cloning and expression of murine thrombopoietin cDNA and stimulation of platelet production in vivo. *Nature* 369, 565–568. doi: 10.1038/369565a0
- Lue, I., and Asahina, K. (2016). The role of mesothelial cells in liver development. *Injury Regener. Gut Liver* 10:166. doi: 10.5009/gnl15226
- Lyman, S. D., James, L., Bos, T., Vanden de Vries, P., Brasel, K., Gliniak, B., et al. (1993). Molecular cloning of a ligand for the flt3flk-2 tyrosine kinase receptor: a proliferative factor for primitive hematopoietic cells. *Cell* 75, 1157–1167. doi: 10.1016/0092-8674(93)90325-K
- Manesia, J. K., Xu, Z., Broekaert, D., Boon, R., van Vliet, A., Eelen, G., et al. (2015). Highly proliferative primitive fetal liver hematopoietic stem cells are fueled by oxidative metabolic pathways. *Stem Cell Res.* 15, 715–721. doi: 10.1016/j.scr.2015.11.001
- Margagliotti, S., Clotman, F., Pierreux, C. E., Beaudry, J. B., Jacquemin, P., Rousseau, G. G., et al. (2007). The oncut transcription factors HNF-6/OC-1 and OC-2 regulate early liver expansion by controlling hepatoblast migration. *Dev. Biol.* 311, 579–589. doi: 10.1016/j.ydbio.2007.09.013
- Matsumoto, K., Yoshitomi, H., Rossant, J., and Zaret, K. S. (2001). Liver organogenesis promoted by endothelial cells prior to vascular function. *Science* 294, 559–563. doi: 10.1126/science.1063889
- Matthews, W., Jordan, C. T., Wiegand, G. W., Pardoll, D., and Lemischka, I. R. (1991). A receptor tyrosine kinase specific to hematopoietic stem and progenitor cell-enriched populations. *Cell* 65, 1143–1152. doi: 10.1016/0092-8674(91)90010-V
- McDermott, D. H., Gao, J.-L., Liu, Q., Siwicki, M., Martens, C., Jacobs, P., et al. (2015). Chromothrptic cure of WHIM syndrome. *Cell* 160, 686–699. doi: 10.1016/j.cell.2015.01.014
- McGrath, K. E., Frame, J. M., Fegan, K. H., Bowen, J. R., Conway, S. J., Catherman, S. C., et al. (2015). Distinct sources of hematopoietic progenitors emerge before HSCs and provide functional blood cells in the mammalian Embryo. *Cell Rep.* 11, 1892–1904. doi: 10.1016/j.celrep.2015.05.036
- Medvinsky, A., Dzierzak, E., Ridgeway, T., and Hill, M. (1996). Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell* 86, 897–906. doi: 10.1016/S0092-8674(00)80165-8
- Méndez-Ferrer, S., Michurina, T. V., Ferraro, F., Mazloom, A. R., MacArthur, B. D., Lira, S. A., et al. (2010). Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* 466, 829–834. doi: 10.1038/nature09262
- Migliaccio, G., Migliaccio, A. R., Petti, S., Mavilio, F., Russo, G., Lazzaro, D., et al. (1986). Human embryonic hemopoiesis. Kinetics of progenitors and precursors underlying the yolk sac-liver transition. *J. Clin. Invest.* 78, 51–60. doi: 10.1172/JCI112572
- Miller, C. L., Rebel, V. I., Helgason, C. D., Lansdorp, D., and Eaves, C. J. (1997). Impaired steel factor responsiveness differentially affects the detection and long-term maintenance of fetal liver hematopoietic stem cells in vivo. *Blood* 89, 1214–1223. doi: 10.1182/blood.v89.4.1214
- Miyajima, A., Kinoshita, T., Tanaka, M., Kamiya, A., Mukoyama, Y., and Hara, T. (2000). Role of oncostatin M in hematopoiesis and liver development. *Cytokine Growth. Factor. Rev.* 11, 177–183. doi: 10.1016/S1359-6101(00)00003-4
- Mizoguchi, T., Pinho, S., Ahmed, J., Kunisaki, Y., Hanoun, M., Mendelson, A., et al. (2014). Osterix marks distinct waves of primitive and definitive stromal progenitors during bone marrow development. *Dev. Cell* 29, 340–349. doi: 10.1016/j.devcel.2014.03.013
- Moore, K. A., Pytowski, B., Witte, L., Hicklin, D., and Lemischka, I. R. (1997). Hematopoietic activity of a stromal cell transmembrane protein containing epidermal growth factor-like repeat motifs. *Proc. Natl. Acad. Sci. U.S.A.* 94, 4011–4016. doi: 10.1073/pnas.94.8.4011
- Morgan, D. O., Edman, J. C., Standring, D. N., Fried, V. A., Smith, M. C., Roth, R. A., et al. (1987). Insulin-like growth factor II receptor as a multifunctional binding protein. *Nature* 329, 301–307. doi: 10.1038/329301a0
- Morrison, S. J., Hemmati, H. D., Wandycz, A. M., and Weissman, I. L. (1995). The purification and characterization of fetal liver hematopoietic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 92, 10302–10306. doi: 10.1073/pnas.92.22.10302
- Mucenski, M. L., McLain, K., Kier, A., Swerdlow, S. H., Schreiner, C. M., Miller, T. A., et al. (1991). A functional c-myc Gene is required for normal murine fetal hepatic hematopoiesis. *Cell* 65, 677–689.
- Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S. I., Kitamura, Y., et al. (1996). Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature* 382, 635–638. doi: 10.1038/382635a0
- Nakamura, Y., Hagiwara, T., Hosokawa, K., Iwasaki, H., Matsuo, S., Takahashi, T., et al. (2007). Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. *Cell Stem Cell* 1, 685–697. doi: 10.1016/j.stem.2007.10.020
- Nakashima, K., Zhou, X., Kunkel, G., Zhang, Z., Deng, J. M., Behringer, R. R., et al. (2002). The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* 108, 17–29. doi: 10.1016/S0092-8674(01)00622-5
- Nierhoff, D., Ogawa, A., Oertel, M., Chen, Y.-Q., and Shafritz, D. A. (2005). Purification and characterization of mouse fetal liver epithelial cells with high in vivo repopulation capacity. *Hepatology* 42, 130–139. doi: 10.1002/hep.20735
- Nishina, H., Vaz, C., Billia, P., Nghiem, M., Sasaki, T., De la Pompa, J. L., et al. (1999). Defective liver formation and liver cell apoptosis in mice lacking the stress signaling kinase SEK1/MKK4. *Development* 126, 505–516.
- Nitou, M., Ishikawa, K., and Shiojiri, N. (2000). Immunohistochemical analysis of development of desmin-positive hepatic stellate cells in mouse liver. *J. Anat.* 197, 635–646. doi: 10.1046/j.1469-7580.2000.19740635.x
- Nitou, M., Sugiyama, Y., Ishikawa, K., and Shiojiri, N. (2002). Purification of fetal mouse hepatoblasts by magnetic beads coated with monoclonal anti-E-cadherin antibodies and their in vitro culture. *Exper. Cell Res.* 279, 330–343. doi: 10.1006/excr.2002.5615
- Nomura, S., Ogami, K., Kawamura, K., Tsukamoto, I., Kudo, Y., Kanakura, Y., et al. (1997). Cellular localization of thrombopoietin mRNA in the liver by in situ hybridization. *Exp. Hematol.* 25, 565–572.
- Nonaka, H., Tanaka, M., Suzuki, K., and Miyajima, A. (2007). Development of murine hepatic sinusoidal endothelial cells characterized by the expression of hyaluronan receptors. *Dev. Dyn.* 236, 2258–2267. doi: 10.1002/dvdy.21227

- Omatsu, Y., Sugiyama, T., Kohara, H., Kondoh, G., Fujii, N., Kohno, K., et al. (2010). The essential functions of adipo-osteogenic progenitors as the hematopoietic stem and progenitor cell niche. *Immunity* 33, 387–399. doi: 10.1016/j.immuni.2010.08.017
- Onitsuka, I., Tanaka, M., and Miyajima, A. (2010). Characterization and functional analyses of hepatic mesothelial cells in mouse liver development. *Gastroenterology* 138, 1525–1535. doi: 10.1053/j.gastro.2009.12.059
- Ottersbach, K., and Dzierzak, E. (2005). The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. *Dev. Cell* 8, 377–387. doi: 10.1016/j.devcel.2005.02.001
- Palis, J., Chan, R. J., Koniski, A., Patel, R., Starr, M., and Yoder, M. C. (2001). Spatial and temporal emergence of high proliferative potential hematopoietic precursors during murine embryogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 98, 4528–4533. doi: 10.1073/pnas.071002398
- Palis, J., Robertson, S., Kennedy, M., Wall, C., and Keller, G. (1999). Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. *Development* 126, 5073–5084.
- Park, L. S., Friend, D. J., Schmierer, A. E., Dower, S. K., and Namen, A. E. (1990). Murine interleukin 7 (IL-7) receptor: characterization on an IL-7-dependent Cell Line. *J. Exper. Med.* 171, 1073–1089. doi: 10.1084/jem.171.4.1073
- Pei, W., Feyerabend, T. B., Rössler, J., Wang, X., Postrach, D., Busch, K., et al. (2017). Polylox barcoding reveals hematopoietic stem cell fates realized *in vivo*. *Nature* 548, 456–460. doi: 10.1038/nature23653
- Peschon, J. J., Morrissey, P. J., Grabstein, K. H., Ramsdell, F. J., Maraskovsky, E., Gliniak, B. C., et al. (1994). Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J. Exp. Med.* 180, 1955–1960. doi: 10.1084/jem.180.5.1955
- Petit-Cocault, L., Volle-Challier, C., Fleury, M., Peault, B., and Souyri, M. (2007). Dual role of Mpl receptor during the establishment of definitive hematopoiesis. *Development* 134, 3031–3040. doi: 10.1242/dev.001818
- Pinho, S., and Frenette, P. S. (2019). Hematopoietic stem cell activity and interactions with the niche. *Nat. Rev. Mol. Cell Biol.* 20, 303–320. doi: 10.1038/s41580-019-0103-9
- Pinho, S., Lacombe, J., Hanoun, M., Mizoguchi, T., Bruns, I., Kunisaki, Y., et al. (2013). PDGFR α and CD51 mark human Nestin+ sphere-forming mesenchymal stem cells capable of hematopoietic progenitor cell expansion. *J. Exper. Med.* 210, 1351–1367. doi: 10.1084/jem.20122252
- Ploemacher, R. E., and van Soest, P. L. (1977). Morphological investigation on phenylhydrazine-induced erythropoiesis in the adult mouse liver. *Cell Tissue Res.* 178, 435–461. doi: 10.1007/bf00219567
- Prior, N., Hindley, C. J., Rost, F., Esteban, E. M., Lau, W. W. Y., Göttgens, B., et al. (2018). Lgr5+ stem/progenitor cells reside at the apex of the embryonic hepatoblast pool. *bioRxiv* [Preprint]. doi: 10.1101/485870
- Qian, H., Buza-Vidas, N., Hyland, C. D., Jensen, C. T., Antonchuk, J., Månsson, R., et al. (2007). Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells. *Cell Stem Cell* 1, 671–684. doi: 10.1016/j.stem.2007.10.008
- Ramond, C., Berthault, C., Burlen-Defranoux, O., De Sousa, A. P., Guy-Grand, D., Vieira, P., et al. (2014). Two waves of distinct hematopoietic progenitor cells colonize the fetal thymus. *Nat. Immunol.* 15, 27–35. doi: 10.1038/ni.2782
- Ramsfjell, V., Borge, O. J., Veiby, O. P., Cardier, J., Murphy, M. J., Lyman, S. D., et al. (1996). Thrombopoietin, but not erythropoietin, directly stimulates multilineage growth of primitive murine bone marrow progenitor cells in synergy with early acting cytokines: distinct interactions with the ligands for c-kit and FLT3. *Blood* 88, 4481–4492.
- Rhodes, K. E., Gekas, C., Wang, Y., Lux, C. T., Francis, C. S., Chan, D. N., et al. (2008). The emergence of hematopoietic stem cells is initiated in the placental vasculature in the absence of circulation. *Cell Stem Cell* 2, 252–263. doi: 10.1016/j.stem.2008.01.001
- Rieger, M. A., Hoppe, P. S., Smejkal, B. M., Eitelhuber, A. C., and Schroeder, T. (2009). Hematopoietic cytokines can instruct lineage choice. *Science* 325, 217–218. doi: 10.1126/science.1171461
- Robin, C., Bollerot, K., Mendes, S., Haak, E., Crisan, M., Cerisoli, F., et al. (2009). Human placenta is a potent hematopoietic niche containing hematopoietic stem and progenitor cells throughout development. *Cell Stem Cell* 5, 385–395. doi: 10.1016/j.stem.2009.08.020
- Rodriguez-Fraticelli, A. E., Wolock, S. L., Weinreb, C. S., Panero, R., Patel, S. H., Jankovic, M., et al. (2018). Clonal analysis of lineage fate in native hematopoiesis. *Nature* 553, 212–216. doi: 10.1038/nature25168
- Roy, V., and Verfaillie, C. M. (1999). Expression and function of cell adhesion molecules on fetal liver, cord blood and bone marrow hematopoietic progenitors: implications for anatomical localization and developmental stage specific regulation of hematopoiesis. *Exp. Hematol.* 27, 302–312. doi: 10.1016/S0301-472X(98)00031-9
- Rubin, J. B., Shia, M. A., and Pilch, P. F. (1983). Stimulation of tyrosine-specific phosphorylation *in vitro* by insulin-Like growth factor I. *Nature* 305, 438–440. doi: 10.1038/305438a0
- Ruggeri, A., Labopin, M., Sormani, M. P., Sanz, G., Sanz, J., Volt, F., et al. (2014). Engraftment kinetics and graft failure after single umbilical cord blood transplantation using a myeloablative conditioning regimen. *Haematologica* 99, 1509–1515. doi: 10.3324/haematol.2014.109280
- Ryu, K. Y., Park, H., Rossi, D. J., Weissman, I. L., and Kopito, R. R. (2012). Perturbation of the hematopoietic system during embryonic liver development due to disruption of polyubiquitin gene Ubc in mice. *PLoS One* 7:32956. doi: 10.1371/journal.pone.0032956
- Sacchetti, B., Funari, A., Michienzi, S., Di Cesare, S., Piersanti, S., Saggio, I., et al. (2007). Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* 131, 324–336. doi: 10.1016/j.cell.2007.08.025
- Sauvageau, G., Iscove, N. N., and Humphries, R. K. (2004). *In vitro* and *in vivo* expansion of hematopoietic stem cells. *Oncogene* 23, 7223–7232. doi: 10.1038/sj.onc.1207942
- Sawyer, S. T., Krantz, S. B., and Luna, J. (1987). Identification of the receptor for erythropoietin by cross-linking to Friend virus-infected erythroid cells. *Proc. Natl. Acad. Sci. U.S.A.* 84, 3690–3694. doi: 10.1073/pnas.84.11.3690
- Schofield, R. (1978). The relationship between the spleen colony-forming cell and the hematopoietic stem cell. *Blood Cells* 4, 7–25.
- Schumann, K., Lämmermann, T., Brückner, M., Legler, D. F., Polleux, J., Spatz, J. P., et al. (2010). Immobilized chemokine fields and soluble chemokine gradients cooperatively shape migration patterns of dendritic cells. *Immunity* 32, 703–713. doi: 10.1016/j.immuni.2010.04.017
- Schuster, J. A., Stupnikov, M. R., Ma, G., Liao, W., Lai, R., Ma, Y., et al. (2012). Expansion of hematopoietic stem cells for transplantation: current perspectives. *Exper. Hematol. Oncol.* 1:12. doi: 10.1186/2162-3619-1-12
- Semenza, G. L., Koury, S. T., Neffelt, M. K., Gearhart, J. D., and Antonarakis, S. E. (1991). Cell-type-specific and hypoxia-inducible expression of the human erythropoietin gene in transgenic mice. *Proc. Natl. Acad. Sci. U.S.A.* 88, 8725–8729. doi: 10.1073/pnas.88.19.8725
- Severn, C. B. (1971). A morphological study of the development of the human liver. I. Development of the hepatic diverticulum. *Am. J. Anat.* 131, 133–158. doi: 10.1002/aja.1001310202
- Shiojiri, N., and Sugiyama, Y. (2004). Immunolocalization of extracellular matrix components and integrins during mouse liver development. *Hepatology* 40, 346–355. doi: 10.1002/hep.20303
- Sigurdsson, V., Takei, H., Soboleva, S., Radulovic, V., Galeev, R., and Siva, K. (2016). Bile acids protect expanding hematopoietic stem cells from unfolded protein stress in fetal liver short article bile acids protect expanding hematopoietic stem cells from unfolded protein stress in fetal liver. *Stem Cell* 18, 522–532. doi: 10.1016/j.stem.2016.01.002
- Soares-da-Silva, F., Burlen-Defranoux, O., Elsaid, R., Iturri, L., Freyer, L., Sismeiro, O., et al. (2020). Yolk sac erythromyeloid progenitors sustain erythropoiesis throughout embryonic life. *bioRxiv* [Preprint]. doi: 10.1101/2020.02.27.968230
- Solar, G. P., Kerr, W. G., Zeigler, F. C., Hess, D., Donahue, C., Sauvage, F. J., et al. (1998). Role of c-mpl in early hematopoiesis. *Blood* 92, 4–10.
- Stier, S., Ko, Y., Forkert, R., Lutz, C., Neuhaus, T., Grunewald, E., et al. (2005). Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. *J. Exper. Med.* 201, 1781–1791. doi: 10.1084/jem.20041992
- Sudo, T., Ito, M., Ogawa, Y., Iizuka, M., Kodama, H., Kunisada, T., et al. (1989). Interleukin 7 production and function in stromal cell-dependent B cell development. *J. Exp. Med.* 170, 333–338. doi: 10.1084/jem.170.1.333

- Sugiyama, D., Kulkeaw, K., and Mizuochi, C. (2013). TGF-beta-1 up-regulates extra-cellular matrix production in mouse hepatoblasts. *Mech. Dev.* 130, 195–206. doi: 10.1016/j.mod.2012.09.003
- Sugiyama, D., Kulkeaw, K., Mizuochi, C., Horio, Y., and Okayama, S. (2011). Hepatoblasts comprise a niche for fetal liver erythropoiesis through cytokine production. *Biochem. Biophys. Res. Commun.* 410, 301–306. doi: 10.1016/j.bbrc.2011.05.137
- Sugiyama, T., Kohara, H., Noda, M., and Nagasawa, T. (2006). Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* 25, 977–988. doi: 10.1016/j.immuni.2006.10.016
- Sugiyama, Y., Koike, T., and Shiojiri, N. (2010a). Developmental changes of cell adhesion molecule expression in the fetal mouse liver. *Anat. Rec.* 293, 1698–1710. doi: 10.1002/ar.21204
- Sugiyama, Y., Takabe, Y., Nakakura, T., Tanaka, S., Koike, T., and Shiojiri, N. (2010b). Sinusoid development and morphogenesis may be stimulated by VEGF-Flk-1 signaling during fetal mouse liver development. *Dev. Dyn.* 239, 386–397. doi: 10.1002/dvdy.22162
- Sun, J., Ramos, A., Chapman, B., Johnnidis, J. B., Le, L., Ho, Y.-J., et al. (2014). Clonal dynamics of native haematopoiesis. *Nature* 514, 322–327. doi: 10.1038/nature13824
- Suzuki, A., Zheng, Y. W., Kondo, R., Kusakabe, M., Takada, Y., Fukao, K., et al. (2000). Flow-cytometric separation and enrichment of hepatic progenitor cells in the developing mouse liver. *Hepatology* 32, 1230–1239. doi: 10.1053/jhep.2000.20349
- Swartley, O. M., Foley, J. F., Livingston, D. P., Cullen, J. M., and Elmore, S. A. (2016). Histology atlas of the developing mouse hepatobiliary hemolymphatic vascular system with emphasis on embryonic days 11.5–18.5 and early postnatal development. *Toxicol. Pathol.* 44, 705–725. doi: 10.1177/0192623316630836
- Tajer, P., Pike-Overzet, K., Arias, S., Havenga, M., and Staal, F. (2019). Ex vivo expansion of hematopoietic stem cells for therapeutic purposes: lessons from development and the niche. *Cells* 8:169. doi: 10.3390/cells8020169
- Takabe, Y., Yagi, S., Koike, T., and Shiojiri, N. (2012). Immunomagnetic exclusion of E-cadherin-positive hepatoblasts in fetal mouse liver cell cultures impairs morphogenesis and gene expression of sinusoidal endothelial cells. *J. Anat.* 221, 229–239. doi: 10.1111/j.1469-7580.2012.01532.x
- Takashima, Y., Terada, M., Kawabata, M., and Suzuki, A. (2015). Dynamic three-dimensional morphogenesis of intrahepatic bile ducts in mouse liver development. *Hepatology* 61, 1003–1011. doi: 10.1002/hep.27436
- Takebe, T., Enomura, M., Yoshizawa, E., Kimura, M., Koike, H., Ueno, Y., et al. (2015). Vascularized and complex organ buds from diverse tissues via mesenchymal cell-driven condensation. *Cell Stem Cell* 16, 556–565. doi: 10.1016/j.stem.2015.03.004
- Tamplin, O. J., Durand, E. M., Carr, L. A., Childs, S. J., Hagedorn, E. J., Li, P., et al. (2015). Hematopoietic stem cell arrival triggers dynamic remodeling of the perivascular niche. *Cell* 160, 241–252. doi: 10.1016/j.cell.2014.12.032
- Tan, K. S., Kulkeaw, K., Nakanishi, Y., and Sugiyama, D. (2017). Expression of cytokine and extracellular matrix mRNAs in fetal hepatic stellate cells. *Genes Cells* 22, 836–844. doi: 10.1111/gtc.12517
- Tanaka, M., Okabe, M., Suzuki, K., Kamiya, Y., Tsukahara, Y., Saito, S., et al. (2009). Mouse hepatoblasts at distinct developmental stages are characterized by expression of EpCAM and DLK1: drastic change of EpCAM expression during liver development. *Mech. Dev.* 126, 665–676. doi: 10.1016/j.mod.2009.06.939
- Tanaka, Y., Kurosawa, S., Tajima, K., Tanaka, T., Ito, R., Inoue, Y., et al. (2016). Analysis of non-relapse mortality and causes of death over 15 years following allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant.* 51, 553–559. doi: 10.1038/bmt.2015.330
- Taniguchi, H., Toyoshima, T., Fukao, K., and Nakauchi, H. (1996). Presence of hematopoietic stem cells in the adult liver. *Nat. Med.* 2, 198–203. doi: 10.1038/nm0296-198
- Tanimizu, N., Kaneko, K., Itoh, T., Ichinohe, N., Ishii, M., Mizuguchi, T., et al. (2016). Intrahepatic bile ducts are developed through formation of homogeneous continuous luminal network and its dynamic rearrangement in mice. *Hepatology* 64, 175–188. doi: 10.1002/hep.28521
- Tanimizu, N., Miyajima, A., and Mostov, K. E. (2009). Liver progenitor cells fold up a cell monolayer into a double-layered structure during tubular morphogenesis. *Mol. Biol. Cell* 20, 2486–2494. doi: 10.1091/mbc.e08-02-0177
- Tanimizu, N., Nishikawa, M., Saito, H., Tsujimura, T., and Miyajima, A. (2003). Isolation of hepatoblasts based on the expression of Dlk/Pref-1. *J. Cell Sci.* 116, 1775–1786. doi: 10.1242/jcs.00388
- Tanimizu, N., Saito, H., Mostov, K., and Miyajima, A. (2004). Long-term culture of hepatic progenitors derived from mouse Dlk+ hepatoblasts. *J. Cell Sci.* 117, 6425–6434. doi: 10.1242/jcs.01572
- Taoudi, S., Gonneau, C., Moore, K., Sheridan, J. M., Blackburn, C. C., Taylor, E., et al. (2008). Extensive hematopoietic stem cell generation in the AGM region via maturation of VE-Cadherin+CD45+ pre-definitive HSCs. *Cell Stem Cell* 3, 99–108. doi: 10.1016/j.stem.2008.06.004
- Taoudi, S., Morrison, A. M., Inoue, H., Gribi, R., Ure, J., and Medvinsky, A. (2005). Progressive divergence of definitive haematopoietic stem cells from the endothelial compartment does not depend on contact with the foetal liver. *Development* 132, 4179–4191. doi: 10.1242/dev.01974
- Tavian, M., Coulombel, L., Luton, D., Clemente, H. S., Dieterlen-Lièvre, F., and Péault, B. (1996). Aorta-associated CD34+ hematopoietic cells in the early human embryo. *Blood* 87, 67–72.
- Tavian, M., Hallais, M. F., and Péault, B. (1999). Emergence of intraembryonic hematopoietic precursors in the pre-liver human embryo. *Development* 126, 793–803.
- Terada, T., and Nakanuma, Y. (1995). Detection of apoptosis and expression of apoptosis-related proteins during human intrahepatic bile duct development. *Am. J. Pathol.* 146, 67–74.
- Tremblay, K. D., and Zaret, K. S. (2005). Distinct populations of endoderm cells converge to generate the embryonic liver bud and ventral foregut tissues. *Dev. Biol.* 280, 87–99. doi: 10.1016/j.ydbio.2005.01.003
- Tsai, S., Emerson, S. G., Sieff, C. A., and Nathan, D. G. (1986). Isolation of a human stromal cell strain secreting hemopoietic growth factors. *J. Cell. Physiol.* 127, 137–145. doi: 10.1002/jcp.1041270117
- Tsuneto, M., Tokoyoda, K., Kajikhina, E., Hauser, A. E., Hara, T., Tani-ichi, S., et al. (2013). B-cell progenitors and precursors change their microenvironment in fetal liver during early development. *Stem Cells* 31, 2800–2812. doi: 10.1002/stem.1421
- Van Eyken, P., Sciort, R., Callea, F., Steen, K., Van Der Moerman, P., and Desmet, V. J. (1988). The development of the intrahepatic bile ducts in man: a keratin-immunohistochemical study. *Hepatology* 8, 1586–1595. doi: 10.1002/hep.1840080619
- Vestentoft, P. S., Jørgensen, P., Hopkinson, B. M., Vainer, B., Møllgård, K., Quistorff, B., et al. (2011). Three-dimensional reconstructions of intrahepatic bile duct tubulogenesis in human liver. *BMC Dev. Biol.* 11:56. doi: 10.1186/1471-213X-11-56
- Vigon, I., Mornon, J. P., Cocault, L., Mitjavila, M. T., Tambourin, P., Gisselbrecht, S., et al. (1992). Molecular cloning and characterization of MPL, the human homolog of the v-mpl oncogene: identification of a member of the hematopoietic growth factor receptor superfamily. *Proc. Natl. Acad. Sci. U.S.A.* 89, 5640–5644. doi: 10.1073/pnas.89.12.5640
- Wake, K. (1971). “Sternzellen” in the liver: perisinusoidal cells with special reference to storage of vitamin A. *Am. J. Anat.* 132, 429–461. doi: 10.1002/aja.1001320404
- Walasek, M. A., van Os, R., and de Haan, G. (2012). Hematopoietic stem cell expansion: challenges and opportunities. *Ann. N. Y. Acad. Sci.* 1266, 138–150. doi: 10.1111/j.1749-6632.2012.06549.x
- Wang, H. U., Chen, Z. F., and Anderson, D. J. (1998). Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell* 93, 741–753. doi: 10.1016/S0092-8674(00)81436-1
- Watanabe, H., Miyaji, C., Seki, S., and Abo, T. (1996). c-kit+ stem cells and thymocyte precursors in the livers of adult mice. *J. Exp. Med.* 184, 687–693. doi: 10.1084/jem.184.2.687
- Wei, Q., and Frenette, P. S. (2018). Niches for hematopoietic stem cells and their progeny. *Immunity* 48, 632–648. doi: 10.1016/j.immuni.2018.03.024
- Weidemann, A., and Johnson, R. S. (2009). Nonrenal regulation of EPO synthesis. *Kidney Int.* 75, 682–688. doi: 10.1038/ki.2008.687

- Wilkinson, A. C., Igarashi, K. J., and Nakauchi, H. (2020). Haematopoietic stem cell self-renewal in vivo and ex vivo. *Nat. Rev. Genet.* 27, 273–278. doi: 10.1038/s41576-020-0241-0
- Wilkinson, A. C., Ishida, R., Kikuchi, M., Sudo, K., Morita, M., Crisostomo, R. V., et al. (2019). Long-term ex vivo haematopoietic-stem-cell expansion allows nonconditioned transplantation. *Nature* 571, 117–121. doi: 10.1038/s41586-019-1244-x
- Wilson, A., Laurenti, E., Oser, G., van der Wath, R. C., Blanco-Bose, W., Jaworski, M., et al. (2008). Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. *Cell* 135, 1118–1129. doi: 10.1016/j.cell.2008.10.048
- Wilson, W., Groat, C. S., and Leduc, E. H. (2006). Histogenesis of the liver. *Ann. N. Y. Acad. Sci.* 111, 8–24. doi: 10.1111/j.1749-6632.1963.tb36945.x
- Wineman, J., Moore, K., Lemischka, I., and Müller-Sieburg, C. (1996). Functional heterogeneity of the hematopoietic microenvironment: rare stromal elements maintain long-term repopulating stem cells. *Blood* 87, 4082–4090.
- Wittig, O., Paez-Cortez, J., and Cardier, J. E. (2010). Liver sinusoidal endothelial cells promote b lymphopoiesis from primitive hematopoietic cells. *Stem Cells Dev.* 19, 341–350. doi: 10.1089/scd.2009.0300
- Wohrer, S., Knapp, D. J. H. F., Copley, M. R., Benz, C., Kent, D. G., Rowe, K., et al. (2014). Distinct stromal cell factor combinations can separately control hematopoietic stem cell survival, proliferation, and self-renewal. *Cell Rep.* 7, 1956–1967. doi: 10.1016/j.celrep.2014.05.014
- Wolber, F. M., Leonard, E., Michael, S., Orschell-Traycoff, C. M., Yoder, M. C., and Srour, E. F. (2002). Roles of spleen and liver in development of the murine hematopoietic system. *Exp. Hematol.* 30, 1010–1019. doi: 10.1016/S0301-472X(02)00881-0
- Yang, L., Li, L. C., Lamaoqiezhong, L., Wang, X., Wang, W. H., Wang, Y. C., et al. (2019). The contributions of mesoderm-derived cells in liver development. *Semin. Cell Dev. Biol.* 92, 63–76. doi: 10.1016/j.semdb.2018.09.003
- Yang, L., Wang, W. H., Qiu, W. L., Guo, Z., Bi, E., and Xu, C. R. (2017). A single-cell transcriptomic analysis reveals precise pathways and regulatory mechanisms underlying hepatoblast differentiation. *Hepatology* 66, 1387–1401. doi: 10.1002/hep.29353
- Yarden, Y., Kuang, W. J., Yang-Feng, T., Coussens, L., Munemitsu, S., Dull, T. J., et al. (1987). Human proto-oncogene c-kit: a new cell surface receptor tyrosine kinase for an unidentified ligand. *EMBO J.* 6, 3341–3351. doi: 10.1002/j.1460-2075.1987.tb02655.x
- Yu, V. W. C., and Scadden, D. T. (2016). Hematopoietic stem cell and its bone marrow niche. *Curr. Top. Dev. Biol.* 118, 21–44. doi: 10.1016/bs.ctdb.2016.01.009
- Zhang, C. C., Kaba, M., Ge, G., Xie, K., Tong, W., Hug, C., et al. (2006). Angiopoietin-like proteins stimulate ex vivo expansion of hematopoietic stem cells. *Nat. Med.* 12, 240–245. doi: 10.1038/nm1342
- Zhang, C. C., and Lodish, H. F. (2004). Insulin-like growth factor 2 expressed in a novel fetal liver cell population is a growth factor for hematopoietic stem cells. *Blood* 103, 2513–2521. doi: 10.1182/blood-2003-08-2955
- Zheng, J., Huynh, H., Umikawa, M., Silvany, R., and Zhang, C. C. (2011). Angiopoietin-like protein 3 supports the activity of hematopoietic stem cells in the bone marrow niche. *Blood* 117, 470–479. doi: 10.1182/blood-2010-06-291716
- Zheng, J., Umikawa, M., Cui, C., Li, J., Chen, X., Zhang, C. C., et al. (2012). Inhibitory receptors bind ANGPTLs and support blood stem cells and leukaemia development. *Nature* 485, 656–660. doi: 10.1038/nature11095
- Zhou, B. O., Yue, R., Murphy, M. M., Peyer, J. G., and Morrison, S. J. (2014). Leptin-receptor-expressing mesenchymal stromal cells represent the main source of bone formed by adult bone marrow. *Cell Stem Cell* 15, 154–168. doi: 10.1016/j.stem.2014.06.008
- Zorn, A. M. (2008). Liver development. *Stembook* 2, 1–26. doi: 10.3824/stembook.1.25.1

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Soares-da-Silva, Peixoto, Cumano and Pinto-do-Ó. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

ANNEX II

Article accepted for publication, currently under editorial production:

Hematopoiesis: A Layered Organization Across Chordate Species

Ramy Elsaid^{1-3†}, Francisca Soares-da-Silva^{1-6†}, Marcia Peixoto¹⁻⁵, Dali Amiri¹⁻³, Nathan Mackowski¹⁻³, Pablo Pereira¹⁻³, Antonio Bandeira^{1-3*}, Ana Cumano^{1-3*}

¹Unit of Lymphocytes and Immunity, Immunology Department, Institut Pasteur, Paris, France. ²INSERM U1223, Paris, France. ³Université de Paris, Célule Pasteur, Paris, France. ⁴I3S – Instituto de Investigação e Inovação em Saúde & INEB – Instituto Nacional de Engenharia Biomédica, Universidade do Porto, Porto, Portugal. ⁵Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal. ⁶Graduate Program in Areas of Basic and Applied Biology, Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal

*Corresponding authors. Ana Cumano ana.cumano@pasteur.fr; Antonio Bandeira antonio.bandeira-ferreira@pasteur.fr

†These authors have contributed equally to this work.

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 $\gamma\delta$ 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.

1. 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 versus 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.

2. 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 (Hartenstein, 2006 Muñoz-Chápuli et al., 2005). However, there are several exceptions to this rule. For example, annelids have a closed 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 *Scf*, 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.

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, $\alpha\beta$ -T, and $\gamma\delta$ -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 1**).

Similar to other vertebrates, zebrafish hematopoiesis develops through successive waves emerging at different locations (**Table 2, Figure 2**). Contrary to mammals, birds, and other teleosts, zebrafish primitive hematopoiesis initiates intra-embryonically, 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 towards 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 IL1 β -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 towards 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 $\alpha\beta$ 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 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-versus-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 congeneric 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 (Yvernogeu 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 (Tober et al., 2007 Xu M et al., 2001) 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 (Bertrand et al., 2010 Frame et al., 2016 Goldie et al., 2008 Kasaai et al., 2017 McGrath et al., 2015). 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 (Ajami et al., 2007 Jenkins et al., 2011 Merad et al., 2002).

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 (Boisset et al., 2010 de Bruijn et al., 2000 Yzaguirre and Speck, 2016 Zovein et al., 2008). 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 (Ganuza et al., 2017 Godin et al., 1999). 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) ($V\gamma 5^+$) (Ikuta et al., 1990), lymphoid tissue inducer (LTi) cells (Eberl et al., 2004), and a subset of IL-17-producer $\gamma\delta$ T cells ($V\gamma 6^+$) (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 (Ivanovs et al., 2011 Tavian et al., 1996). 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 towards 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).

3. 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 Elsaid et al., 2019 Herzenberg, 2000 Montecino-Rodriguez and Dorshkind, 2012). 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 $\alpha\beta$ 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 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 Dunon et al., 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 $V\gamma 5^+$ T cells and LTi cells. A second wave of more immature hematopoietic progenitors colonize the thymus after E15 and ensure thymopoiesis thereafter (**Figure 5**) (Elsaid et al., 2020 Ramond et al., 2014).

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**). $V\gamma 5^+$ 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 $\gamma\delta$ 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 $\alpha\beta$ 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 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 $\gamma\delta$ 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 $\gamma\delta$ 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 $V\gamma5^+$ and $V\gamma6^+$ 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 $V\gamma5$ -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 $V\gamma5^+$ T cells in the skin, wound healing is delayed (Jameson et al., 2002). Similarly, in the absence of $V\gamma6^+$ 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, $\gamma\delta$ T cells might only be crucial in neonates and juveniles before conventional T cell immunity is mature. Both $V\gamma5^+$ 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. LT α i are no longer found in the adult thymus, suggesting that, together with $\gamma\delta$ 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.

4. 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

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

Funding

This work was financed by the PPU Pasteur Ph.D. program. This work was financed by the Institut Pasteur, INSERM, Pasteur-Weizmann Foundation and ANR (grant Twothyme and Epi-Dev) through grants to AC; by REVIVE (Investissement d'Avenir; ANR-10-LABX-73) through grants to AC and RE; by FCT through the grants "PD/BD/114128/2015" and "POCI-0100145-FEDER-01638" to FS-d-S; and the grant "SFRH/BD/143605/2019" to MP.

Acknowledgments

We are grateful to L. du Pasquier for the advice and critical reading of the manuscript. We are also grateful to Eliza Gomez-Perdiguero (Institut Pasteur) and the members of her laboratory for stimulating discussions.

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

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, TCR β and TCR δ chains, or from V and J elements in Ig light chains, TCR α and TCR γ 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 towards 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.

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	<i>In vitro</i> 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 <i>In vivo</i> 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 <i>In vivo</i> assays are time-consuming	Characterization of hematopoietic populations by surface markers expression—flow cytometry (Notta et al., 2011 Notta et al., 2016) Evaluation of differentiation potential—<i>in vitro</i> colony-forming assays (Notta et al., 2016) <i>In vivo</i> functional repopulation assays in immunodeficient mice—xenograft models (Beer and Eaves, 2015 Kamel-Reid et al., 1989) 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	Lack of antibodies for phenotypic characterization Lack of knock-in technologies Need to establish breeding standards;	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)

	Easy high-resolution optical imaging in live animals Large-scale genetic and chemical screens Several transgenic lines available (reviewed in (Stachura and Traver, 2016))	Inbreed and outbreed depression	Hematopoietic cell transplantation (Hess et al, 2013 Traver et al., 2003 Traver et al., 2004) 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) <i>In vivo</i> lineage tracing (e.g., He et al., 2020 Jin et al., 2007 Murayama et al., 2006)
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 <i>in vitro</i> cultures Gene manipulation technologies difficult to perform	Quail–chicken and chicken–chicken chimeras (Le Douarin, 1969) Corio-allantoid transplantation (Yvernogeu 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 (Bertrand et al., 2005 Palis et al., 1999)
			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 (Yvernogeu 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

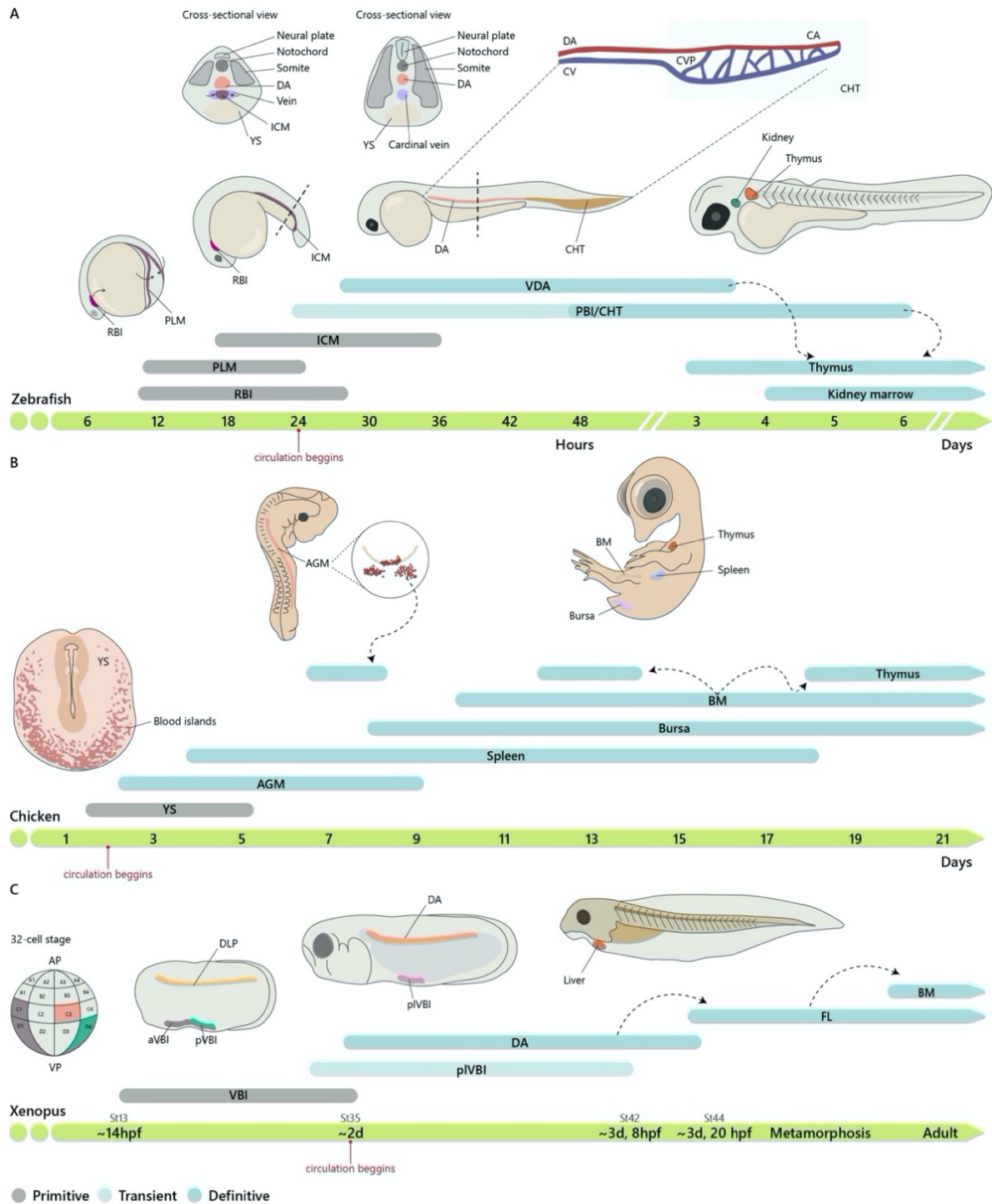


FIGURE 2 | Embryonic origin of the hematopoietic system. (A) Timeline of hematopoietic development in zebrafish. In zebrafish, primitive hematopoiesis occurs in the RBI and ICM region generating primitive macrophages and erythrocytes, respectively. The CHT, consisting of the CA (the continuation of the 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; pIVBI, posterior-lateral VBI; RBI, rostral blood islands; YS, yolk sac; VBI, ventral blood island.

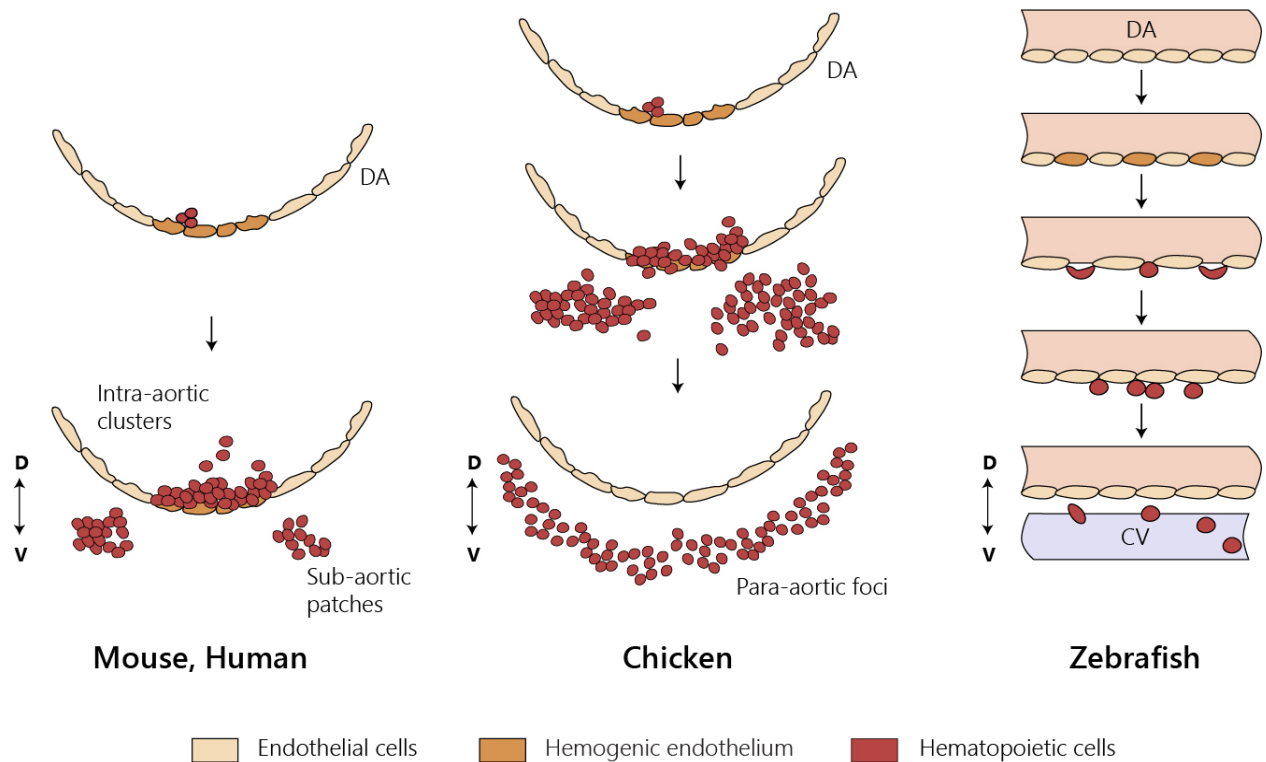


FIGURE 3 | HSC emergence at the dorsal aorta in mice, chicken, and zebrafish. Intra-embryonic hematopoietic clusters (IAHCs) containing newly formed HSCs in birds and in mammals. IAHC originate from PAF in chicken and sub-aortic patches in mice. Contrary to mammals and birds, IAHCs are not observed in 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.

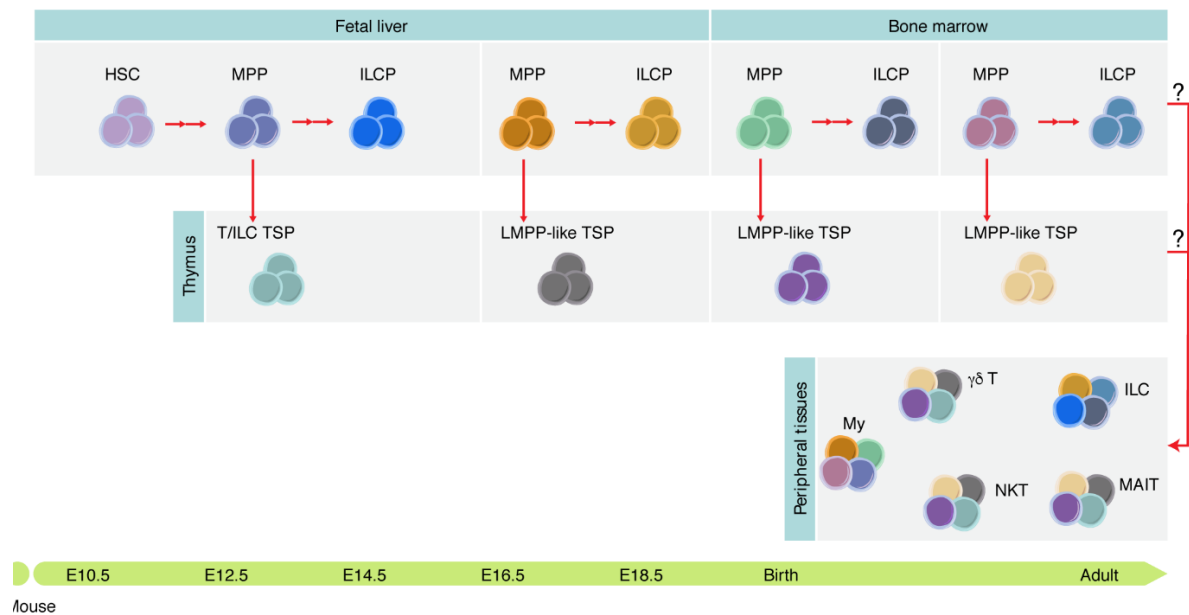


FIGURE 4 | Tissue-resident immune cells are mosaic of cells derived at different developmental stages. Different hematopoietic progenitors are generated during development, some of which contribute to the adult tissue-resident immune cell compartment diversity. It is still unknown which functions these 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.

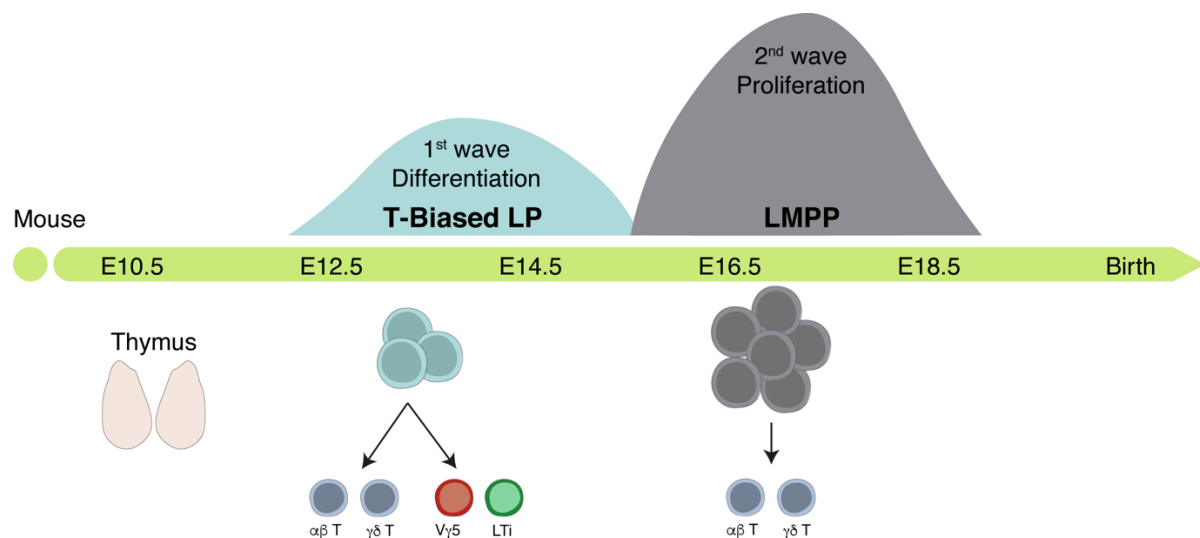


FIGURE 5 | The fetal thymus is colonized by two successive waves of hematopoietic progenitors. Scheme of the timeline of thymus colonization in the mouse. Area of the Gaussian curves is proportional to the number of progenitors in each wave. The first wave generates few cells whereas the second expands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

REFERENCES

- Choi K, Kennedy M, Kazarov A, Papadimitriou JC, Keller G (1998) A common precursor for hematopoietic and endothelial cells. *Development* 125:725-732
- Moore MA, Owen JJ (1967) Chromosome marker studies in the irradiated chick embryo. *Nature* 215:1081-108210.1038/2151081a0
- Moore MA, Metcalf D (1970) Ontogeny of the haemopoietic system: yolk sac origin of in vivo and in vitro colony forming cells in the developing mouse embryo. *Br J Haematol* 18:279-29610.1111/j.1365-2141.1970.tb01443.x
- Dieterlen-Lievre F (1975) On the origin of haemopoietic stem cells in the avian embryo: an experimental approach. *J Embryol Exp Morphol* 33:607-619
- Lassila O, Eskola J, Toivanen P, Martin C, Dieterlen-Lievre F (1978) The origin of lymphoid stem cells studied in chick yolk sac-embryo chimaeras. *Nature* 272:353-35410.1038/272353a0
- Cumano A, Dieterlen-Lievre F, Godin I (1996) Lymphoid potential, probed before circulation in mouse, is restricted to caudal intraembryonic splanchnopleura. *Cell* 86:907-916
- Godin I, Cumano A (2002) The hare and the tortoise: an embryonic haematopoietic race *Nature Reviews Immunology*
- Nat Rev Immunol* 2:593-60410.1038/nri857
- Frame JM, Fegan KH, Conway SJ, McGrath KE, Palis J (2016) Definitive Hematopoiesis in the Yolk Sac Emerges from Wnt-Responsive Hemogenic Endothelium Independently of Circulation and Arterial Identity. *Stem Cells* 34:431-44410.1002/stem.2213
- Ema H, Nakauchi H (2000) Expansion of hematopoietic stem cells in the developing liver of a mouse embryo. *Blood* 95:2284-2288
- Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, Mehler MF, Conway SJ, Ng LG, Stanley ER, Samokhvalov IM, Merad M (2010) Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science* 330:841-84510.1126/science.1194637
- Gomez Perdiguero E, Klapproth K, Schulz C, Busch K, Azzoni E, Crozet L, Garner H, Trouillet C, de Bruijn MF, Geissmann F, Rodewald HR (2015) Tissue-resident macrophages originate from yolk-sac-derived erythromyeloid progenitors. *Nature* 518:547-55110.1038/nature13989
- Evans CJ, Hartenstein V, Banerjee U (2003) Thicker than blood: conserved mechanisms in *Drosophila* and vertebrate hematopoiesis. *Dev Cell* 5:673-69010.1016/s1534-5807(03)00335-6
- Hartenstein V (2006) Blood cells and blood cell development in the animal kingdom. *Annu Rev Cell Dev Biol* 22:677-71210.1146/annurev.cellbio.22.010605.093317
- Muñoz-Chápuli R, Carmona R, Guadix JA, Macías D, Pérez-Pomares JM (2005) The origin of the endothelial cells: an evo-devo approach for the invertebrate/vertebrate transition of the circulatory system. *Evol Dev* 7:351-35810.1111/j.1525-142X.2005.05040.x
- Delsuc F, Brinkmann H, Chourrout D, Philippe H (2006) Tunicates and not cephalochordates are the closest living relatives of vertebrates. *Nature* 439:965-96810.1038/nature04336
- Pascual-Anaya J, Albuixech-Crespo B, Somorjai IM, Carmona R, Oisi Y, Alvarez S, Kuratani S, Muñoz-Chápuli R, García-Fernández J (2013) The evolutionary origins of chordate hematopoiesis and vertebrate endothelia. *Dev Biol* 375:182-19210.1016/j.ydbio.2012.11.015
- Rosental B, Raveh T, Voskoboynik A, Weissman IL (2020) Evolutionary perspective on the hematopoietic system through a colonial chordate: allogeneic immunity and hematopoiesis. *Curr Opin Immunol* 62:91-9810.1016/j.coi.2019.12.006
- Rosental B, Kowarsky M, Seita J, Corey DM, Ishizuka KJ, Palmeri KJ, Chen SY, Sinha R, Okamoto J, Mantalas G, Manni L, Raveh T, Clarke DN, Tsai JM, Newman AM, Neff NF, Nolan GP, Quake SR, Weissman IL, Voskoboynik A (2018) Complex mammalian-like haematopoietic system found in a colonial chordate. *Nature* 564:425-42910.1038/s41586-018-0783-x
- Pancer Z, Amemiya CT, Ehrhardt GR, Ceitlin J, Gartland GL, Cooper MD (2004) Somatic diversification of variable lymphocyte receptors in the agnathan sea lamprey. *Nature* 430:174-18010.1038/nature02740
- Bajoghli B, Guo P, Aghaallaei N, Hirano M, Strohmeier C, McCurley N, Bockman DE, Schorpp M, Cooper MD, Boehm T (2011) A thymus candidate in lampreys. *Nature* 470:90-9410.1038/nature09655
- Boehm T, Hirano M, Holland SJ, Das S, Schorpp M, Cooper MD (2018) Evolution of Alternative Adaptive Immune Systems in Vertebrates. *Annu Rev Immunol* 36:19-4210.1146/annurev-immunol-042617-053028
- Amemiya CT, Saha NR, Zapata A (2007) Evolution and development of immunological structures in the lamprey. *Curr Opin Immunol* 19:535-54110.1016/j.coi.2007.08.003
- Manca R, Glomski CA, Pica A (2018) Evolutionary intraembryonic origin of vertebrate hematopoietic stem cells in the elasmobranch spleen. *Eur J Histochem* 6210.4081/ejh.2018.2987
- Manca R, Glomski C, Pica A (2019) Hematopoietic stem cells debut in embryonic lymphomyeloid tissues of elasmobranchs. *Eur J Histochem* 6310.4081/ejh.2019.3060
- Detrich HW, Kieran MW, Chan FY, Barone LM, Yee K, Rundstadler JA, Pratt S, Ransom D, Zon LI (1995) Intraembryonic hematopoietic cell migration during vertebrate development. *Proc Natl Acad Sci U S A* 92:10713-1071710.1073/pnas.92.23.10713
- Thompson MA, Ransom DG, Pratt SJ, MacLennan H, Kieran MW, Detrich HW, Vail B, Huber TL, Paw B, Brownlie AJ, Oates AC, Fritz A, Gates MA, Amores A, Bahary N, Talbot WS, Her H, Beier DR,

- Postlethwait JH, Zon LI (1998) The cloche and spadetail genes differentially affect hematopoiesis and vasculogenesis. *Dev Biol* 197:248-26910.1006/dbio.1998.8887
- Herbomel P, Thisse B, Thisse C (1999) Ontogeny and behaviour of early macrophages in the zebrafish embryo. *Development* 126:3735-3745
- Kalev-Zylinska ML, Horsfield JA, Flores MV, Postlethwait JH, Vitas MR, Baas AM, Crosier PS, Crosier KE (2002) Runx1 is required for zebrafish blood and vessel development and expression of a human RUNX1-CBF2T1 transgene advances a model for studies of leukemogenesis. *Development* 129:2015-2030
- Bertrand JY, Chi NC, Santoso B, Teng S, Stainier DY, Traver D (2010) Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature* 464:108-11110.1038/nature08738
- Kissa K, Herbomel P (2010) Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *Nature* 464:112-11510.1038/nature08761
- Kobayashi I, Kobayashi-Sun J, Kim AD, Pouget C, Fujita N, Suda T, Traver D (2014) Jam1a-Jam2a interactions regulate haematopoietic stem cell fate through Notch signalling. *Nature* 512:319-32310.1038/nature13623
- Wilkinson RN, Pouget C, Gering M, Russell AJ, Davies SG, Kimelman D, Patient R (2009) Hedgehog and Bmp polarize hematopoietic stem cell emergence in the zebrafish dorsal aorta. *Dev Cell* 16:909-91610.1016/j.devcel.2009.04.014
- Espín-Palazón R, Stachura DL, Campbell CA, García-Moreno D, Del Cid N, Kim AD, Candel S, Meseguer J, Mulero V, Traver D (2014) Proinflammatory signaling regulates hematopoietic stem cell emergence. *Cell* 159:1070-108510.1016/j.cell.2014.10.031
- Frame JM, Kubaczka C, Long TL, Esain V, Soto RA, Hachimi M, Jing R, Shwartz A, Goessling W, Daley GQ, North TE (2020) Metabolic Regulation of Inflammasome Activity Controls Embryonic Hematopoietic Stem and Progenitor Cell Production. *Dev Cell* 55:133-149.e610.1016/j.devcel.2020.07.015
- Gomes AM, Kurochkin I, Chang B, Daniel M, Law K, Satija N, Lachmann A, Wang Z, Ferreira L, Ma'ayan A, Chen BK, Papatsenko D, Lemischka IR, Moore KA, Pereira CF (2018) Cooperative Transcription Factor Induction Mediates Hemogenic Reprogramming. *Cell Rep* 25:2821-2835.e710.1016/j.celrep.2018.11.032
- Murayama E, Kissa K, Zapata A, Mordet E, Briolat V, Lin HF, Handin RI, Herbomel P (2006) Tracing hematopoietic precursor migration to successive hematopoietic organs during zebrafish development. *Immunity* 25:963-97510.1016/j.immuni.2006.10.015
- Bertrand JY, Kim AD, Violette EP, Stachura DL, Cisson JL, Traver D (2007) Definitive hematopoiesis initiates through a committed erythromyeloid progenitor in the zebrafish embryo. *Development* 134:4147-415610.1242/dev.012385
- Tamplin OJ, Durand EM, Carr LA, Childs SJ, Hagedorn EJ, Li P, Yzaguirre AD, Speck NA, Zon LI (2015) Hematopoietic stem cell arrival triggers dynamic remodeling of the perivascular niche. *Cell* 160:241-25210.1016/j.cell.2014.12.032
- Blaser BW, Moore JL, Hagedorn EJ, Li B, Riquelme R, Lichtig A, Yang S, Zhou Y, Tamplin OJ, Binder V, Zon LI (2017) CXCR1 remodels the vascular niche to promote hematopoietic stem and progenitor cell engraftment. *J Exp Med* 214:1011-102710.1084/jem.20161616
- Xu J, Zhu L, He S, Wu Y, Jin W, Yu T, Qu JY, Wen Z (2015) Temporal-Spatial Resolution Fate Mapping Reveals Distinct Origins for Embryonic and Adult Microglia in Zebrafish. *Dev Cell* 34:632-64110.1016/j.devcel.2015.08.018
- Tian Y, Xu J, Feng S, He S, Zhao S, Zhu L, Jin W, Dai Y, Luo L, Qu JY, Wen Z (2017) The first wave of T lymphopoiesis in zebrafish arises from aorta endothelium independent of hematopoietic stem cells. *J Exp Med* 214:3347-336010.1084/jem.20170488
- Lopez D, Lin L, Monaghan JR, Cogle CR, Bova FJ, Maden M, Scott EW (2014) Mapping hematopoiesis in a fully regenerative vertebrate: the axolotl. *Blood* 124:1232-124110.1182/blood-2013-09-526970
- Golub R, André S, Hassanin A, Affaticati P, Larijani M, Fella JS (2004) Early expression of two TdT isoforms in the hematopoietic system of the Mexican axolotl. Implications for the evolutionary origin of the N-nucleotide addition. *Immunogenetics* 56:204-21310.1007/s00251-004-0681-2
- Ciau-Uitz A, Patient R (2016) The embryonic origins and genetic programming of emerging haematopoietic stem cells. *FEBS Lett* 590:4002-401510.1002/1873-3468.12363
- Du Pasquier L, Schwager J, Flajnik MF (1989) The immune system of *Xenopus*. *Annu Rev Immunol* 7:251-27510.1146/annurev.iy.07.040189.001343
- Chen XD, Turpen JB (1995) Intraembryonic origin of hepatic hematopoiesis in *Xenopus laevis*. *J Immunol* 154:2557-2567
- Ciau-Uitz A, Walmsley M, Patient R (2000) Distinct origins of adult and embryonic blood in *Xenopus*. *Cell* 102:787-79610.1016/s0092-8674(00)00067-2
- Smith PB, Flajnik MF, Turpen JB (1989) Experimental analysis of ventral blood island hematopoiesis in *Xenopus* embryonic chimeras. *Dev Biol* 131:302-31210.1016/s0012-1606(89)80003-x
- Turpen JB, Kelley CM, Mead PE, Zon LI (1997) Bipotential primitive-definitive hematopoietic progenitors in the vertebrate embryo. *Immunity* 7:325-33410.1016/s1074-7613(00)80354-4
- Ciau-Uitz A, Liu F, Patient R (2010) Genetic control of hematopoietic development in *Xenopus* and zebrafish. *Int J Dev Biol* 54:1139-114910.1387/ijdb.093055ac
- Imai Y, Ishida K, Nemoto M, Nakata K, Kato T, Maéno M (2017) Multiple origins of embryonic and tadpole myeloid cells in *Xenopus laevis*. *Cell Tissue Res* 369:341-35210.1007/s00441-017-2601-4
- Yaparla A, Reeves P, Grayfer L (2019) Myelopoiesis of the Amphibian *Xenopus laevis* Is Segregated to the Bone Marrow, Away From Their Hematopoietic Peripheral Liver. *Front Immunol* 10:301510.3389/fimmu.2019.03015

- Greenhalgh P, Olesen CE, Steiner LA (1993) Characterization and expression of recombination activating genes (RAG-1 and RAG-2) in *Xenopus laevis*. *J Immunol* 151:3100-3110
- Le Douarin N (1969) [Details of the interphase nucleus in Japanese quail (*Coturnix coturnix japonica*)]. *Bull Biol Fr Belg* 103:435-452
- Le Douarin NM, Dieterlen-Lièvre F (2013) How studies on the avian embryo have opened new avenues in the understanding of development: a view about the neural and hematopoietic systems. *Dev Growth Differ* 55:1-1410.1111/dgd.12015
- Manaia A, Lemarchandel V, Klaine M, Max-Audit I, Romeo P, Dieterlen-Lièvre F, Godin I (2000) Lmo2 and GATA-3 associated expression in intraembryonic hemogenic sites. *Development* 127:643-653
- Dunon D, Allioli N, Vainio O, Ody C, Imhof BA (1998) Renewal of thymocyte progenitors and emigration of thymocytes during avian development. *Dev Comp Immunol* 22:279-28710.1016/s0145-305x(98)00010-x
- Yvernogeu L, Robin C (2017) Restricted intra-embryonic origin of bona fide hematopoietic stem cells in the chicken. *Development* 144:2352-236310.1242/dev.151613
- Cuadros MA, Martin C, Coltey P, Almendros A, Navascués J (1993) First appearance, distribution, and origin of macrophages in the early development of the avian central nervous system. *J Comp Neurol* 330:113-12910.1002/cne.903300110
- Pardanaud L, Luton D, Prigent M, Bourcheix LM, Catala M, Dieterlen-Lièvre F (1996) Two distinct endothelial lineages in ontogeny, one of them related to hemopoiesis. *Development* 122:1363-1371
- Pouget C, Gautier R, Teillet MA, Jaffredo T (2006) Somite-derived cells replace ventral aortic hemangioblasts and provide aortic smooth muscle cells of the trunk. *Development* 133:1013-102210.1242/dev.02269
- Richard C, Drevon C, Canto PY, Villain G, Bollérot K, Lempereur A, Teillet MA, Vincent C, Rosselló Castillo C, Torres M, Piwarzyk E, Speck NA, Souyri M, Jaffredo T (2013) Endothelio-mesenchymal interaction controls runx1 expression and modulates the notch pathway to initiate aortic hematopoiesis. *Dev Cell* 24:600-61110.1016/j.devcel.2013.02.011
- Jaffredo T, Gautier R, Brajeul V, Dieterlen-Lièvre F (2000) Tracing the progeny of the aortic hemangioblast in the avian embryo. *Dev Biol* 224:204-21410.1006/dbio.2000.9799
- Tober J, Koniski A, McGrath KE, Vemishetti R, Emerson R, de Mesy-Bentley KK, Waugh R, Palis J (2007) The megakaryocyte lineage originates from hemangioblast precursors and is an integral component both of primitive and of definitive hematopoiesis. *Blood* 109:1433-144110.1182/blood-2006-06-031898
- Xu M J, Matsuoka S, Yang FC, Ebihara Y, Manabe A, Tanaka R, Eguchi M, Asano S, Nakahata T, Tsuji K (2001) Evidence for the presence of murine primitive megakaryocytopoiesis in the early yolk sac. *Blood* 97:2016-202210.1182/blood.v97.7.2016
- Palis J, Robertson S, Kennedy M, Wall C, Keller G (1999) Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. *Development* 126:5073-5084
- Wong PM, Chung SW, Reicheld SM, Chui DH (1986) Hemoglobin switching during murine embryonic development: evidence for two populations of embryonic erythropoietic progenitor cells. *Blood* 67:716-721
- Kingsley PD, Malik J, Fantauzzo KA, Palis J (2004) Yolk sac-derived primitive erythroblasts enucleate during mammalian embryogenesis. *Blood* 104:19-2510.1182/blood-2003-12-4162
- Bertrand JY, Jalil A, Klaine M, Jung S, Cumano A, Godin I (2005) Three pathways to mature macrophages in the early mouse yolk sac. *Blood* 106:3004-301110.1182/blood-2005-02-0461
- Goldie LC, Lucitti JL, Dickinson ME, Hirschi KK (2008) Cell signaling directing the formation and function of hemogenic endothelium during murine embryogenesis. *Blood* 112:3194-320410.1182/blood-2008-02-139055
- Kasaai B, Caolo V, Peacock HM, Lehoux S, Gomez-Perdiguero E, Luttun A, Jones EA (2017) Erythro-myeloid progenitors can differentiate from endothelial cells and modulate embryonic vascular remodeling. *Sci Rep* 7:4381710.1038/srep43817
- McGrath KE, Frame JM, Fegan KH, Bowen JR, Conway SJ, Catherman SC, Kingsley PD, Koniski AD, Palis J (2015) Distinct Sources of Hematopoietic Progenitors Emerge before HSCs and Provide Functional Blood Cells in the Mammalian Embryo. *Cell Rep* 11:1892-190410.1016/j.celrep.2015.05.036
- Hoeffel G, Chen J, Lavin Y, Low D, Almeida FF, See P, Beaudin AE, Lum J, Low I, Forsberg EC, Poidinger M, Zolezzi F, Larbi A, Ng LG, Chan JK, Greter M, Becher B, Samokhvalov IM, Merad M, Ginhoux F (2015) C-Myb(+) erythro-myeloid progenitor-derived fetal monocytes give rise to adult tissue-resident macrophages. *Immunity* 42:665-67810.1016/j.immuni.2015.03.011
- Ajami B, Bennett JL, Krieger C, Tetzlaff W, Rossi FM (2007) Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nat Neurosci* 10:1538-154310.1038/nn2014
- Jenkins SJ, Ruckerl D, Cook PC, Jones LH, Finkelman FD, van Rooijen N, MacDonald AS, Allen JE (2011) Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. *Science* 332:1284-128810.1126/science.1204351
- Merad M, Manz MG, Karsunky H, Wagers A, Peters W, Charo I, Weissman IL, Cyster JG, Engleman EG (2002) Langerhans cells renew in the skin throughout life under steady-state conditions. *Nat Immunol* 3:1135-114110.1038/ni852
- Boisset JC, van Cappellen W, Andrieu-Soler C, Galjart N, Dzierzak E, Robin C (2010) In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. *Nature* 464:116-12010.1038/nature08764
- Zovein AC, Hofmann JJ, Lynch M, French WJ, Turlo KA, Yang Y, Becker MS, Zanetta L, Dejana E, Gasson JC, Tallquist MD, Iruela-Arispe ML (2008) Fate tracing reveals the endothelial origin of hematopoietic stem cells. *Cell Stem Cell* 3:625-63610.1016/j.stem.2008.09.018

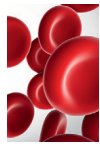
- de Bruijn MF, Speck NA, Peeters MC, Dzierzak E (2000) Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. *EMBO J* 19:2465-2474.10.1093/emboj/19.11.2465
- Yzaguirre AD, Speck NA (2016) Extravascular endothelial and hematopoietic islands form through multiple pathways in midgestation mouse embryos. *Dev Biol* 415:111-121.10.1016/j.ydbio.2016.04.004
- Medvinsky A, Dzierzak E (1996) Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell* 86:897-906
- Kumano K, Chiba S, Kunisato A, Sata M, Saito T, Nakagami-Yamaguchi E, Yamaguchi T, Masuda S, Shimizu K, Takahashi T, Ogawa S, Hamada Y, Hirai H (2003) Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells. *Immunity* 18:699-711.10.1016/s1074-7613(03)00117-1
- Mariani SA, Li Z, Rice S, Krieg C, Frangkogianni S, Robinson M, Vink CS, Pollard JW, Dzierzak E (2019) Pro-inflammatory Aorta-Associated Macrophages Are Involved in Embryonic Development of Hematopoietic Stem Cells. *Immunity* 50:1439-1452.e510.1016/j.immuni.2019.05.003
- Godin I, Garcia-Porrero JA, Dieterlen-Lièvre F, Cumano A (1999) Stem cell emergence and hemopoietic activity are incompatible in mouse intraembryonic sites. *J Exp Med* 190:43-52
- Ganuza M, Hall T, Finkelstein D, Chabot A, Kang G, McKinney-Freeman S (2017) Lifelong haematopoiesis is established by hundreds of precursors throughout mammalian ontogeny. *Nat Cell Biol* 19:1153-1163.10.1038/ncb3607
- Gekas C, Dieterlen-Lièvre F, Orkin SH, Mikkola HK (2005) The placenta is a niche for hematopoietic stem cells. *Dev Cell* 8:365-375.10.1016/j.devcel.2004.12.016
- Ottersbach K, Dzierzak E (2005) The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. *Dev Cell* 8:377-387.10.1016/j.devcel.2005.02.001
- Ikuta K, Kina T, MacNeil I, Uchida N, Peault B, Chien YH, Weissman IL (1990) A developmental switch in thymic lymphocyte maturation potential occurs at the level of hematopoietic stem cells. *Cell* 62:863-874
- Eberl G, Marmon S, Sunshine MJ, Rennert PD, Choi Y, Littman DR (2004) An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells. *Nat Immunol* 5:64-73.10.1038/ni1022
- Haas JD, Ravens S, Düber S, Sandrock I, Oberdörfer L, Kashani E, Chennupati V, Föhse L, Naumann R, Weiss S, Krueger A, Förster R, Prinz I (2012) Development of interleukin-17-producing $\gamma\delta$ T cells is restricted to a functional embryonic wave. *Immunity* 37:48-59.10.1016/j.immuni.2012.06.003
- Tavian M, Hallais MF, Péault B (1999) Emergence of intraembryonic hematopoietic precursors in the pre-liver human embryo. *Development* 126:793-803
- Ivanovs A, Rybtsov S, Ng ES, Stanley EG, Elefanti AG, Medvinsky A (2017) Human haematopoietic stem cell development: from the embryo to the dish. *Development* 144:2323-2337.10.1242/dev.134866
- Tavian M, Coulombel L, Luton D, Clemente HS, Dieterlen-Lièvre F, Péault B (1996) Aorta-associated CD34+ hematopoietic cells in the early human embryo. *Blood* 87:67-72
- Ivanovs A, Rybtsov S, Welch L, Anderson RA, Turner ML, Medvinsky A (2011) Highly potent human hematopoietic stem cells first emerge in the intraembryonic aorta-gonad-mesonephros region. *J Exp Med* 208:2417-2427.10.1084/jem.20111688
- Ueno H, Weissman IL (2006) Clonal analysis of mouse development reveals a polyclonal origin for yolk sac blood islands. *Dev Cell* 11:519-533.10.1016/j.devcel.2006.08.001
- Rhodes KE, Gekas C, Wang Y, Lux CT, Francis CS, Chan DN, Conway S, Orkin SH, Yoder MC, Mikkola HK (2008) The emergence of hematopoietic stem cells is initiated in the placental vasculature in the absence of circulation. *Cell Stem Cell* 2:252-263.10.1016/j.stem.2008.01.001
- Herzenberg LA (1989) Toward a layered immune system. *Cell* 59:953-954.10.1016/0092-8674(89)90748-4
- Herzenberg LA (2000) B-1 cells: the lineage question revisited. *Immunol Rev* 175:9-22
- Montecino-Rodriguez E, Dorshkind K (2012) B-1 B cell development in the fetus and adult. *Immunity* 36:13-21.10.1016/j.immuni.2011.11.017
- Elsaid R, Yang J, Cumano A (2019) The influence of space and time on the establishment of B cell identity. *Biomed J* 42:209-217.10.1016/j.bj.2019.07.008
- Beaudin AE, Boyer SW, Perez-Cunningham J, Hernandez GE, Derderian SC, Jujavarapu C, Aaserude E, MacKenzie T, Forsberg EC (2016) A Transient Developmental Hematopoietic Stem Cell Gives Rise to Innate-like B and T Cells. *Cell Stem Cell* 19:768-783.10.1016/j.stem.2016.08.013
- Kristiansen TA, Jaensson Gyllenbäck E, Zriwil A, Björklund T, Daniel JA, Sitnicka E, Soneji S, Bryder D, Yuan J (2016) Cellular Barcoding Links B-1a B Cell Potential to a Fetal Hematopoietic Stem Cell State at the Single-Cell Level. *Immunity* 45:346-357.10.1016/j.immuni.2016.07.014
- Graf R, Seagal J, Otipoby KL, Lam KP, Ayoub S, Zhang B, Sander S, Chu VT, Rajewsky K (2019) BCR-dependent lineage plasticity in mature B cells. *Science* 363:748-753.10.1126/science.aau8475
- Vanhee S, Åkerstrand H, Kristiansen TA, Datta S, Montano G, Vergani S, Lang S, Ungerback J, Doyle A, Olsson K, Beneventi G, Jensen CT, Bellodi C, Soneji S, Sigvardsson M, Gyllenbäck EJ, Yuan J (2019) Lin28b controls a neonatal to adult switch in B cell positive selection. *Sci Immunol* 4:1126/sciimmunol.aax4453
- Wong JB, Hewitt SL, Heltemes-Harris LM, Mandal M, Johnson K, Rajewsky K, Koralov SB, Clark MR, Farrar MA, Skok JA (2019) B-1a cells acquire their unique characteristics by bypassing the pre-BCR selection stage. *Nat Commun* 10:4768.10.1038/s41467-019-12824-z
- Abramson J, Anderson G (2017) Thymic Epithelial Cells. *Annu Rev Immunol* 35:85-118.10.1146/annurev-immunol-051116-052320
- Hosokawa H, Rothenberg EV (2020) How transcription factors drive choice of the T cell fate. *Nat Rev Immunol* 10.1038/s41577-020-00426-6

- Elsaid R, Meunier S, Burlen-Defranoux O, Soares-da-Silva F, Perchet T, Iturri L, Freyer L, Vieira P, Pereira P, Golub R, Bandeira A, Gomez Perdiguero E, Cumano A (2020) A wave of bipotent T/ILC-restricted progenitors shapes the embryonic thymus microenvironment in a time-dependent manner. *Blood* 10.1182/blood.2020006779
- Hess I, Boehm T (2012) Intravital imaging of thymopoiesis reveals dynamic lympho-epithelial interactions. *Immunity* 36:298-309 10.1016/j.immuni.2011.12.016
- Lee A, Hsu E (1994) Isolation and characterization of the *Xenopus* terminal deoxynucleotidyl transferase. *J Immunol* 152:4500-4507
- Turpen JB, Smith PB (1989) Precursor immigration and thymocyte succession during larval development and metamorphosis in *Xenopus*. *J Immunol* 142:41-47
- Reynaud CA, Anquez V, Grimal H, Weill JC (1987) A hyperconversion mechanism generates the chicken light chain preimmune repertoire. *Cell* 48:379-388 10.1016/0092-8674(87)90189-9
- Coltey M, Jotereau FV, Le Douarin NM (1987) Evidence for a cyclic renewal of lymphocyte precursor cells in the embryonic chick thymus. *Cell Differ* 22:71-82
- Dunon D, Allio N, Vainio O, Ody C, Imhof BA (1999) Quantification of T-cell progenitors during ontogeny: thymus colonization depends on blood delivery of progenitors. *Blood* 93:2234-2243
- Ramond C, Berthault C, Burlen-Defranoux O, de Sousa AP, Guy-Grand D, Vieira P, Pereira P, Cumano A (2014) Two waves of distinct hematopoietic progenitor cells colonize the fetal thymus. *Nat Immunol* 15:27-35 10.1038/ni.2782
- Takahama Y, Ohigashi I, Baik S, Anderson G (2017) Generation of diversity in thymic epithelial cells. *Nat Rev Immunol* 17:295-305 10.1038/nri.2017.12
- Roberts NA, White AJ, Jenkinson WE, Turchinovich G, Nakamura K, Withers DR, McConnell FM, Desanti GE, Benezech C, Parnell SM, Cunningham AF, Paolino M, Penninger JM, Simon AK, Nitta T, Ohigashi I, Takahama Y, Caamano JH, Hayday AC, Lane PJ, Jenkinson EJ, Anderson G (2012) Rank signaling links the development of invariant $\gamma\delta$ T cell progenitors and Aire(+) medullary epithelium. *Immunity* 36:427-437 10.1016/j.immuni.2012.01.016
- Berthault C, Ramond C, Burlen-Defranoux O, Soubigou G, Chea S, Golub R, Pereira P, Vieira P, Cumano A (2017) Asynchronous lineage priming determines commitment to T cell and B cell lineages in fetal liver. *Nat Immunol* 18:1139-1149 10.1038/ni.3820
- Böiers C, Carrelha J, Lutteropp M, Luc S, Green JC, Azzoni E, Woll PS, Mead AJ, Hultquist A, Swiers G, Perdiguero EG, Macaulay IC, Melchiori L, Luis TC, Kharazi S, Bouriez-Jones T, Deng Q, Pontén A, Atkinson D, Jensen CT, Sitnicka E, Geissmann F, Godin I, Sandberg R, de Bruijn MF, Jacobsen SE (2013) Lymphomyeloid contribution of an immune-restricted progenitor emerging prior to definitive hematopoietic stem cells. *Cell Stem Cell* 13:535-548 10.1016/j.stem.2013.08.012
- Luis TC, Luc S, Mizukami T, Boukarabila H, Thongjuea S, Woll PS, Azzoni E, Giustacchini A, Lutteropp M, Bouriez-Jones T, Vaidya H, Mead AJ, Atkinson D, Böiers C, Carrelha J, Macaulay IC, Patient R, Geissmann F, Nerlov C, Sandberg R, de Bruijn MF, Blackburn CC, Godin I, Jacobsen SE (2016) Initial seeding of the embryonic thymus by immune-restricted lympho-myeloid progenitors. *Nat Immunol* 17:1424-1435 10.1038/ni.3576
- Zhu Q, Gao P, Tober J, Bennett L, Chen C, Uzun Y, Li Y, Howell ED, Mumau M, Yu W, He B, Speck NA, Tan K (2020) Developmental trajectory of prehematopoietic stem cell formation from endothelium. *Blood* 136:845-856 10.1182/blood.2020004801
- Tieppo P, Papadopoulou M, Gatti D, McGovern N, Chan JKY, Gosselin F, Goetzeluk G, Weening K, Ma L, Dauby N, Cogan A, Donner C, Ginhoux F, Vandekerckhove B, Vermijlen D (2020) The human fetal thymus generates invariant effector $\gamma\delta$ T cells. *J Exp Med* 217:10.1084/jem.20190580
- Mold JE, Venkatasubrahmanyam S, Burt TD, Michaëlsson J, Rivera JM, Galkina SA, Weinberg K, Stoddart CA, McCune JM (2010) Fetal and adult hematopoietic stem cells give rise to distinct T cell lineages in humans. *Science* 330:1695-1699 10.1126/science.1196509
- Ng MSF, Roth TL, Mendoza VF, Marson A, Burt TD (2019) Helios enhances the preferential differentiation of human fetal CD4⁺ naïve T cells into regulatory T cells. *Sci Immunol* 4:10.1126/sciimmunol.aav5947
- Jameson J, Ugarte K, Chen N, Yachi P, Fuchs E, Boismenu R, Havran WL (2002) A role for skin $\gamma\delta$ T cells in wound repair. *Science* 296:747-749 10.1126/science.1069639
- Guo XJ, Dash P, Crawford JC, Allen EK, Zamora AE, Boyd DF, Duan S, Bajracharya R, Awad WA, Apiwattanakul N, Vogel P, Kanneganti TD, Thomas PG (2018) Lung $\gamma\delta$ T Cells Mediate Protective Responses during Neonatal Influenza Infection that Are Associated with Type 2 Immunity. *Immunity* 49:531-544. e610.1016/j.immuni.2018.07.011
- Guerau-de-Arellano M, Martinic M, Benoist C, Mathis D (2009) Neonatal tolerance revisited: a perinatal window for Aire control of autoimmunity. *J Exp Med* 206:1245-1252 10.1084/jem.20090300
- Sun J, Ramos A, Chapman B, Johnnidis JB, Le L, Ho YJ, Klein A, Hofmann O, Camargo FD (2014) Clonal dynamics of native haematopoiesis. *Nature* 514:322-327 10.1038/nature13824
- Notta F, Doulatov S, Laurenti E, Poepl A, Jurisica I, Dick JE (2011) Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science* 333:218-221 10.1126/science.1201219
- Notta F, Zandi S, Takayama N, Dobson S, Gan OI, Wilson G, Kaufmann KB, McLeod J, Laurenti E, Dunant CF, McPherson JD, Stein LD, Dror Y, Dick JE (2016) Distinct routes of lineage development reshape the human blood hierarchy across ontogeny. *Science* 351:aab2116 10.1126/science.aab2116

- Kamel-Reid S, Letarte M, Sirard C, Doedens M, Grunberger T, Fulop G, Freedman MH, Phillips RA, Dick JE (1989) A model of human acute lymphoblastic leukemia in immune-deficient SCID mice. *Science* 246:1597-160010.1126/science.2595371
- Beer PA, Eaves CJ (2015) Modeling Normal and Disordered Human Hematopoiesis. *Trends Cancer* 1:199-21010.1016/j.trecan.2015.09.002
- Scala S, Aiuti A (2019) In vivo dynamics of human hematopoietic stem cells: novel concepts and future directions. *Blood Adv* 3:1916-192410.1182/bloodadvances.2019000039
- Stachura DL, Traver D (2016) Cellular dissection of zebrafish hematopoiesis. *Methods Cell Biol* 133:11-5310.1016/bs.mcb.2016.03.022
- Sertori R, Trengove M, Basheer F, Ward AC, Liongue C (2016) Genome editing in zebrafish: a practical overview. *Brief Funct Genomics* 15:322-33010.1093/bfpg/elv051
- Traver D, Paw BH, Poss KD, Penberthy WT, Lin S, Zon LI (2003) Transplantation and in vivo imaging of multilineage engraftment in zebrafish bloodless mutants. *Nat Immunol* 4:1238-124610.1038/ni1007
- Hess I, Iwanami N, Schorpp M, Boehm T (2013) Zebrafish model for allogeneic hematopoietic cell transplantation not requiring preconditioning. *Proc Natl Acad Sci U S A* 110:4327-433210.1073/pnas.1219847110
- Traver D, Winzeler A, Stern HM, Mayhall EA, Langenau DM, Kutok JL, Look AT, Zon LI (2004) Effects of lethal irradiation in zebrafish and rescue by hematopoietic cell transplantation. *Blood* 104:1298-130510.1182/blood-2004-01-0100
- Stachura DL, Svoboda O, Lau RP, Balla KM, Zon LI, Bartunek P, Traver D (2011) Clonal analysis of hematopoietic progenitor cells in the zebrafish. *Blood* 118:1274-128210.1182/blood-2011-01-331199
- Wolf A, Aggio J, Campbell C, Wright F, Marquez G, Traver D, Stachura DL (2017) Zebrafish Caudal Haematopoietic Embryonic Stromal Tissue (CHEST) Cells Support Haematopoiesis. *Sci Rep* 7:4464410.1038/srep44644
- Demy DL, Ranta Z, Giorgi JM, Gonzalez M, Herbolme P, Kissa K (2013) Generating parabiotic zebrafish embryos for cell migration and homing studies. *Nat Methods* 10:256-25810.1038/nmeth.2362
- Hess I, Boehm T (2016) Stable multilineage xenogeneic replacement of definitive hematopoiesis in adult zebrafish. *Sci Rep* 6:1963410.1038/srep19634
- Parada-Kusz M, Penaranda C, Hagedorn EJ, Clatworthy A, Nair AV, Henninger JE, Ernst C, Li B, Riquelme R, Jijon H, Villablanca EJ, Zon LI, Hung D, Allende ML (2018) Generation of mouse-zebrafish hematopoietic tissue chimeric embryos for hematopoiesis and host-pathogen interaction studies. *Dis Model Mech* 1110.1242/dmm.034876
- He S, Tian Y, Feng S, Wu Y, Shen X, Chen K, He Y, Sun Q, Li X, Xu J, Wen Z, Qu JY (2020) In vivo single-cell lineage tracing in zebrafish using high-resolution infrared laser-mediated gene induction microscopy. *Elife* 910.7554/eLife.52024
- Jin H, Xu J, Wen Z (2007) Migratory path of definitive hematopoietic stem/progenitor cells during zebrafish development. *Blood* 109:5208-521410.1182/blood-2007-01-069005
- Migliaccio G, Migliaccio AR, Petti S, Mavilio F, Russo G, Lazzaro D, Testa U, Marinucci M, Peschle C (1986) Human embryonic hemopoiesis. Kinetics of progenitors and precursors underlying the yolk sac----liver transition. *J Clin Invest* 78:51-6010.1172/JCI112572

ANNEX III

Author's proofs version:



HEMATOPOIESIS AND STEM CELLS

A wave of bipotent T/ILC-restricted progenitors shapes the embryonic thymus microenvironment in a time-dependent manner

Ramy Elsaid,^{1,3} Sylvain Meunier,^{1,3} Odile Buren-Defranoux,^{1,3} Francisca Soares-da-Silva,^{1,2,4,5} Thibaut Perchet,^{1,3} Lorea Iturri,^{6,7} Laina Freyer,⁶ Paulo Vieira,^{1,3} Pablo Pereira,^{1,3} Rachel Golub,^{1,3} Antonio Bandeira,^{1,3} Elisa Gomez Perdiguerro,⁶ and Ana Cumano^{1,3}

¹Unit of Lymphopoiesis, Immunology Department, Institut Pasteur, Paris, France; ²Unité 1223, INSERM, Paris, France; ³Université Paris Diderot, Sorbonne Paris Cité, Paris, France; ⁴Instituto de Investigação e Inovação em Saúde (I3S) and ⁵Instituto Nacional de Engenharia Biomédica (INEB), Universidade do Porto, Porto, Portugal; ⁶Macrophages and Endothelial Cells Group, Development and Stem Cell Biology Department, Institut Pasteur, Paris, France; and ⁷Cellule Pasteur, University Pierre et Marie Curie (UPMC), Paris, France

Q:1,2,3,4
Q:5,6,7,8

KEY POINTS

- Embryonic-restricted bipotent T/innate lymphoid cell progenitors shape the thymic architecture by controlling mTEC maturation.
- These cells do not originate from YS but rather from intraembryonic-derived progenitors.

Q:9,10

During embryonic development, multiple waves of hematopoietic progenitors with distinct lineage potential are differentially regulated in time and space. Two different waves of thymic progenitors colonize the fetal thymus where they contribute to thymic organogenesis and homeostasis. The origin, the lineage differentiation potential of the first wave, and their relative contribution in shaping the thymus architecture, remained, however, unclear. Here, we show that the first wave of thymic progenitors comprises a unique population of bipotent cells generating a lymphoid tissue inducer (LTi), in addition to invariant $V\gamma 5^+$ T cells. Transcriptional analysis revealed that innate lymphoid gene signatures and, more precisely, the LTi-associated transcripts were expressed in the first, but not in the second, wave of thymic progenitors. Depletion of early thymic progenitors in a temporally controlled manner showed that the progeny of the first wave is indispensable for the differentiation of autoimmune regulator-expressing medullary thymic epithelial cells (mTECs). We further show that these progenitors are of strict hematopoietic stem cell origin, despite the overlap between lymphopoiesis initiation and the transient expression of lymphoid-associated transcripts in yolk sac (YS) erythromyeloid-restricted precursors. Our work highlights the relevance of the developmental timing on the emergence of different lymphoid subsets, required for the establishment of a functionally diverse immune system. (*Blood*. 2020;00(00):1-13)

Introduction

Q:11,12,13 The embryonic thymus is colonized by 2 waves of distinct hematopoietic progenitors, named early thymic progenitors (ETPs).¹ The first wave of ETP follows a unique developmental program as they share phenotype, transcriptional signature, and restricted T-cell differentiation potential with HSA^{hi} $\alpha 4\beta 7^+$ fetal liver (FL) common lymphoid progenitors (CLPs).^{2,3} After E15.5, the thymus is colonized by a second wave of ETPs that share phenotype, transcriptional signature, and differentiation potential with lymphomyeloid-primed progenitors (LMPPs).²⁻⁵

Three lymphoid subsets are exclusively developed during embryonic life: the skin-resident $V\gamma 5^+$ T cells, the lungs and the genital urinary tract resident $V\gamma 6^+$, and a subset of group 3 innate lymphoid cells (ILCs; ILC3) known as lymphoid tissue inducers (LTi's).¹ Both $V\gamma 5^+$ T cells and LTi's are required for *Aire*⁺ medullary thymic epithelial cell (mTEC) development before birth.⁶⁻⁹ *Aire* expression is concomitant with that of CD80,

a marker of mTEC maturation, and neonatal *Aire* expression is necessary for inducing lifelong T-cell tolerance throughout life.^{8,10,11} LTi's are also required for secondary lymphoid tissue organization^{12,13} whereas $V\gamma 6^+$ T cells contribute to tissue remodeling.¹⁴

The majority of embryonic thymic ILCs are LTi's gradually lost after birth and only a small population of ILC2 is maintained throughout life.¹⁵ The first ETPs generate $V\gamma 5^+$ T cells,^{3,16} however, it is important to determine their origin, whether they generate other embryonic lymphoid cells such as thymic LTi's, and what is their contribution to shaping the thymic architecture in a time-dependent manner.⁸

Most hematopoietic cells are constantly differentiating from hematopoietic stem cells (HSCs) although few lineages are HSC independent and exclusively produced during embryonic development.^{1,17} For example, yolk sac (YS) erythromyeloid

progenitors (EMPs) contribute to tissue-resident macrophages that persist throughout life.¹⁸ This layered organization of the hematopoietic system raises the possibility that, similar to tissue macrophages, the first innate lymphocytes could also be YS derived. Accordingly, cells identified as lymphomyeloid-restricted progenitors (LMPs) were identified in E9.5 YS before HSC activity is detected.¹⁸ They were shown to express lymphoid-associated genes (*Il7r*, *Rag2*, *Rag1*) and were proposed as the origin of the first wave of ETP.¹⁹ Independent reports have also converged to support the notion that $V\gamma 5^+$ and the $V\gamma 6^+$ T cells might originate from YS progenitors, independent of HSCs.^{20,21} In contrast, $V\gamma 5^+$ and B1a B cells were shown to be preferentially derived from a particular FL-transient HSC-like subset marked by a history of *Flk2* expression.²²

In this report, we studied the developmental origin, differentiation potential, and the temporal requirement of the first ETPs for shaping the thymus microenvironment. We showed, in single-cell differentiation assays, that the first ETPs comprise bipotent T/LTi progenitors that generate the thymic LTi's. Colonization of *Rag2*^{-/-} γc ^{-/-} thymic lobes with the first, but not the second, wave ETPs reconstituted the LTi compartment. Depletion of the first wave of lymphoid progenitors resulted in lower numbers of $V\gamma 5^+$ T cells, LTi's, and severely reduced numbers of CD80⁺ mTECs after birth. Therefore, the major role of the first ETPs appear to be providing cells that drive thymic organogenesis and tissue homeostasis.^{1,10,23}

We further demonstrated, using an inducible lineage tracer model, that the first ETPs originate from HSCs. YS-derived progenitors, although showing a history of expression of lymphoid-associated genes such as *Il7r*, *Rag2* and *Rag1*, failed to generate a lymphoid progeny in vivo or in vitro. This transient expression, also found in other FL myeloid progenitors, is restricted to embryonic hematopoiesis and uncoupled from differentiation potential. Altogether, our data highlight the impact of embryonic developmental timing on lymphocyte production, gene expression, organogenesis, and heterogeneity of the immune system.

Methods

Detailed methods are available in supplemental Methods (available on the Blood Web site).

Animals

Mice were bred in house under specific pathogen-free conditions. *Il7r*^{Cre},²⁴ *Rag2*^{-/-} γc ^{-/-} CD45.1,²⁵ *ROSA26*^{Tomato},²⁶ and *ROSA26*^{YFP} mice were on C57BL/6 background, *Csf1*^{MerCreMer} mice²⁷ were on FVB background. All animal manipulations were performed according to the French Agriculture ministry and to the European Parliament Directive 2010/63/EU.

Fate mapping of *Il7r*⁺ hematopoietic progenitors

Il7r^{Cre} female mice were crossed to homozygous *ROSA26*^{YFP}. Indicated embryonic tissues and adult tissues were analyzed by flow cytometry.

Lineage tracing of *Csf1r*⁺ progenitors

Csf1^{MerCreMer} were crossed with either *ROSA26*^{YFP} or *ROSA26*^{Tomato} reporter mice. A single dose of 75 $\mu g/g$ (body weight) of 4-Hydroxytamoxifen (4OHT; Sigma) injected either at

embryonic day 8.5 (E8.5) or E9.5 induced reporter recombination and was supplemented with 37.5 $\mu g/g$ (body weight) progesterone (Sigma) (to counteract the mixed estrogen agonist effects of 4OHT).²⁸ To induce recombination at E10.5, female mice received a single dose of 1.2 mg of 4OHT and 0.6 mg of progesterone.

In vivo anti-IL7R α (A7R34) antibody injections

Interleukin 7 receptor α (IL-7R α) blockade was performed by 3 successive IV injections of 1 mg of anti-IL7R α antibody (A7R34)²⁹ (a gift from Shin Nishikawa) in pregnant female mice at E10.5, E12.5, and E14.5 of gestation.

Results

E13 ETP retain T/LTi lineage potential in vitro and generate LTi cells in a fetal thymic microenvironment

The analysis of the transcriptional profiles of the first (E13) and the second (E18) ETP waves identified the overexpression, in the former, of LTi-associated transcripts (*Rorc*, *Cd4*, *Cxcr5*, *Il1r1*, and *Ltb*)^{3,30} (supplemental Figure 1a). Genes involved in $\gamma\delta$ T-cell development (*Bhlhe40*, *Cited4*, *B4galnt2*, *Tgm2*, and *Txk*) were also differentially expressed between E13 and E18 ETPs (supplemental Figure 1a). Of note, *Sell* and *Ly6a* were upregulated in E18 ETPs, in line with their LMPP affiliation.^{2,3} The E14 DN3 thymocytes were devoid of TdT activity (supplemental Figure 1b) generating T cells with a restricted T-cell repertoire, suggesting that they do not contribute significantly to the adaptive T cell.

We performed a 2-step culture^{2,5} (Figure 1A) to detect the potential of a single ETP (sorted as in Ramond et al,³ Lin⁻CD117⁺CD44⁺CD24^{low}) to generate all major lymphoid lineages (T, B, ILC, natural killer [NK]) and myeloid cells (granulocyte macrophage [GM]) (Figure 1B). We found that E13 ETPs only generated T and ILC whereas E18 ETPs retained B and myeloid potential, consistent with our previous observations.³ Most ETPs gave rise to T and ILCs, although a few (6% to 15%) only generated T cells and none gave ILCs only. Approximately 59% of the clones derived from E18 ETPs generate all subsets of ILC and NK cells in contrast to 24% of E13 ETPs (Figure 1B-C), suggesting that E13 ETPs have a more restricted differentiation potential.

The striking difference between the 2 subsets was the high frequency of bipotent T/LTi precursors (22%) only observed in E13 ETPs (Figure 1C). Accordingly, a subset of E13-derived ILC3 expressed the LTi marker CCR6 (Figure 1D).

We colonized *Rag2*^{-/-} γc ^{-/-} non-irradiated fetal thymic organ culture (FTOC) with limited numbers of E13 or E18 ETPs and found that only the former could generate thymic LTi's (Figure 1D). In addition, only E13 ETPs generated $V\gamma 5^+$ $\gamma\delta$ T cells and both waves generated double-positive (DP) thymocytes (Figure 1D), in line with previous report from our laboratory.³ To ensure E13 ETP bipotency in a thymic environment, we repeated the experiment in Figure 1A, and used the expanded progeny of single E13 ETPs in *Rag2*^{-/-} γc ^{-/-} FTOCs. Results indicate that 50% of ETPs generated $V\gamma 5$ expressing $\gamma\delta$ T cells and LTi's in fetal thymic (Figure 1E).

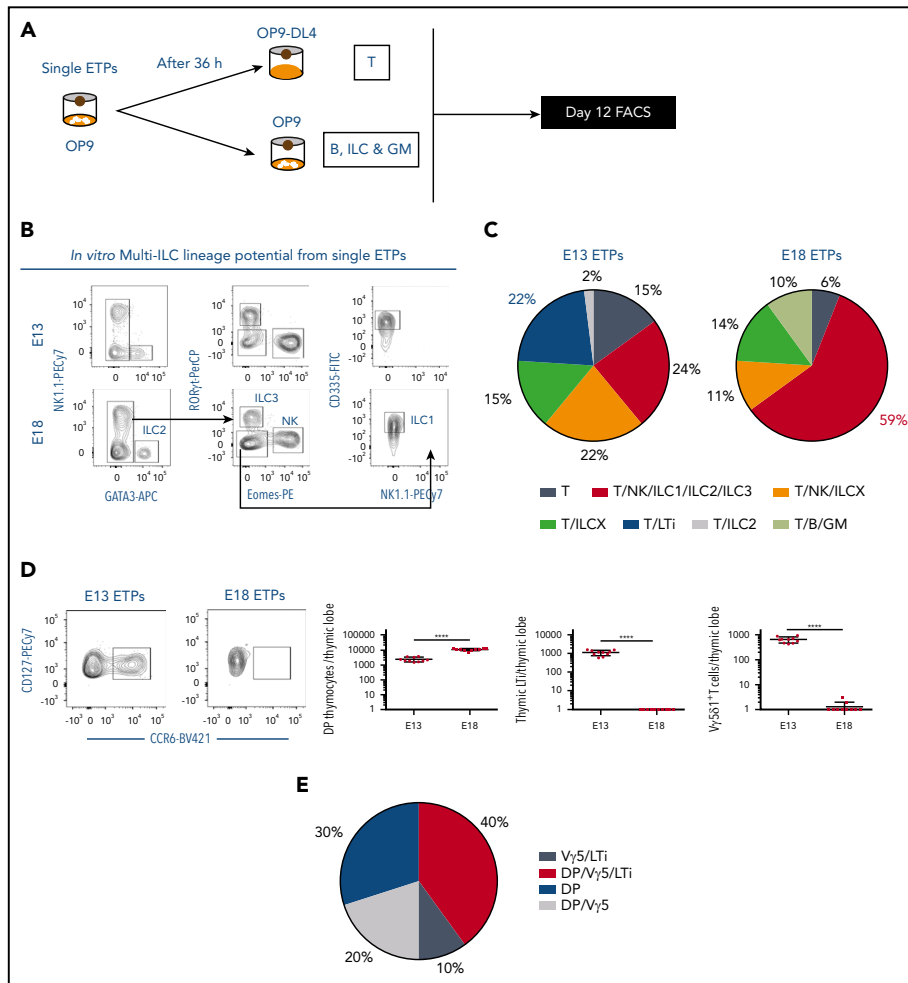


Figure 1. E13 ETPs retain T/LTI lineage potential in vitro and generate LTI cells in a fetal thymic microenvironment. (A) Experimental strategy for combined T, B, and ILC lineage potential analysis of single ETPs. Single ETPs from either E13 or E18 were sorted onto OP9 stromal cells supplemented with IL-7, Flt3L, KitL, and IL-2. After 36 hours, clones were subdivided into conditions that sustain differentiation of T cells (OP9-DL4 supplemented with IL-7, Flt3L, KitL, and IL-2) or B, myeloid, and ILC/LTI (OP9 supplemented with IL-7, Flt3L, KitL, and GM-CSF). (B) Representative flow cytometry plots outlining the gating strategy used to identify ILC1, ILC2, ILC3, and NK cells of E13 and E18 ETP single-cell-derived multi-ILC lineage clones. (C) Pie chart indicating all phenotypic combinations in individual clones detected after *in vitro* differentiation of E13 (81 clones analyzed of 240 sorted cells; cloning efficiency [CE], 34%) or E18 ETPs (86 clones analyzed of 240 sorted cells; CE, 36%) in 2 independent experiments. Frequency of clones generating only T cells (black); T, NK, ILC1, ILC2, and ILC3 (red) (different in E13 and E18 clones; $P < .0001$); T and LTI (RORγt⁺CCR6⁺) (blue) (different in E13 and E18 clones; $P < .0001$); T, NK, ILC2, 2 other ILC subsets (orange); T and ILC2 (gray); T and 2 other ILC subsets (green); T, B, and myeloid cells (olive) (different in E13 and E18 clones; $P = .0036$). All other combinations were not significantly different in E13 and E18 clones. (D) Flow cytometry plots of Rag2^{-/-}γc^{-/-} E14.5 thymic lobes reconstituted with 500 E13 ETPs or 500 E18 ETPs per lobe and gated CD3⁻ and stained for the expression of CD127 and CCR6 (left plots). Numbers of double-positive (DP) thymocytes per lobe (right plot), thymic LTI per lobe (middle plot) Vγ5Vδ1 γδT cells (left plot). FTOCs were analyzed at day 12 after reconstitution. **** $P < .0001$. (E) Single E13 ETP were processed as in panel A. After 36 to 48 hours in culture, clones were harvested and colonized single Rag2^{-/-}γc^{-/-} E14.5 thymic lobes. After 24 to 48 hours in hanging, drop lobes were transferred into filters and analyzed at day 12 ($n = 10$ lobes).

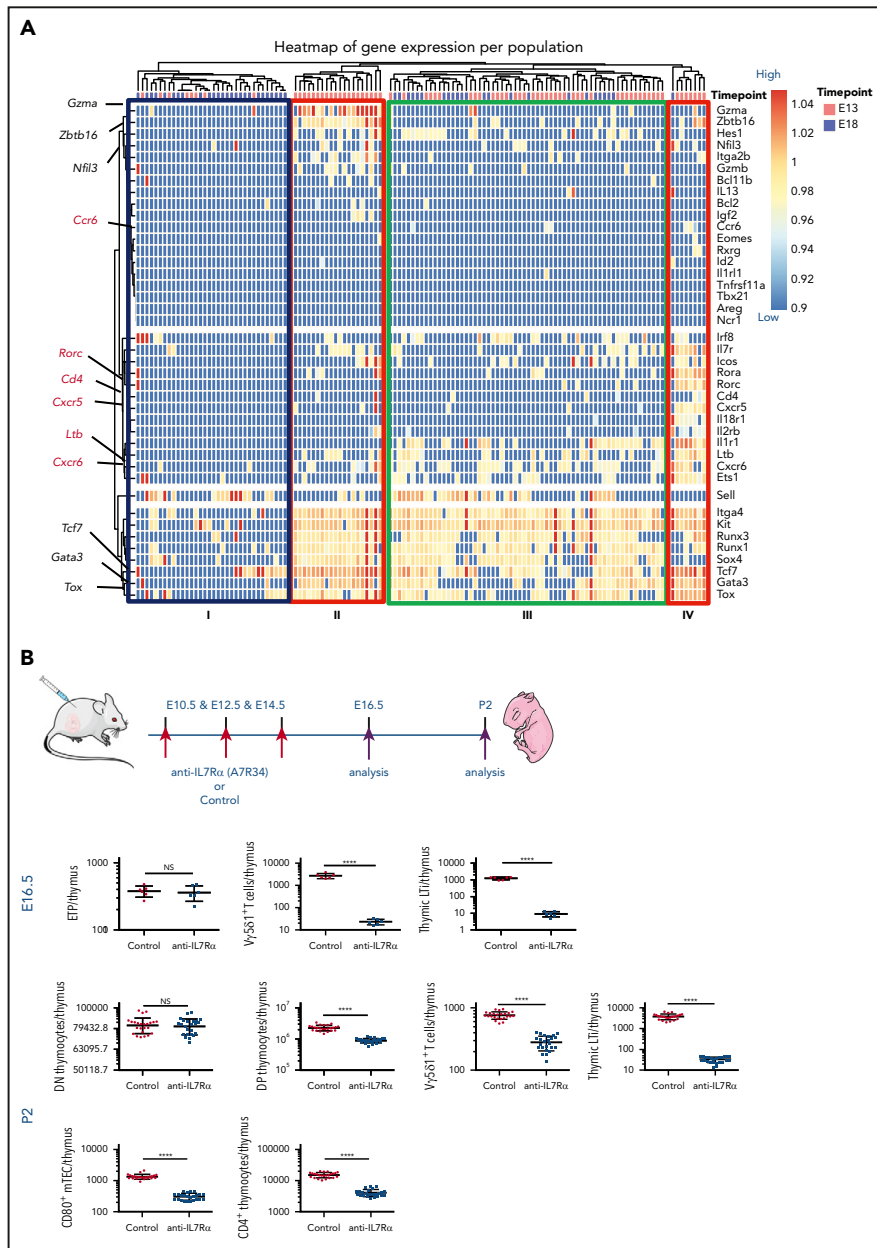


Figure 2. E13 ETPs have a transcriptional LTI signature and are required for the maturation (CD80⁺) of mTECs. (A) Single-cell multiplex qPCR analyzed by hierarchical clustering of single E13 and E18 ETPs (80 cells each) for the expression of ILC lineage-specific transcripts (right margin). Each column represents a cell (E13 ETP in salmon, E18 ETP

Altogether, E12.5-15.5 ETPs comprise unique bipotent progenitors, LT α biased, that generate thymic LT α and V γ 5 $^{+}$ $\gamma\delta$ T cells³ within a thymic microenvironment.

First-wave ETPs have an LT α -primed transcriptional profile

Q:21

To understand the molecular basis of the E13 ETP LT α lineage potential, we performed single-cell transcriptional analysis by multiplex quantitative reverse transcription polymerase chain reaction for the expression of 41 lymphoid-associated genes. Unsupervised hierarchical clustering identified 4 distinct clusters (Figure 2A). Cluster I, comprising a majority (76%) of E18 ETPs, showed no ILC transcript expression, indicating that they are not primed toward a specific ILC lineage, consistent with their multipotent ILC differentiation capacity. E13 ETPs, found in clusters II and IV, expressed the ILC-associated transcripts *Tox*, *Tcf7*, and *Gata3*. Expression of LT α transcripts *Cxcr6*, *Cd4*, *Rorc*, *Cxcr5*, *Il1r1*, and *Ltb* was found in cells within cluster IV, consistent with LT α -differentiation potential.

Cells in cluster II expressed *Zbtb16*, *Gzmb*, *Nfil3*, *Itga2b*, and *Gzma* associated with innate-like T cells.³¹ Cluster III comprised two-thirds of E13 and one-third of E18 ETPs and was characterized by the lower expression of *Tox*, *Gata3*, *Tcf7*, *Sox4*, *Runx1*, and *Runx3* indicative of common ILC priming and consistent with a broad ILC-differentiation potential (Figure 2A). Altogether, these results indicated that the first ETPs are unique in expressing LT α and invariant T cells transcripts, indicative of transcriptional priming.

First-wave ETPs contribute to the maturation of the thymic mTECs

Q:22

To assess the role of the first ETPs and their progeny in mTEC maturation, we used *in vivo* injection of A7R34 anti-IL7R α ³²⁻³⁴ antibody in pregnant female mice. We injected A7R34 antibody at E10.5, E12.5, and E14.5, and analyzed the embryos at E16.6 and P2 newborn mice. At E16.5, we observed severely reduced numbers of V γ 5 $^{+}$ T and LT α cells in the thymus although the second-wave ETPs³ were not altered (Figure 2B).

P2-treated mice had normal numbers of DN thymocytes consistent with an unaffected second wave of thymic colonization. P2 had significantly lower numbers of V γ 5 $^{+}$ T, and drastically lower numbers of LT α , compared with controls, indicating efficient depletion of the first ETPs and their progeny (Figure 2B). Interestingly, CD80 $^{+}$ mTECs (mature Aire-expressing cells)⁸ were reduced approximately threefold, indicating that the first ETPs are required for mTEC maturation, around birth. CD4 $^{+}$ CD8 $^{+}$ DP cells were also reduced by less than threefold. Our results indicate that the progeny of the first-wave ETPs contribute to mTEC maturation, required for conventional T-cell negative selection.

Q:29

Figure 2 (continued) in blue) and each row represents 1 gene (of 41 genes). Highlighted are ILC-associated transcripts (black) and LT α -associated transcripts (in red). Analysis was done by normalizing expression to 2 independent housekeeping genes (*Gapdh* and *Actinb*) in R package Phenograph as in ■■■ and represented in a code color where red represents high expression and blue low expression. Data are pooled from 2 independent experiments. (B) Pregnant female mice were injected at E10.5, E12.5, and E14.5 with 1 mg of anti-IL-7R α antibody A7R34 (red arrows indicate time points of anti-IL-7R α injections and violet arrows indicate time points of analysis). Thymic lobes were analyzed at E16.5 for the presence of ETP (Lin $^{-}$ CD117 $^{+}$ CD44 $^{+}$ CD24 int), for V γ 5V δ 1 $\gamma\delta$ T cells, and for LT α (CD127 $^{+}$ CCR6 $^{+}$ TcR $^{-}$ CD3 $^{-}$ cells) (n = 6), or at P2 for the presence of DN (CD4 $^{-}$ CD8 $^{-}$), DP(CD4 $^{+}$ CD8 $^{+}$), CD4 $^{+}$ thymocytes, and of V γ 5V δ 1 $\gamma\delta$ T cells, for LT α (CD127 $^{+}$ CCR6 $^{+}$ TcR $^{-}$ CD3 $^{-}$ cells), and for CD80 $^{+}$ mTECs (EpCAM $^{+}$ Ly51 $^{-}$ UEA.1 $^{-}$ CD45 $^{-}$) (n = 24). Plots show numbers of the respective populations per thymus. Data from 2 independent experiments. ****P < .0001.

Neonatal thymectomy does not impact numbers of tissue-resident V γ 5 $^{+}$ and V γ 6 $^{+}$ $\gamma\delta$ T cells

V γ 5 $^{+}$ and V γ 6 $^{+}$ $\gamma\delta$ T cells in the skin and in the lymph nodes (LNs), respectively, are of embryonic origin,^{16,35} although they develop asynchronously. To assess the dependency of these $\gamma\delta$ subsets on thymic output, indicating their origin in the first or second wave of ETPs, we performed neonatal thymectomy. Six weeks after neonatal thymectomy or sham operation as control (Figure 3A), complete thymectomy was ascertained by the absence of DP CD4/CD8 cells in the tissue around the thymus location (Figure 3B). Thymectomized mice (n = 6) were severely lymphopenic as shown by the numbers of CD4 and CD8 $\alpha\beta$ T cells in the inguinal (i) LN (Figure 3D) and in the spleen. Splenic CD44 $^{+}$ $\alpha\beta$ T cells were severely reduced (Figure 3E), a sign of activation of the T-cell compartment consequent to the lymphopenia and consistent with the absence of a functional thymus in these mice. The numbers of V γ 5 $^{+}$ $\gamma\delta$ T cells in the skin and of iLN V γ 4 $^{-}$ IL17-producing $\gamma\delta$ T cells (considered as V γ 6 $^{+}$) were similar in sham and in thymectomized animals (Figure 3C-D). By contrast, iLN V γ 4 $^{+}$ IL-17-producing $\gamma\delta$ T cells were severely reduced (Figure 3D). These experiments suggest that these 2 embryonic $\gamma\delta$ T-cell subsets are likely derived from the first wave ETPs. All other $\alpha\beta$ or $\gamma\delta$ T depend on postnatal thymic output and thus are issued from the second wave of ETPs.

Embryonic ETPs have multi-ILC lineage potential *in vivo*

To assess E13 and E18 ETP ILC lineage potential *in vivo*, CD45.2 $^{+}$ E13 or E18 ETPs were IV transferred into 1-day old CD45.1 $^{+}$ Rag2 $^{-/-}$ γ c $^{-/-}$ mice and E13 FL HSCs (Lin $^{-}$ CD117 $^{+}$ Sca1 $^{+}$ CD150 $^{+}$ CD48 $^{-}$) were used as a positive control (supplemental Figure 2a). Five weeks after transfer,³⁶ mice engrafted with HSCs and E18 ETPs developed B cells, ILCs, and myeloid cells (supplemental Figure 2c and data not shown). By contrast, mice engrafted with E13 ETPs generated only ILC (supplemental Figure 2b), consistent with the E13 ETP-restricted lymphoid potential. Analysis of PB 3 and 6 weeks or splenic cells 5 weeks after transplantation show a total absence of B and myeloid progeny from E13 ETPs (supplemental Figure 2b,e). Few T cells were generated in these chimeras because Rag2 $^{-/-}$ γ c $^{-/-}$ mice have an atrophic thymus and ETPs downregulate CCR7 and CCR9 once in the thymus, consequently losing their ability to home back to the thymus.³⁷

Analysis of intestinal lamina propria, where all ILC lineages coexist, showed that both waves of ETPs generated NK cells (EOMES $^{+}$), ILC1 (EOMES $^{-}$), ILC3 (ROR γ t $^{+}$), and ILC2 (GATA3 $^{+}$). In the lung, we found ILC2; in the spleen, NK cells; and in the liver, NK cells and ILC1 (supplemental Figure 2c-d). These results indicated that E13 and E18 ETPs have the capacity to generate all ILC subsets *in vivo* recapitulating the expected tissue distribution.

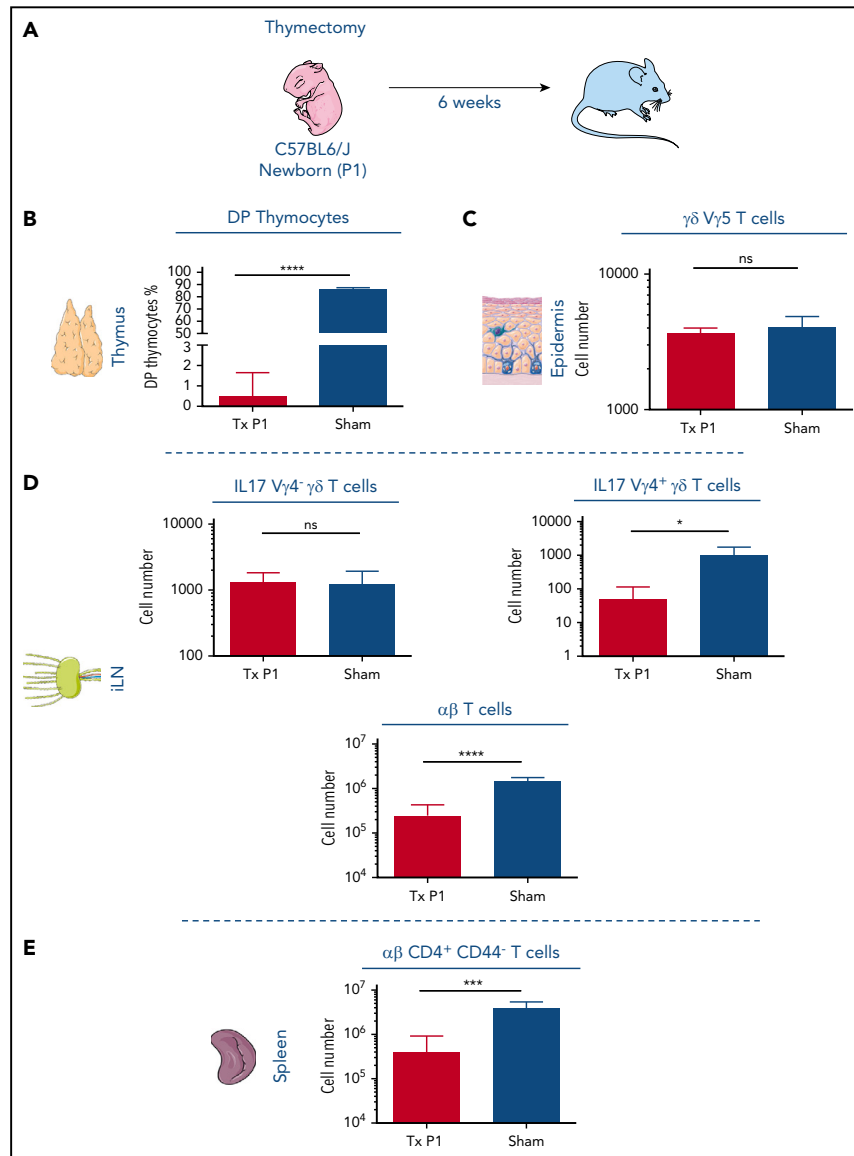


Figure 3. Neonatal thymectomy has no effect on embryonic-derived V γ 5 and V γ 6 $\gamma\delta$ T cells. (A) Schematic diagram of neonatal thymectomy experiment. P1 newborn mice were thymectomized and analyzed 6 weeks later. (B) Frequencies of double-positive CD4/CD8 T cells found in the thymus and perithymic tissue isolated from sham and thymectomized mice, respectively. (C) Number of V γ 5⁺ $\gamma\delta$ T cells in the epidermis isolated from 1 ear of sham and thymectomized mice. (D) Number of V γ 6 IL17 $\gamma\delta$ T cells, V γ 4 IL17 $\gamma\delta$ T cells, and $\alpha\beta$ T cells in 1 inguinal lymph node, isolated from sham and thymectomized mice. (E) Number of $\alpha\beta$ CD4⁺ CD44⁻ T cells in the spleen. * P < .05, ** P < .01, and *** P < .001 (Student t test). Data are representative of at least 4 mice in each group from 2 independent experiments. Data are depicted as mean plus or minus standard error of the mean (SEM).

Q:30

Emergence of *Il7r*-expressing YS progenitors in E9.5 embryos

Q:23 We used a lineage tracer mouse line, *Il7ra*^{Cre}Rosa26^{YFP},²⁴ to identify the origin of the first *Il7r*⁺ ETPs. YFP-expressing cells were undetectable in E8.5 embryos, in YS and embryo proper, in either the Kit⁺ or Kit⁻ fraction (data not shown). Consistent with previous studies, YFP⁺ progenitors were first detected in Lin⁻ Kit⁺ CD41⁺ E9.5 YS (Figure 4A-C) and undetected in the AGM or in the placenta (Figure 4D). Five percent to 8% of the c-kit⁺ YS cells were YFP⁺ (Figure 4B-C), expressed CD31, CD16/32, and lacked surface expression of CD115, CD127, CD135, the phenotype of YS EMPs (Lin⁻ Kit⁺ CD41⁺ YFP⁻; Figure 4E).^{38,39}

Q:24

To investigate the lineage potential of YS YFP⁺ cells, single cells were cultured into monolayers of OP9-DL4 (T/GM) or OP9 (B/GM) stroma (Figure 4F). Although YFP⁺ YS cells showed myeloid potential, they lacked detectable T- or B-cell potential (Figure 4G-H). Liquid-culture colony assays showed that both YFP⁺ and YFP⁻ Lin⁻ Kit⁺ YS cells exhibited comparable GM and MkE potential (Figure 4I). These results indicated that YFP expression in the E9.5 YS embryos does not equate with lymphoid potential, and both YFP⁺ and YFP⁻ E9.5 YS Kit⁺ cells showed a lineage potential similar to EMP.^{28,38,39}

E9.5 YS-derived cells did not generate V γ 5⁺ T cells or initiate thymopoesis, whereas E10.5 YS-Kit⁺ progenitors and E12 FL LSK readily generated T cells including V γ 5⁺ T cells in FTOCs of thymic lobes (Figure 4J). E10.5 YS-Kit⁺ progenitor-derived T cells originate from AGM pre-HSCs, known to enter circulation between E9.5 and E11.5^{40,41} and were used to probe the sensitivity of the T-cell assay.

These experiments showed that E9.5 YS progenitors are devoid of lymphoid potential under culture conditions sufficiently sensitive to detect few circulating T-cell progenitors.

Expression of lymphoid-associated transcripts in YS progenitors is transient

To further characterize YFP⁺ YS progenitors, we analyzed the expression of transcripts associated with multiple hematopoietic lineages in single E9.5 YS YFP⁺ and YFP⁻ Lin⁻ CD117⁺ CD41⁺ cells. Unsupervised hierarchical clustering did not segregate YFP⁺ and YFP⁻ cells, indicating that they do not differ significantly in the expression of the analyzed transcripts (Figure 5A). Cluster I-associated *Pu-1*, *Irf8* and *Csf1r* expressions with low or no *Kdr*, *Tek*, *Runx1*, *Mpl*, *Foxo1*, *Il7ra*, and *Flk2*-expressing cells, a pattern of expression corresponding to EMPs⁴² (Figure 5A). Cluster II singled out EMP-expressing *Pu-1*, *Irf8*, and *Csf1r* but also *Kdr*, *Tek*, *Runx1*, *Mpl*, and *Foxo1*, suggesting recent specification from hemogenic endothelium.⁴²⁻⁴⁴ These cells also exhibited *Il7ra* and *Flk2*. Clusters III and IV comprised YFP⁺ and YFP⁻ cells with high levels of *Gata1* indicating their erythroid lineage engagement. Cells in cluster IV also expressed *Klf1*, indicating that they are more advanced into the erythroid lineage differentiation. As expected, no lymphoid-associated transcripts were detected in cells from cluster III and IV. *Ebf1*, *Pax5*, or *Rag1* were not detected in any of the analyzed YS cells. Within the 47 YFP⁺ cells analyzed only 6 coexpressed *Il7ra* transcripts reinforcing the notion that *Il7ra* expression is transient in E9.5 YS progenitors (Figure 5B).

Interestingly, analyzed at the population level, YFP⁺ cells expressed significantly higher levels of *Rag1*, *Rag2*, and *Il7r* than YFP⁻ cells albeit 10-fold lower than CLPs (supplemental Figure 3).

These experiments indicated that expression of lymphoid-associated genes in emerging YS-derived EMPs is transient and dissociated from differentiation potential.

Multiple FL hematopoietic progenitors express lymphoid-associated genes

We further assessed whether YFP expression also occurs in other embryonic hematopoietic cells. We found that unlike in adult bone marrow (BM),²⁴ YFP expression in the FL was found in multipotent and myeloid progenitors (supplemental Figure 4a-b). FL and fetal blood (FB), short-term HSCs (ST-HSCs), multipotent progenitors (MPPs), GM progenitors (GMPs), common myeloid progenitors (CMPs) and megakaryocytes-erythroid progenitors (MEPs) were labeled at frequencies ranging from 10% to 40% (supplemental Figure 4a-b; supplemental Figure 5 for gating strategy). The frequency of YFP⁺ ST-HSCs and myeloid progenitors progressively decreased after birth and was undetectable in adult mice²⁴ (supplemental Figure 4b). FL YFP⁺ GMPs and CMPs also lacked any detectable B-cell potential in vitro (supplemental Figure 4c).

We found that 40% of E18 brain microglia, the FL Kupffer cells, and the skin mast and Langerhans cells also expressed YFP, in line with a recent report⁴⁵ (supplemental Figure 6a-b). These observations indicated that this model does not faithfully trace the origin of embryonic lymphoid cells.

Thymopoiesis-initiating cells develop exclusively from HSC-derived progenitors

To define the origin of the first ETPs, we used a fate-mapping mouse model expressing the tamoxifen-inducible Cre (*Mer-CreMer*) under the control of the *Csf1r* promoter²⁷ (Figure 6A). Injection of hydroxy-tamoxifen (4-OH-TAM) in either *Csf1r*^{MerCreMer}Rosa26^{YFP} or Rosa26^{Tomato} (depending on the availability of fluorochrome-labeled antibodies for the analysis) induced recombination and permanently labeled *Csf1r*-expressing cells and their progeny. *Csf1r* is first expressed in E8.5 YS progenitors,²⁸ later it is expressed in E9.5 YS *Il7ra*^{Cre}Rosa26^{YFP} (Figure 5A; supplemental Figure 3)¹⁸ and at E10.5 *Csf1r* is also expressed in pre-HSC⁴³ (supplemental Figure 7d).

In embryos pulsed at E8.5, YFP⁺ cells were detected in the E9.5 YS, expressed *Csf1r*, *Il7r*, and were devoid of lymphoid potential (supplemental Figure 7a-c). In embryos pulsed at either E8.5 or E9.5 and analyzed at E12.5, YFP⁺ HSCs, HSC-derived lymphoid progenitors, and ETPs were either not detectable or represented <1% in FL and thymus, respectively (Figure 6B-C). In contrast, 30% to 50% of microglia cells were labeled, indicating the labeling efficiency. Embryos pulsed at E10.5 and analyzed at E12.5 showed significant labeling in populations of FL HSCs, multipotent, lymphoid, and myeloid progenitors (Figure 6C). Interestingly, the frequency of Tomato⁺ cells in E12.5 ETPs (25%) and in FL LTi (22%) (supplemental Figure 7e for gating strategy) paralleled that of HSCs (31%).

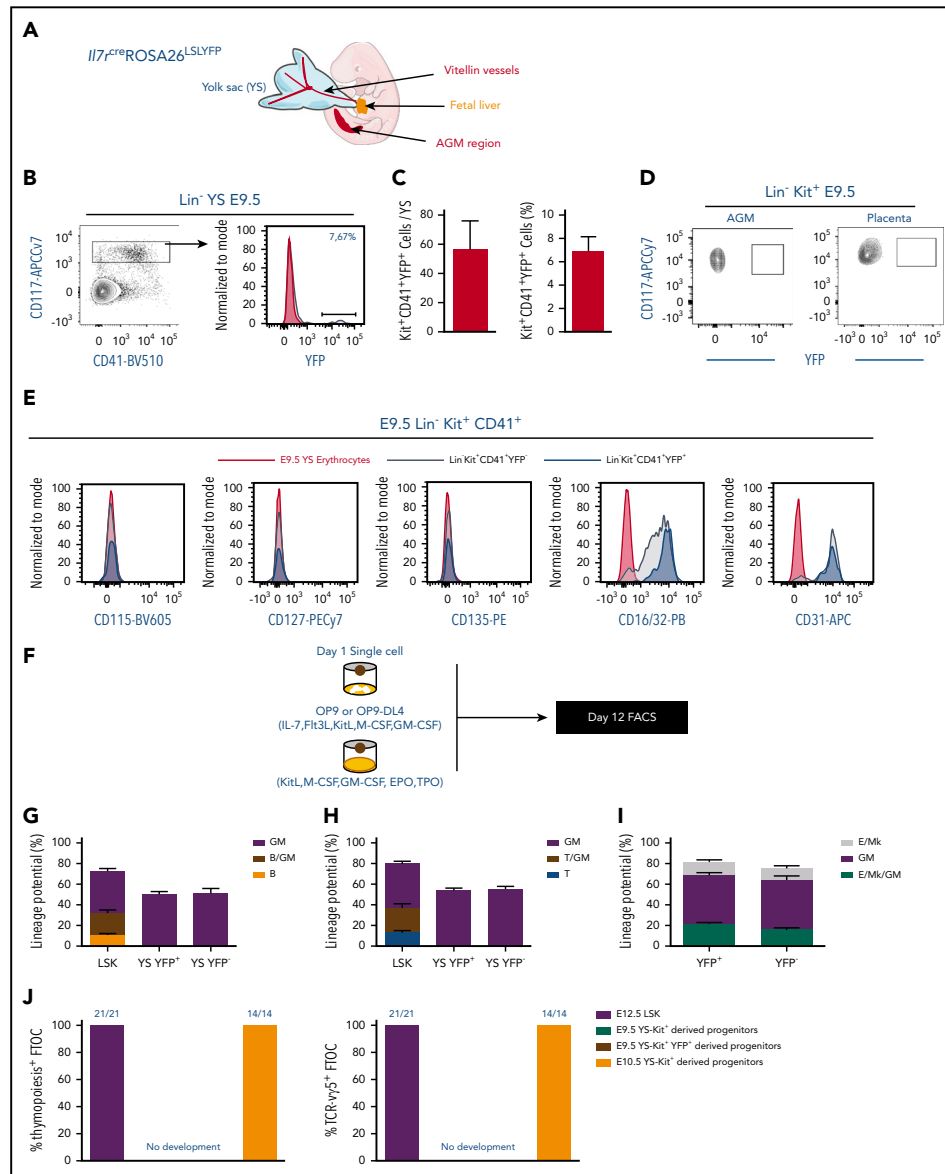
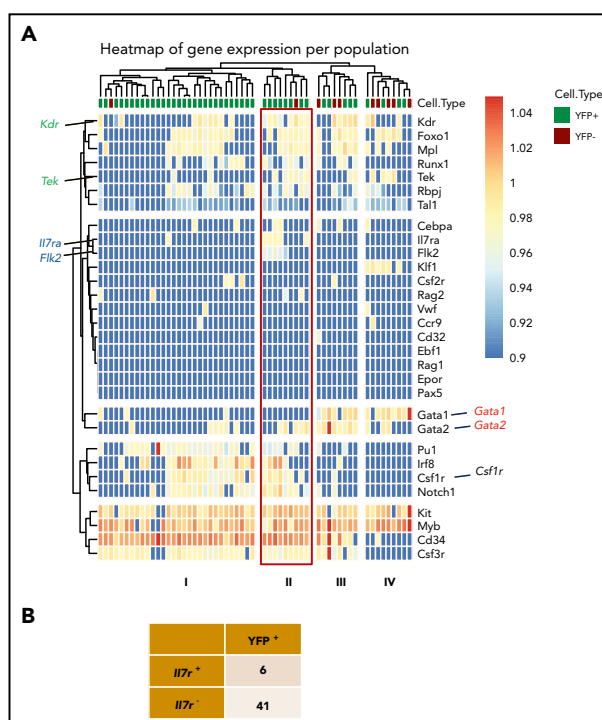


Figure 4. Emergence of *Il7Rα*⁺ expressing YS progenitors in E9.5 embryos. (A) Schematic representation of the anatomical sites analyzed in mouse embryos: YS, head, FL and the AGM region. (B) Representative flow cytometry plot showing YFP expression on live Lin[−]CD117⁺CD41⁺ E9.5 YS cells from *Il7^{cre}ROSA26^{LSL}YFP*. (C) Number and percentage of YFP⁺ per YS (mean plus or minus SEM). Data representative of 14 embryos from 4 independent experiments. (D) Representative flow cytometry plot showing YFP expression on live Lin[−]CD117⁺ E9.5 placenta and AGM region cells. (E) Expression of CD115, CD127, CD135, CD16/32, and CD31 within the E9.5 YS Lin[−]CD117⁺CD41⁺ YFP⁺ and YFP[−] populations. (F) Experimental strategy for single-cell lineage potential assay of E9.5 YS Lin[−]CD117⁺CD41⁺ YFP⁺ and YFP[−] populations. Frequency of wells containing (G) B cells or (H) T cells in cultures of single-sorted E9.5 YS Lin[−]CD117⁺CD41⁺ YFP⁺ and YFP[−] cells (180 cells from each population analyzed in 3 independent experiments). (I) Frequency of erythroid and megakaryocytic (E/Mk), myeloid (GM), or mixed E/Mk/GM colonies of single-sorted E9.5 YS Lin[−]CD117⁺CD41⁺ YFP⁺ and YFP[−] cells. (J) Frequency of thymic lobes irradiated and reconstituted with CD117⁺ E9.5 YS YFP⁺ or YFP[−], or with E12.5 LSK or E10.5 YS as controls that contained CD4/CD8 DP cells (left plot) or TCR-Vγ5⁺ T cells (right plot). FTOCs were analyzed at day 12 after reconstitution.

Figure 5. Gene-expression analysis of single E9.5 YS Lin⁺ CD117⁺ CD41⁺ YFP⁺ and YFP⁻ cells. (A) Single-cell multiplex qPCR analyzed by hierarchical clustering of single YFP⁺ (47 cells) and YFP⁻ (10 cells) E9.5 Lin⁺ CD117⁺ CD41⁺ cells analyzed for the expression of lymphoid-associated (*Il7r*, *Rag1*, *Rag2*, *Flt3*), myeloid-associated (*Cebpa*, *Cd32*, *Csf1r*, *Csf2r*, *Csf3r*, *Irf8*, *Pu1*), B-cell-associated (*Pax5*, *Ebf1*), E/Mk-associated (*EpoR*, *Gata1*, *Mpl*, *Tal1*, *Vwf*, *Klf1*), and hematopoiesis-associated (*Gata2*, *Myb*, *Runx1*, *Kit*, *Cd34*) genes. (B) Number of *Il7r*⁺ and *Il7r*⁻ cells within E9.5 YS YFP⁻ expressing cells.



These fate-mapping experiments demonstrated that under physiological conditions, no detectable ETPs or lymphoid progenitors originate from YS cells, rather their origin is coincident in time with that of pre-HSCs.

Innate-like V γ 5⁺ T cells or B1-a cells that differentiate during a limited time window of embryonic development have been proposed to derive from HSC-independent precursors.

Adult *Csf1*^{MerCreMer}*Rosa26*^{YFP} or *Csf1*^{MerCreMer}*Rosa26*^{Tomato} mice pulsed at E8.5 showed no YFP-labeled lymphocytes and those pulsed at E9.5 showed <1% of V γ 5⁺ T or B-1a-labeled cells. In both cases, 30% to 40% of microglia cells were YFP labeled (Figure 6C). Embryos pulsed at E10.5 showed a frequency of V γ 5⁺ T- or B-1a-labeled cells similar to that found in HSCs (Figure 6C) (supplemental Figure 7f for gating strategy). These results are consistent with a strict HSC origin of all lymphoid cells including the first wave of ETPs and innate-like lymphocytes of embryonic origin.

Discussion

Here, we show that the first ETPs comprise a unique and novel population of bipotent cells that generate the invariant T and LTi lineages, and together induce the maturation of medullary thymic epithelial cells.^{8,9} This observation was further supported

by a transcriptional analysis showing a cluster of E13.5 ETPs that are primed toward the LTi lineage with high expression of *Rorc*, *Cd4*, *Cxcr5*, *Il1r1*, and *Ltb*.³⁰ The first ETPs were devoid of TdT activity giving rise to T cells without N sequences and thus with a restricted T-cell repertoire. Low TdT expression, together with the poor proliferative capacity,³ suggested that the contribution of the first ETPs to the conventional T-cell compartment is limited.¹ Together, these observations indicate that the first wave of ETPs is dedicated to induce early maturation of the thymic epithelia and to generate invariant T cells that ensure tissue homeostasis. Neonatal Aire expression is necessary for inducing lifelong T-cell tolerance¹¹ and it was shown that both V γ 5⁺ $\gamma\delta$ cells and LTi's play an important role in the maturation of CD80⁺ mTECs.⁸ Our results highlight the necessity of a normal development of the first ETPs and their progeny to ensure Aire expression at birth and therefore induction of neonatal thymic tolerance and avoiding autoimmunity. A recent report analyzing human embryonic thymopoiesis suggests that similar sequence of events might be shared between mouse and human.⁴⁶

Using *Rag1*-GFP mice, it was argued that E11.5 ETPs retained myeloid potential and resembled PIRA/B⁺ LMPPs in phenotype, lineage potential, and molecular signature.¹⁹ Our data show that the first ETPs, isolated using generally accepted surface markers^{3,47} and not based on reporter mouse models, are T/ILC restricted with poor B and myeloid potential.

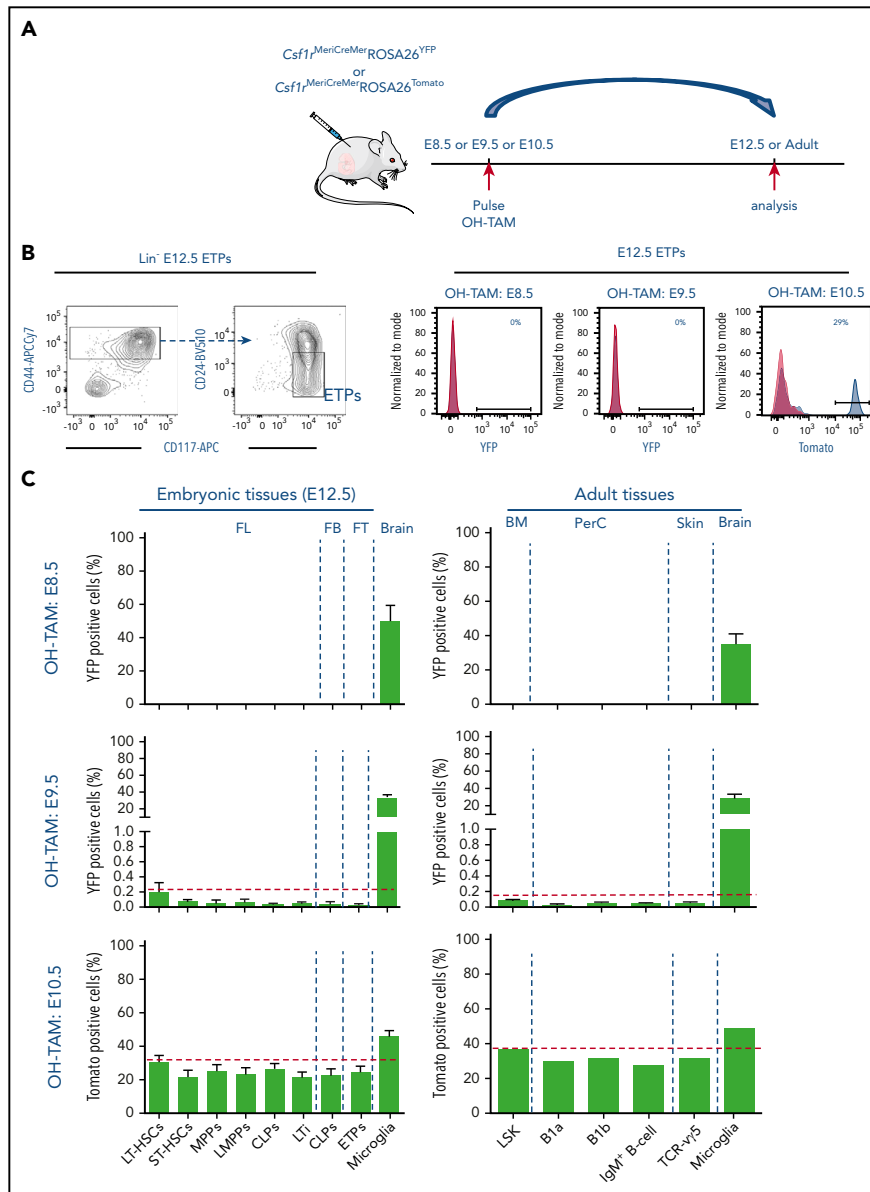
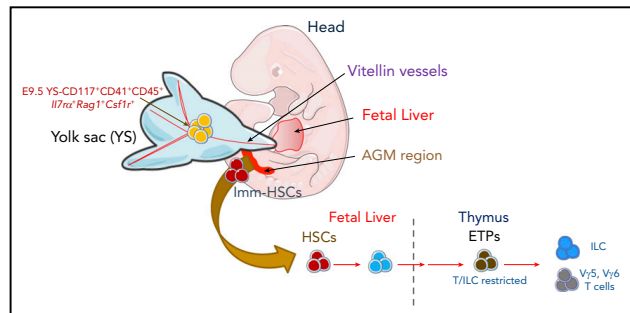


Figure 6. Thymopoiesis-initiating cells develop exclusively from HSC-derived progenitors. (A) Experimental design of lineage-tracing analysis of *Csf1l*-expressing cells after OH tamoxifen (OH-TAM) administration at E8.5, E9.5, or E10.5. Arrows indicate pulse and analysis time points. (B) Flow cytometry analysis of E12.5 early thymic progenitors (ETPs). E12.5 thymic lobes of embryos pulsed at indicated time points were analyzed. Left plots show the gating strategy to identify ETP. Right representative histograms show the frequency of Tomato⁺ or YFP⁺ cells in ETP. For each experiment, 1 representative analysis is shown. (C) Frequencies of YFP- or Tomato-labeled hematopoietic progenitors and LTI's in E12.5 FL, fetal blood (FB), and thymi (left plots) and of LSK, B1 B cells, B cells, and Vγ5⁺ T cells in adult tissues (right plots) of animals pulsed at E8.5, E9.5, or E10.5. Microglia served as controls for labeling efficiency. All data are pooled from minimally 2 independent experiments for embryos analyzed at E12.5 (pulsed at E8.5, n = 8; E9.5, n = 10; E10.5, n = 10); for adult tissues, animals were analyzed between 10 and 12 weeks of age (pulsed at E8.5, n = 6; E9.5, n = 6; E10.5, n = 2). Data are depicted as mean plus or minus SEM except for adult animals pulsed at E10.5 (mean). BM, bone marrow; FT, fetal thymus; PerC, peritoneal cavity.

Figure 7. Model depicting the origin and the lineage potential of thymopoiesis-initiating progenitors.



The analysis of YFP⁺ cells under the control of the *Il7r*-regulatory sequences throughout embryonic life indicated that, unlike adult hematopoietic progenitors, multipotent- and myeloid-restricted progenitors express lymphoid-associated genes, albeit in a transient manner, without any consequent restriction in their differentiation potential.^{45,48,49} These data argue against the notion that the embryonic thymus is initially seeded at E11.5 by Rag1-expressing lymphomyeloid-restricted progenitors.¹⁹ These observations established that in hematopoiesis, gene expression does not necessarily equate with lineage potential and care should be taken before extrapolating to embryonic development findings obtained by the analysis of adult hematopoiesis.

Q:25

By analogy with tissue-resident macrophages,^{17,28} it was proposed that also early developing innate-like T cells, and therefore the first ETPs, were HSC independent.^{18-21,50-52} Inducible lineage tracing of *Cdh5*- and *Runx1*-expressing cells²⁰ suggested that Vγ5⁺ T cells were generated from YS-derived hematopoietic progenitors. *Cdh5*, *Runx1*, and *Tie2* are expressed in endothelial cells of both the YS, that produces EMPs, and the AGM that is the source of HSCs.⁵³ Therefore, endothelial cells and their hematopoietic progeny will be labeled in both generation sites with different frequencies at different induction time points.^{20,28,54-56} To unambiguously assign the contribution of each anatomical site to blood cell formation, we used the *Csf1r*^{MerCreMer} lineage-tracing model that marks the progeny of cells generated in each of these hematopoietic sites in a nonoverlapping spatiotemporal manner. Recombination induced at E8.5-E9.5 marks the YS-derived progenitors (including lymphoid-associated gene-expressing progenitors), in which virtually no Vγ5⁺ T cells or early ETPs were labeled. Recombination induced at E10.5 traced both YS-derived *Csf1r*⁺ myeloid cells, HSCs, and also E12.5 ETPs and CLPs, and adult Vγ5⁺ γδ T cells and B1a B cells. These experiments are the first in vivo evidence that ETPs, innate-like lymphocytes, and lymphoid progenitors originate in the embryo from *Csf1r*⁺ HSC-derived progeny. However compelling, these results are also compatible with a late expression of *Csf1r* in subsets of YS cells. We think that this is unlikely because induction at E9.5 leads to virtually no contribution of *Csf1r*⁺ cells to the lymphoid compartment, which would be expected from an ongoing process and because it was previously reported that between E9 and E11 the frequency of *Csf1r*⁺ YS cells continuously decrease.⁵⁴ It is, however, possible that AGM-derived

MPPs bypass the HSC stage and directly differentiate and contribute to the first-wave ETPs, as recently reported in zebrafish⁵⁷ and in mice.⁵⁰ Altogether, these data indicate that lymphopoiesis is a hallmark of HSCs and HSC-derived progenitors.

The ability of adult ETPs to generate ILCs in vitro has been previously documented,^{31,58,59} however, the capacity of E13 ETPs to generate thymic LTi's within a thymic microenvironment is the first evidence that these cells can originate also from hematopoietic progenitors other than the classical ILCP.^{60,61} and in organs other than the FL where hematopoiesis occurs⁶² (Figure 7).

Q:26

This highly orchestrated sequence of events highlights the requirement of a layered immune cell production whereby the first wave of ETPs is essential for thymic organogenesis and T-cell tolerance.

Acknowledgments

The authors thank S. Novault, S. Megharba, and S. Schmutz from the flow cytometry core facility of the Institut Pasteur for technical support and the staff of the animal facility of the Institut Pasteur for mouse care. The authors thank H.-R. Rodewald for providing *Il7ra*^{Cre}*Rosa26*^{YFP} mice and S. E. W. Jacobsen for fruitful discussions.

This work was supported by the Institut Pasteur, INSERM, the Pasteur-Weizmann Foundation, and Agence Nationale de la Recherche (ANR; grant TwoThyme and Epi-Dev) through grants (A.C.); by REVIVE (Investissement d'Avenir, ANR-10-LABX-73) (A.C. and R.E.); by recurrent funding from the Institut Pasteur, the Centre National de la Recherche Scientifique (CNRS), Revive (Investissement d'Avenir, ANR-10-LABX-73), and by European Research Council (ERC) investigator award 2016-StG-715320; E.G.P.).

Q:27

Authorship

Contribution: R.E. designed and performed most experiments, analyzed data, and wrote the manuscript; S.M. analyzed chimeric mice and contributed to discussions; F.S.-d.-S. and T.P. performed Biomark analysis; O.B.-D. performed neonatal thymectomy; E.G.P., L.F., and L.I. performed fate-mapping experiments; A.B. performed adoptive transfer in neonatal mice; R.G., P.V., A.B., and P.P. contributed to the discussions; A.C. directed the research, designed experiments, analyzed data, and wrote the manuscript; and all authors contributed to the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Q:28

ORCID profiles: F.S.-d.-S., 0000-0003-1312-7601; L.F., 0000-0002-1997-4150; E.G.P., 0000-0002-7717-7897; A.C., 0000-0002-4578-959X.

Correspondence: Ana Cumano, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris Cedex 15, France; e-mail: ana.cumano@pasteur.fr.

Footnotes

Submitted 1 May 2020; accepted 10 September 2020; prepublished online on *Blood* First Edition 6 October 2020. DOI 10.1182/blood.202006779.

For original data, please contact the corresponding author.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

REFERENCES

- Cumano A, Berthault C, Ramond C, et al. New molecular insights into immune cell development. *Annu Rev Immunol*. 2019;37:497-519.
- Berthault C, Ramond C, Buren-Defranoux O, et al. Asynchronous lineage priming determines commitment to T cell and B cell lineages in fetal liver. *Nat Immunol*. 2017;18(10):1139-1149.
- Ramond C, Berthault C, Buren-Defranoux O, et al. Two waves of distinct hematopoietic progenitor cells colonize the fetal thymus. *Nat Immunol*. 2014;15(1):27-35.
- Bhandoola A, von Boehmer H, Petrie HT, Zúñiga-Pflücker JC. Commitment and developmental potential of extrathymic and intrathymic T cell precursors: plenty to choose from. *Immunity*. 2007;26(6):678-689.
- Luc S, Luis TC, Boukarabila H, et al. The earliest thymic T cell progenitors sustain B cell and myeloid lineage potential. *Nat Immunol*. 2012;13(4):412-419.
- Alves NL, Takahama Y, Ohgashi I, et al. Serial progression of cortical and medullary thymic epithelial microenvironments. *Eur J Immunol*. 2014;44(1):16-22.
- Boyden LM, Lewis JM, Barbee SD, et al. Skint1, the prototype of a newly identified immunoglobulin superfamily gene cluster, positively selects epidermal gammadelta T cells. *Nat Genet*. 2008;40(5):656-662.
- Roberts NA, White AJ, Jenkinson WE, et al. Rank signaling links the development of invariant $\gamma\delta$ T cell progenitors and Aire(+) medullary epithelium. *Immunity*. 2012;36(3):427-437.
- Rossi SW, Kim M-Y, Leibbrandt A, et al. RANK signals from CD4(+)3(-) inducer cells regulate development of Aire-expressing epithelial cells in the thymic medulla. *J Exp Med*. 2007;204(6):1267-1272.
- Abramson J, Anderson G. Thymic epithelial cells. *Annu Rev Immunol*. 2017;35:85-118.
- Guerrou-de-Arellano M, Martin M, Benoist C, Mathis D. Neonatal tolerance revisited: a perinatal window for Aire control of autoimmunity. *J Exp Med*. 2009;206(6):1245-1252.
- Cupedo T, Mebius RE. Role of chemokines in the development of secondary and tertiary lymphoid tissues. *Semin Immunol*. 2003;15(5):243-248.
- Cupedo T, Kraal G, Mebius RE. The role of CD45+CD4+CD3- cells in lymphoid organ development. *Immunol Rev*. 2002;189:41-50.
- Papotto PH, Ribot JC, Silva-Santos B. IL-17+ $\gamma\delta$ T cells as kick-starters of inflammation. *Nat Immunol*. 2017;18(6):604-611.
- Jones R, Cosway EJ, Willis C, et al. Dynamic changes in intrathymic ILC populations during murine neonatal development. *Eur J Immunol*. 2018;48(9):1481-1491.
- Ikuta K, Kina T, MacNeil I, et al. A developmental switch in thymic lymphocyte maturation potential occurs at the level of hematopoietic stem cells. *Cell*. 1990;62(5):863-874.
- Perdiguer EG, Geissmann F. The development and maintenance of resident macrophages. *Nat Immunol*. 2016;17(1):2-8.
- Böiers C, Carrelha J, Lutteropp M, et al. Lymphomyeloid contribution of an immune-restricted progenitor emerging prior to definitive hematopoietic stem cells. *Cell Stem Cell*. 2013;13(5):535-548.
- Luis TC, Luc S, Mizukami T, et al. Initial seeding of the embryonic thymus by immune-restricted lympho-myeloid progenitors. *Nat Immunol*. 2016;17(12):1424-1435.
- Gentek R, Ghigo C, Hoeffel G, et al. Epidermal $\gamma\delta$ T cells originate from yolk sac hematopoiesis and clonally self-renew in the adult. *J Exp Med*. 2018;215(12):2994-3005.
- Spidale NA, Sylvia K, Narayan K, et al. Interleukin-17-producing $\gamma\delta$ T cells originate from SOX13⁺ progenitors that are independent of $\gamma\delta$ TCR signaling. *Immunity*. 2018;49(5):857-872.e5.
- Beaudin AE, Boyer SW, Perez-Cunningham J, et al. A transient developmental hematopoietic stem cell gives rise to innate-like B and T cells. *Cell Stem Cell*. 2016;19(6):768-783.
- White AJ, Lucas B, Jenkinson WE, Anderson G. Invariant NKT cells and control of the thymus medulla. *J Immunol*. 2018;200(10):3333-3339.
- Schlenner SM, Madan V, Busch K, et al. Fate mapping reveals separate origins of T cells and myeloid lineages in the thymus. *Immunity*. 2010;32(3):426-436.
- Colucci F, Soudais C, Rosmaraki E, et al. Dissecting NK cell development using a novel alymphoid mouse model: investigating the role of the c-abl proto-oncogene in murine NK cell differentiation. *J Immunol*. 1999;162(5):2761-2765.
- Madisen L, Zwingman TA, Sunkin SM, et al. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat Neurosci*. 2010;13(1):133-140.
- Qian B-Z, Li J, Zhang H, et al. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. *Nature*. 2011;475(7355):222-225.
- Gomez Perdiguer E, Klapproth K, Schulz C, et al. Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors. *Nature*. 2015;518(7540):547-551.
- Sudo T, Nishikawa S, Ohno N, et al. Expression and function of the interleukin 7 receptor in murine lymphocytes. *Proc Natl Acad Sci USA*. 1993;90(19):9125-9129.
- Ishizuka IE, Chea S, Gudjonson H, et al. Single-cell analysis defines the divergence between the innate lymphoid cell lineage and lymphoid tissue-inducer cell lineage. *Nat Immunol*. 2016;17(3):269-276.
- De Obaldia ME, Bhandoola A. Transcriptional regulation of innate and adaptive lymphocyte lineages. *Annu Rev Immunol*. 2015;33:607-642.
- Yoshida H, Kawamoto H, Santee SM, et al. Expression of alpha(4)beta(7) integrin defines a distinct pathway of lymphoid progenitors committed to T cells, fetal intestinal lympho-toxin producer, NK, and dendritic cells. *J Immunol*. 2001;167(5):2511-2521.
- Hashizume T, Togawa A, Nochi T, et al. Peyer's patches are required for intestinal immunoglobulin A responses to *Salmonella* spp. *Infect Immun*. 2008;76(3):927-934.
- Schneider C, Lee J, Koga S, et al. Tissue-resident group 2 innate lymphoid cells differentiate by layered ontogeny and in situ perinatal priming. *Immunity*. 2019;50(6):1425-1438.e5.
- Haas JD, Ravens S, Düber S, et al. Development of interleukin-17-producing $\gamma\delta$ T cells is restricted to a functional embryonic wave. *Immunity*. 2012;37(1):48-59.
- Xu W, Cherrier DE, Chea S, et al. An Id2RFP-reporter mouse redefines innate lymphoid cell precursor potentials. *Immunity*. 2019;50(4):1054-1068.e3.
- Zlotoff DA, Sambandam A, Logan TD, Bell JJ, Schwarz BA, Bhandoola A. CCR7 and CCR9 together recruit hematopoietic progenitors to the adult thymus. *Blood*. 2010;115(10):1897-1905.
- Bertrand JY, Jalil A, Klaine M, Jung S, Cumano A, Godin I. Three pathways to mature macrophages in the early mouse yolk sac. *Blood*. 2005;106(9):3004-3011.
- McGrath KE, Frame JM, Fegan KH, et al. Distinct sources of hematopoietic progenitors emerge before HSCs and provide functional

- blood cells in the mammalian embryo. *Cell Rep.* 2015;11(12):1892-1904.
40. Delassus S, Cumano A. Circulation of hematopoietic progenitors in the mouse embryo. *Immunity.* 1996;4(1):97-106.
 41. McGrath KE, Koniski AD, Malik J, Palis J. Circulation is established in a stepwise pattern in the mammalian embryo. *Blood.* 2003; 101(5):1669-1676.
 42. Mass E, Ballesteros I, Farlik M, et al. Specification of tissue-resident macrophages during organogenesis. *Science.* 2016; 353(6304):aaf4238.
 43. Baron CS, Kester L, Klaus A, et al. Single-cell transcriptomics reveal the dynamic of hematopoietic stem cell production in the aorta. *Nat Commun.* 2018;9(1):2517.
 44. Kasaii B, Caolo V, Peacock HM, et al. Erythromyeloid progenitors can differentiate from endothelial cells and modulate embryonic vascular remodeling. *Sci Rep.* 2017;7:43817.
 45. Leung GA, Cool T, Valencia CH, et al. The lymphoid-associated interleukin 7 receptor (IL7R) regulates tissue-resident macrophage development. *Development.* 2019;146(14): dev176180.
 46. Tieppo P, Papadopoulou M, Gatti D, et al. The human fetal thymus generates invariant effector $\gamma\delta$ T cells. *J Exp Med.* 2020;217(3): e20190580.
 47. Porritt HE, Rumpf LL, Tabrizifard S, Schmitt TM, Zúñiga-Pflücker JC, Petrie HT. Heterogeneity among DN1 prothymocytes reveals multiple progenitors with different capacities to generate T cell and non-T cell lineages. *Immunity.* 2004;20(6):735-745.
 48. Gautier EL, Shay T, Miller J, et al; Immunological Genome Consortium. Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. *Nat Immunol.* 2012;13(11):1118-1128.
 49. Heng TSP, Painter MW; Immunological Genome Project Consortium. The Immunological Genome Project: networks of gene expression in immune cells. *Nat Immunol.* 2008;9(10): 1091-1094.
 50. Kobayashi M, Shelley WC, Seo W, et al. Functional B-1 progenitor cells are present in the hematopoietic stem cell-deficient embryo and depend on Cbfb for their development. *Proc Natl Acad Sci USA.* 2014;111(33): 12151-12156.
 51. Yoshimoto M, Montecino-Rodriguez E, Ferkowicz MJ, et al. Embryonic day 9 yolk sac and intra-embryonic hemogenic endothelium independently generate a B-1 and marginal zone progenitor lacking B-2 potential. *Proc Natl Acad Sci USA.* 2011;108(4):1468-1473.
 52. Yoshimoto M, Porayette P, Glosson NL, et al. Autonomous murine T-cell progenitor production in the extra-embryonic yolk sac before HSC emergence. *Blood.* 2012;119(24): 5706-5714.
 53. Elsaid R, Yang J, Cumano A. The influence of space and time on the establishment of B cell identity. *Biomed J.* 2019;42(4):209-217.
 54. Hoeffel G, Chen J, Lavin Y, et al. C-Myb(+) erythro-myeloid progenitor-derived fetal monocytes give rise to adult tissue-resident macrophages. *Immunity.* 2015;42(4):665-678.
 55. Gentek R, Ghigo C, Hoeffel G, et al. Hemogenic endothelial fate mapping reveals dual developmental origin of mast cells. *Immunity.* 2018;48(6):1160-1171.e5.
 56. Busch K, Klapproth K, Barile M, et al. Fundamental properties of unperturbed hematopoiesis from stem cells in vivo. *Nature.* 2015;518(7540):542-546.
 57. Tian Y, Xu J, Feng S, et al. The first wave of T lymphopoiesis in zebrafish arises from aorta endothelium independent of hematopoietic stem cells. *J Exp Med.* 2017;214(11): 3347-3360.
 58. Wong SH, Walker JA, Jolin HE, et al. Transcription factor ROR α is critical for nuocyte development. *Nat Immunol.* 2012;13(3): 229-236.
 59. Yang Q, Li F, Harly C, et al. TCF-1 upregulation identifies early innate lymphoid progenitors in the bone marrow. *Nat Immunol.* 2015; 16(10):1044-1050.
 60. Diefenbach A, Gnafakis S, Shomrat O. Innate lymphoid cell-epithelial cell modules sustain intestinal homeostasis. *Immunity.* 2020;52(3): 452-463.
 61. Diefenbach A, Colonna M, Koyasu S. Development, differentiation, and diversity of innate lymphoid cells. *Immunity.* 2014;41(3): 354-365.
 62. Cumano A, Godin I. Ontogeny of the hematopoietic system. *Annu Rev Immunol.* 2007; 25:745-785.