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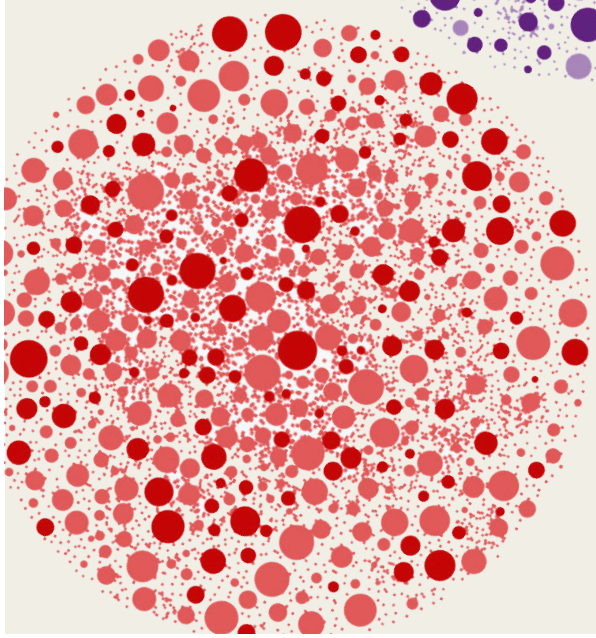

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract

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Key words: Hematopoiesis, Developmental hematopoiesis, Proteomics, Leukemia, Mass Spectrometry

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"We know not what this darkness brings, but the stars all gleam with possibilities."

Benjamin Gibbard

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Manuscript in preparation, 2021

Abstract

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Our initial work resulted in the identification of numerous proteins and biological processes that distinguish fetal and adult Lin^- $Sca\text{-}1^+$ $cKit^+$ (LSK) HSPCs, with particularly strong differences between the two cell types in processes related to immune response, protection against reactive oxygen species (ROS) and proteolysis. We showed that fetal and adult HSPCs exhibit distinct responses to modulation of Type I interferon (IFN) signaling and neutrophil serine protease (NSP) activity. We subsequently investigated redox homeostasis in fetal and adult HSPCs, and showed that in line with the lower expression of proteins involved in antioxidant defense, the fetal HSPC proteome has a significantly higher oxidation level than the adult, and undergoes even further oxidation upon leukemia initiation.

Our subsequent work focused on characterization of ontogenic proteomic and functional changes that occur in lineage-biased hematopoietic progenitor cells (HPCs). We revealed an intrinsically programmed difference in the lineage bias of fetal and adult lymphomyeloid multipotent progenitors (LMPPs), common lymphoid progenitors (CLPs) and granulocyte monocyte progenitors (GMPs), with the adult cells showing a considerably stronger myeloid potential compared to the fetal. The difference in myeloid potential was in part due to a significantly lower expression of Irf8 and a concomitant defect in production of mature monocytes in

fetal relative to adult GMPs, which could be partially rescued by increasing Irf8 expression.

Finally, we focused our investigations on leukemia driven by the MLL-ENL fusion oncogene, a translocation which represents a highly prevalent mutational event in *in utero*-derived infant acute lymphoblastic leukemia (ALL). We showed that fetal MLL-ENL-expressing LMPPs possess an intrinsic bias towards generation of immature lymphoid cells *ex vivo*. In a transplantation model, however, expression of MLL-ENL in fetal as well as adult LMPPs drives acute myeloid leukemia (AML) in adult recipients, suggesting that an interplay between intra- and extracellular factors determines disease progression and outcome in MLLr leukemia. Molecularly, we found that MLL-ENL-mediated leukemia initiation is hallmarked by a differentiation arrest and metabolic reprogramming in fetal as well as adult cells. Remarkably, while expression of proteins associated with inflammation is increased in adult leukemic relative to wild-type (WT) LMPPs, such signatures instead appear to be suppressed in fetal-origin leukemia. Thus, we identified shared as well as ontogeny-specific features of MLL-ENL-mediated leukemogenesis which could potentially be exploited in the development of age-tailored anti-cancer therapies.

Collectively, this thesis work has uncovered numerous novel ontogeny-specific molecular and functional features of fetal and adult HSPCs, both in normal development and in leukemia. The results from the included studies will aid in guiding future efforts aimed at developing novel strategies for prevention and treatment of pediatric and adult acute leukemia.

List of abbreviations

ABM	adult bone marrow
AF4	ALL-1 fused gene from chromosome 4
AF9	ALL-1 fused gene from chromosome 9
AGM	aorta-gonad-mesenephros
ALL	acute lymphoblastic leukemia
AMKL	acute megakaryoblastic leukemia
AML	acute myeloid leukemia
APC	antigen-presenting cell
BCR	B cell receptor
BM	bone marrow
cALL	childhood acute lymphoblastic leukemia
CAR	chimeric antigen receptor
CDP	common DC progenitor
CEBP	CCAAT/enhancer binding protein
CFC	colony-forming cell
CFU	colony-forming unit
CFU-E	colony-forming unit-erythroid
CID	collision-induced dissociation
CLL	chronic lymphocytic leukemia
CLP	common lymphoid progenitor
CML	chronic myelogenous leukemia
CSF	colony-stimulating factor
DC	dendritic cell
DDA	data-dependent acquisition

DIA	data-independent acquisition
DOX	doxycycline
E	embryonic day
EHT	endothelial-to-hematopoietic transition
EILP	early innate lymphoid progenitor
EMP	erythromyeloid progenitor
ENL	eleven-nineteen leukemia
EPO	erythropoietin
ER	endoplasmic reticulum
ETP	early thymic progenitor
ETV6	ETS translocation variant 6
FACS	fluorescence-activated cell sorting
FL	fetal liver
Flt3	fms-like tyrosine kinase 3
Flt3l	Flt3 ligand
FP	fusion protein
G-CSF	granulocyte colony-stimulating factor
GMLP	granulocyte-monocyte lymphoid progenitor
GMP	granulocyte-monocyte progenitor
GP	granulocyte progenitor
GRN	gene regulatory network
GSH	glutathione
HbA	adult hemoglobin
HbF	fetal hemoglobin
HCD	higher-energy collision-induced dissociation
HPC	hematopoietic progenitor cell
HpH-RP	high pH-reverse phase
HSC	hematopoietic stem cell
HSCT	hematopoietic stem cell transplantation

HSPC	hematopoietic stem and progenitor cell
IFN	interferon
IFNaR	interferon α receptor
IL	interleukin
ILC	innate lymphoid cell
iMLL-ENL	inducible MLL-ENL
iPSC	induced pluripotent stem cell
iST	in-StageTip
iTRAQ	isobaric tags for relative and absolute quantitation
LC	liquid chromatography
LFQ	label-free quantification
LIC	leukemia-initiating cell
Lin	lineage
LMPP	lymphomyeloid multipotent progenitor
LSC	leukemia stem cell
LSK	Lin^- $Sca\text{-}1^+$ $cKit^+$
LT	long-term
M-CSF	macrophage colony-stimulating factor
MegE	megakaryocyte-erythroid
MEP	megakaryocyte-erythroid progenitor
MHC	major histocompatibility complex
MLC	myosin light chain
MLL	mixed-lineage leukemia
MLLr	MLL-rearranged
MP	monocyte progenitor
MPP	multipotent progenitor
MS	mass spectrometry
NK	natural killer
NKP	NK progenitor

NKT	natural killer T
NSP	neutrophil serine protease
OXPHOS	oxidative phosphorylation
P-TEFb	positive transcription elongation complex b
pCFU-E	pre-colony-forming unit-erythroid
pGM	pre-granulocyte-monocyte
ROCK	rho kinase
ROS	reactive oxygen species
RP	reverse phase
RUNX1	runt-related transcription factor 1
Sca-1	stem cell antigen-1
SCF	stem cell factor
SCoPE-MS	single-cell proteomics by mass spectrometry
SILAC	stable isotope labeling of amino acids in culture
SOD	superoxide dismutase
SP3	single-pot, solid-phase-enhanced sample preparation
SPS	synchronous precursor selection
ST	short-term
TCR	T cell receptor
TF	transcription factor
TMT	tandem mass tag
TPO	thrombopoietin
WT	wild-type
YS	yolk sac

Introduction

Hematopoiesis

The process by which all blood cells are created throughout life is called *hematopoiesis*, a name derived from Greek and which quite literally translates to, "to make blood". In humans, over 2 million new blood cells are produced every second, making the hematopoietic system subject to one of the highest degrees of cellular turnover in the body [1]. The constant supply of mature blood cells is dependent on a small population of multipotent and self-renewing hematopoietic stem cells (HSCs), which reside in the bone marrow (BM) of adult mammals. The extraordinary regenerative potential of HSCs is best showcased by their ability to rebuild the entire blood and immune system following myeloablation, first demonstrated in mice in 1949 [2] and pioneered in humans in 1957 [3]. Since then, transplantation of BM-derived HSCs has become a life-saving procedure, used to treat an array of hematological and immunological diseases such as leukemia, anemia and autoimmune disorders. To date, HSC transplantation is the only stem cell therapy routinely used in the clinic [4].

The cells of the hematopoietic system

Broadly speaking, the hematopoietic system can be divided into cells involved in innate and adaptive immunity (myeloid and lymphoid cells, respectively), oxygen transport (erythroid cells) and wound healing (platelets) [5]. HSCs give rise to cells belonging to the lymphoid (B, T and natural killer [NK] cells), myeloid (granulocytes, monocytes/macrophages and dendritic cells [DCs]) and megakaryocyte-erythroid (MegE; megakaryocytes/platelets and erythrocytes) lineages through a sequential multi-step differentiation process, which is accompanied by a progressive loss of self-renewal capacity and involves several intermediate populations of hematopoietic progenitor cells (HPCs) [5-9]. Although the exact organization of the so-called hematopoietic hierarchy is subject to debate [10-12], it is generally agreed that the initial steps of HSC differentiation involve a bifurcation of the MegE and lymphomyeloid lineages, followed by separation of the lymphoid and myeloid lineage with the commitment of a bipotent lymphomyeloid multipotent progenitor (LMPP, sometimes referred to as granulocyte-monocyte lymphoid progenitor [GMLP]) lacking MegE potential to either lineage via the

common lymphoid progenitor (CLP) or the pre-granulocyte-monocyte (pGM) progenitor [6-9] (**Figure 1**).

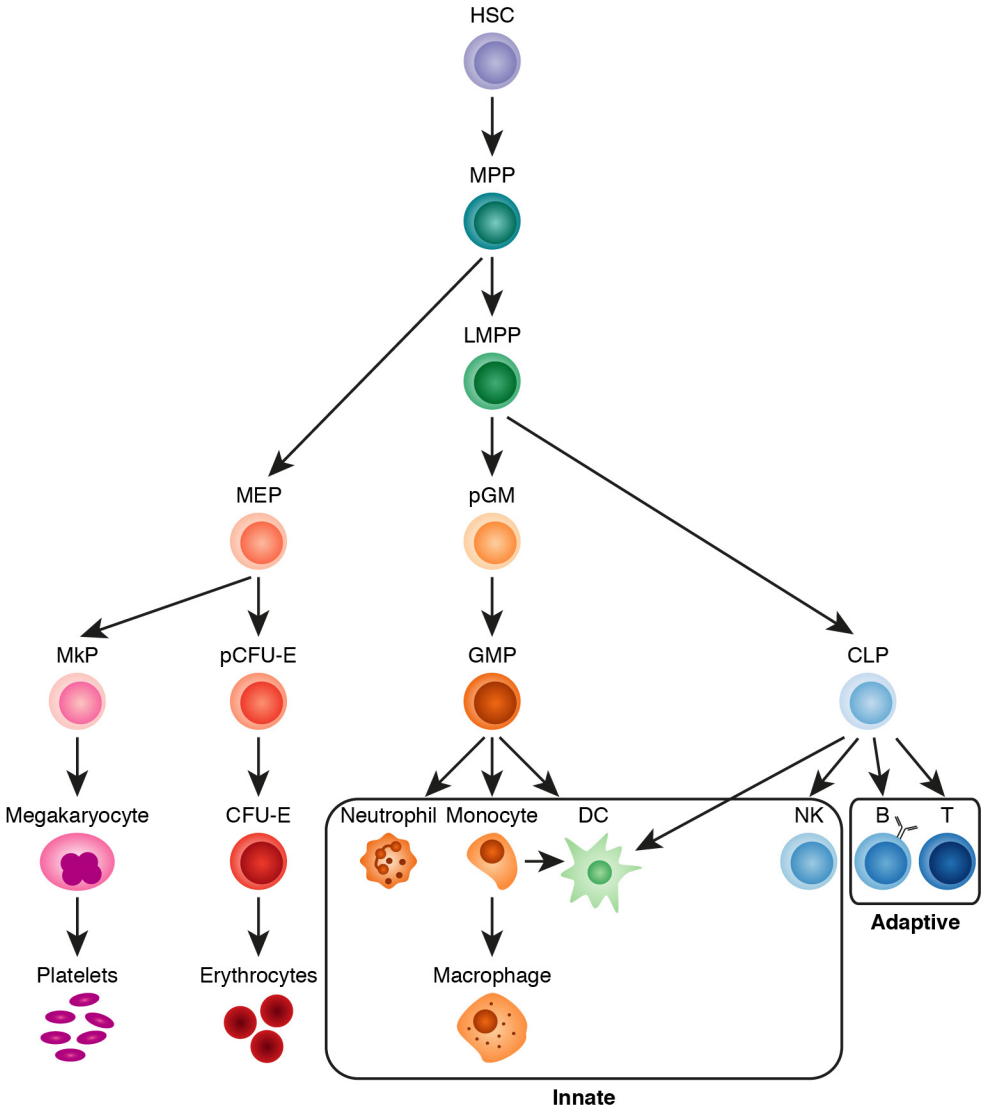

Figure 1. The hematopoietic hierarchy. Hematopoiesis is often depicted as a hierarchical structure, with multipotent, self-renewing HSCs situated at the top. HSCs give rise to mature cells of the lymphoid (NK, B and T cells), myeloid (neutrophils, monocytes/macrophages and DCs) and MegE (platelets and erythrocytes) lineages through a multistep differentiation process which involves several intermediate progenitor populations. The mature cell types of the innate immune system include neutrophils, monocytes/macrophages, DCs and NK cells, whereas the adaptive immune system consists of B and T cells.

The array of different mature cell types of the immune system cover distinct, yet highly complementary, functions. Innate immune cells, which represent the first line of defense within the immune system, function independently of antibody-antigen-mediated interaction and can thus mount a rapid response upon encountering infectious agents or transformed cells. Innate immunity is maintained by neutrophils, monocytes, macrophages, DCs and NK cells, among other cell types. In humans, neutrophils are the most abundant cell type within the pool of circulating leukocytes, and are the first cells recruited upon inflammatory insult. Neutrophils clear pathogens through a number of different mechanisms, including phagocytosis and subsequent reactive oxygen species (ROS)- or neutrophil serine protease (NSP)-mediated elimination of the pathogen, and the release of neutrophil extracellular traps [13].

At least two different populations of monocytes exist in the circulation, which in the mouse are represented by $Ly6C^-$ patrolling monocytes and $Ly6C^+$ inflammatory monocytes, the latter of which is a precursor of macrophages and can differentiate into DCs under conditions of inflammatory stress. Although monocytes have traditionally been considered mere progenitors of phagocyting macrophages and DCs, recent studies have reported a more intricate role of monocytes during infection, where they are involved in processes such as regulation of the inflammatory response via cytokine secretion, and antigen presentation [14]. The main antigen-processing and -presenting cells within the body are however the DCs. DCs are often referred to as "professional antigen-presenting cells (APCs)" and play an important role in bridging innate and adaptive immunity [15]. Of note, while DCs have traditionally been classified as a myeloid cell type, lymphoid progenitors can additionally give rise to DCs with a phenotype indistinguishable from those derived via the myeloid branch of hematopoiesis [16].

The lymphoid axis of the immune system comprises innate as well as adaptive immune cells. NK cells represent the most well-characterized cell type among the innate lymphoid cells (ILCs) [17]. NK cells possess potent cytotoxic activity which allows them to recognize and rapidly eliminate stressed and/or damaged cells, as well as tumor cells which have lost expression of self-identifying surface molecules such as the major histocompatibility complex (MHC) class I [18].

B and T cells are the main effector cells of adaptive immunity, as well as the primary cell types responsible for maintaining immunological memory [19], although some memory of pathogen encounters likely exists also within the innate immune system [20]. A multistep recombination process, termed V(D)J-recombination, enables generation of B an T cells specific for an incredibly broad repertoire of pathogen-associated antigens. A variety of different T cell subsets exist within the body, with functions including direct cell killing ($CD8^+$ cytotoxic T cells and natural killer T [NKT] cells), B cell activation ($CD4^+$ helper T cells) and regulation of the immune response via secretion of cytokines which can either amplify or dampen immune reactions ($CD4^+$ regulatory and helper T cells). The main role of B cells, on the other

hand, is antibody production. In addition to engagement of the B cell receptor (BCR) with antigen presented by APCs, the final step of B cell maturation and production of antibody-producing plasma B cell and long-lived memory B cells typically requires signals mediated though cell-cell contact with T cells [19].

Increasing evidence has highlighted the contribution of hematopoietic cell types other than those traditionally associated with defense against pathogens to the immune response. For example, human megakaryocytes have recently been shown express genes associated with antiviral immunity and secrete cytokines which protect surrounding hematopoietic cells upon infection with influenza and dengue virus [21]. Importantly, even immature HSPCs have now been recognized as potent producers of inflammatory cytokines [22], and HSCs have been reported to retain long-term immunological memory via epigenetic remodeling following exposure to bacterial lipopolysaccharide [23]. Considering the relative recentness of these discoveries, it is highly likely that the novel idea that HSPCs can be directly involved in immune system function has as of yet only been scratched on the surface.

Primitive hematopoiesis

During mammalian embryonic and fetal development, hematopoiesis occurs in several overlapping waves [24] (**Figure 2**). The first wave, often referred to as primitive hematopoiesis, takes place in the extraembryonic yolk sac (YS) and generates primarily cells of the MegE lineage as well as macrophages [25], closely followed by a second wave that gives rise to other myeloid cell types and early lymphoid cells [26]. Both of the initial waves of embryonic hematopoiesis are HSC-independent and occur through distinct populations of transiently existing HPCs with limited or no self-renewal capacity, including erythro-myeloid progenitors (EMPs) and a subset of LMPPs harboring surface expression of interleukin (IL)-7 receptor α (IL-7Rα; IL-7Rα^+ LMPPs) [25-27]. Remarkably, despite the finite time window during which embryonic HPCs are present in the body, EMPs represent the largest source of tissue-resident macrophage populations found in adult organs such as the brain (microglia), skin (Langerhans cells) and liver (Kupffer cells) [28], highlighting a non-redundant role of these primitive blood progenitors in immune system function throughout life.

Definitive hematopoiesis

Approximately 30 days post-conception in humans (corresponding to embryonic day [E] 10.5 in mice), the first cells capable of self-renewal and multi-lineage differentiation, hence representing definitive HSCs, emerge in the aorta-gonad-mesonephros (AGM) region of the embryo and concomitantly travel to other organs such as the YS, the umbilical cord and the placenta via the circulation [29, 30]. The

generation of definitive HSCs occurs through a process termed endothelial-to-hematopoietic transition (EHT) and is dependent on the transcription factors (TFs) runt-related transcription factor 1 (Runx1) and Scl (Tal1) [31, 32]. After the initiation of definitive hematopoiesis in the AGM, the placenta transiently serves as a niche for HSC expansion, after which the cells migrate to the main site of fetal hematopoiesis – the liver [24, 29, 33]. HSCs in the fetal liver (FL) have a high translation rate, cycle actively and undergo frequent self-renewing divisions in order to sustain the growing embryo and to build the foundation of the blood system [24, 34-37]. The largest wave of HSC expansion in the FL occurs between day 40 and 70 of human pregnancy, corresponding to E12-E16 in mice [24, 34]. Starting from E16.5, considerable HSC activity can be detected in the fetal spleen, and splenic hematopoiesis parallels HSC seeding of the BM until two weeks after birth in mice [38].

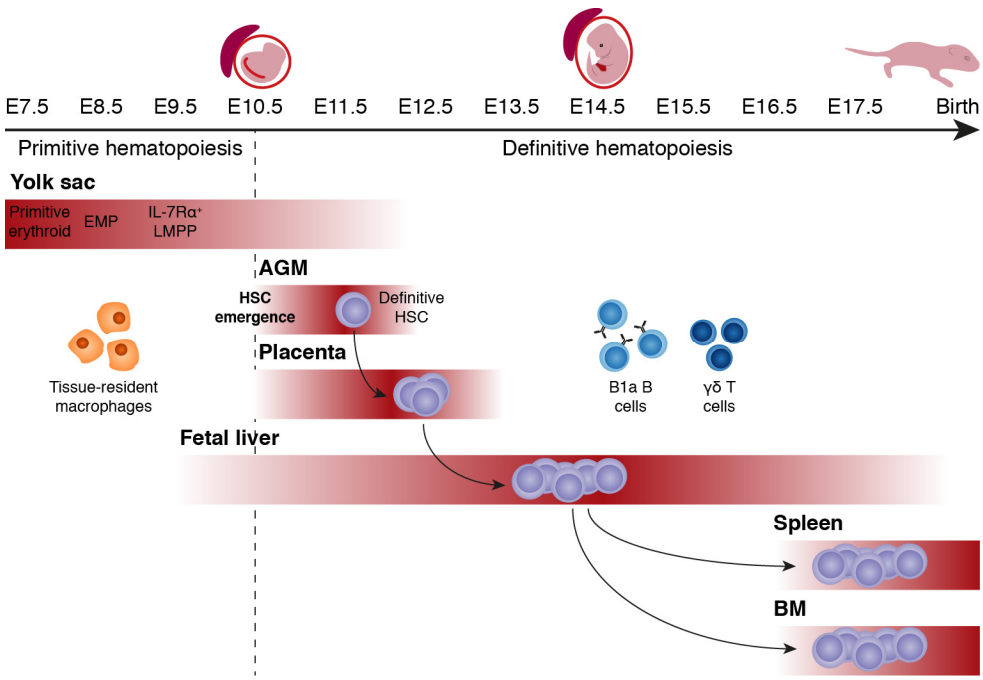

Figure 2. Prenatal development of the mouse hematopoietic system. Hematopoiesis occurs in several overlapping waves and at multiple sites during embryonic and fetal development. Primitive erythroid cells can be detected in the mouse embryo as early as E7.5. Between E8.5-E9.5, EMPs and IL-7Rα$^+$ LMPPs supply the embryo with cells of the erythromyeloid and lymphomyeloid lineages, respectively. EMPs additionally give rise to tissue-resident macrophages which persist throughout life. The emergence of HSCs in the AGM at E10.5 marks the onset of definitive hematopoiesis. HSCs subsequently migrate to and expand in the placenta, the FL, and finally, the spleen and BM. Fetal and early postnatal HSCs give rise to innate-like subsets of B and T cells which persist throughout adulthood.

The differentiation potential of fetal HSCs is similar to that of HSCs in the adult BM (ABM) in the sense that they harbor the capacity to generate progeny of the lymphoid, myeloid and MegE lineages [35, 39]. However, the landscape of the fetal hematopoietic hierarchy is less well-defined than in the adult, and fetal immunophenotypic counterparts of adult HPCs have been shown to exhibit markedly different lineage bias as well as considerable lineage plasticity [40-45]. Elegant lineage-tracing studies have additionally revealed that HPCs derived from the HSC-independent, primitive wave of hematopoiesis contribute to the mature blood cell pool in the fetus even after the emergence of bona fide HSCs [28], which further complicates resolution of fetal HPC populations, as for example EMPs and IL-7Rα^+ LMPPs partially share an immunophenotype with HSC-derived granulocyte-monocyte progenitors (GMPs) and LMPPs, respectively [26, 28].

Although expansion can be regarded as the main focus of fetal hematopoiesis, similarly to embryonic HPCs, fetal hematopoietic stem and progenitor cells (HSPCs) generate immune cells which persist throughout life but are almost exclusively produced during this particular ontogenic stage, including B1a B cells as well as a subclass of γδT cells [46-50]. Despite formally belonging to the adaptive immune system, B1a B cells and fetal-derived γδT cells express a limited repertoire of germline-encoded antigen receptors which mainly recognize conserved patterns of self-antigen expression associated with tissue damage and cellular stress [51]. While their genesis is unique to the pre- and perinatal period, B1a B cells have a life-long function in the first line of defense within the immune system by bridging the gap between innate and antibody-mediated adaptive immunity [47, 48]. This further emphasizes that the adult hematopoietic system is a mosaic of cells generated within time-restricted windows during ontogeny, and establishes that fetal-specific features of hematopoiesis play a crucial part in blood and immune system function throughout postnatal life.

Shifting from a fetal-like to an adult-like state of hematopoiesis

Towards the end of pregnancy, HSCs begin to populate the BM, which remains the primary hematopoietic site for the rest of mammalian life. The HSC niche in the BM consists of multiple cell types, including osteoblasts, endothelial cells and macrophages, which support and regulate adult HSC function through direct cell-cell contact as well as via secretion of a multitude of soluble factors [52].

The ontogeny-related functional changes in HSPCs are believed to be orchestrated by an intrinsically programmed “molecular switch” that is complete approximately 3-4 weeks after birth in mice [53]. During early postnatal life, fetal-specific HSC features are gradually lost as the cells take on an adult phenotype, hallmarked by HSC quiescence, a low translation rate and a balanced output of all blood lineages [36, 54]. Because HSCs are required to sustain hematopoiesis for years, or even

decades in the case of humans, maintenance of a dormant state during homeostatic conditions is crucial for their function as it protects the cells against acquisition of genetic lesions, which would subsequently be propagated within the blood system [55]. Indeed, the remarkable expansion capacity of fetal HSCs [56] comes at the cost of an increased mutational rate in fetal relative to postnatal hematopoiesis [57]. The exact mechanism behind the tolerance for increased mutagenesis in fetal HSCs remains elusive, but may involve a heightened activity of DNA repair pathways during fetal development [58]. Additionally, as maternal-derived bile acids have been shown to protect fetal HSCs from endoplasmic reticulum (ER) stress resulting from high translational rate during fetal life [37], it is plausible that factors stemming from the pregnant mother may provide an additional layer of protection against genotoxic stress in fetal HSCs.

The transition from a fetal to an adult HSC state involves extensive metabolic reprogramming. Whereas rapidly proliferating fetal HSCs utilize oxidative pathways for energy supply [58], quiescent adult HSCs instead primarily rely on anaerobic glycolysis for this purpose [59]. As adult HSCs differentiate towards mature blood cells, energy demands increase drastically and mitochondrial oxidative phosphorylation (OXPHOS) becomes the primary source of ATP from the multipotent progenitor (MPP) state and onwards [60].

In regard to production of differentiated progeny, the rewiring of HSPCs from a fetal-like to an adult-like state includes a shift in lymphoid output from innate-like B1a and γδT cells to the highly antigen-specific B2 B and T cells classically associated with adaptive immunity [47, 48]. In line with this, adult HSPCs express high levels of the enzyme Dntt, which is responsible for N-nucleotide addition during V(D)J-recombination of the BCR and T cell receptor (TCR) [61]. Additionally, during early postnatal life, a switch from expression of fetal hemoglobin (HbF) to expression of adult hemoglobin (HbA) occurs in erythrocyte progenitors, which has important clinical implications as HbF exerts a protective function in sickle cell disease and β-thalassemia [62]. Other differences in lineage potential during fetal and adult life include a stronger bias towards generation of granulocytes in adult relative to fetal HSPCs, which instead are more biased towards the erythroid lineage. In line with this, the transition from fetal to postnatal hematopoiesis is accompanied by an increase in the number of GMPs and a concomitant decrease in size of the megakaryocyte-erythroid progenitor (MEP) population [45].

Intra- and extracellular regulation of hematopoiesis

Fetal and adult-specific regulators of HSPC function

Two of the most potent drivers of a fetal-like state of hematopoiesis are the RNA-binding proteins Lin28b and Igf2bp3 [63, 64]. Lin28b and Ig2bp3 form a complex which stabilizes the expression of other genes highly important for maintaining

fetal-specific features of hematopoiesis, such as Hmga2 [65] and Arid3a [63, 66]. Both Lin28b and Igf2bp3 are rapidly downregulated within the hematopoietic system after birth [50, 63], and ectopic expression of these proteins is sufficient to revert adult HSPCs back to a fetal-like state, which includes reacquisition of a high proliferative rate and erythroid-over-myeloid bias, as well as a significantly increased capacity to generate B1a B cells [45, 63, 64]. Intriguingly, although ectopic expression of Lin28b in adult HSCs increases the cells' proliferative rate, functionality is not compromised [64]. Thus, while exit of quiescence in adult HSCs typically leads to loss of self-renewal capacity and exhaustion, Lin28b confers the cells with the ability to sustain stemness upon entering the cell cycle. Recent work has additionally revealed that Lin28b drives HbF expression during fetal life via suppression of the TF Bcl11a [67], further emphasizing its critical function in maintaining a fetal state in hematopoiesis.

In addition to Lin28b/Igf2bp3, the high self-renewal activity of fetal HSCs is dependent on the genes Sox17 and Ezh2, both of which are largely dispensable for adult HSC function [68, 69]. Fetal hematopoiesis towards all lineages additionally requires intact expression of Runx1 [32], while adult Runx1-deficient HSCs exhibit defects in lymphoid and megakaryocytic differentiation but are viable [70]. Instead, intact expression of, for example, Gfi1, ETS translocation variant 6 (ETV6), Bmi1 and CCAAT/enhancer binding protein (CEBP) α is strictly required for survival and self-renewal of adult, but not fetal, HSCs [71-74]. Loss of either CEBPα or Gfi1 impairs adult hematopoiesis by inducing proliferation and subsequent exhaustion of adult HSCs [72, 73], again highlighting a critical role of strictly enforced quiescence in adult HSC function.

As fetal and adult HSPCs reside in distinct niches, it is perhaps unsurprising that the cells exhibit ontogeny-specific dependencies on extracellular factors for survival and function. Examples include thrombopoietin (TPO), angiopoietin and stem cell factor (SCF) which are required for ABM HSC quiescence and survival, while deficiency of either factor or its receptor (Mpl, Tie2 and cKit, respectively) has little impact on FL hematopoiesis [75-77]. Intriguingly, despite these factors appearing dispensable for fetal HSCs *in vivo*, FL niche cells produce high levels of TPO as well as SCF, and both cytokines are required for *ex vivo* expansion of fetal HSCs [78]. Furthermore, while HSC and MPP numbers are not affected as a consequence of mutations in the SCF receptor cKit, decreased cKit expression results in an overall decrease in FL cellularity [77], suggesting that defective SCF-cKit signaling may affect the genesis of HPCs and other cells downstream of the most immature HSPCs.

In summary, fetal and adult hematopoiesis are regulated by largely distinct sets of gene regulatory networks (GRNs) and extracellular cues which enforce and maintain the key features of hematopoiesis at different developmental stages: in fetus – expansion, and in adult - homeostasis.

Molecular cues governing HSPC differentiation

Differentiation and mature cell production are governed by a complex interplay of TFs and extracellular stimuli in the form of cytokines and receptor-ligand interactions with niche cells. In many cases, lineage commitment is not driven simply by the presence or absence of a particular TF, and one TF may be involved in differentiation towards multiple lineages in a dose-dependent and/or progenitor stage-specific manner [79-82]. An example of such dose-dependency can be found in LMPPs, where high expression of the TF PU.1 (Spi1) drives cells towards myeloid commitment, whereas intermediate expression instead facilitates B lymphopoiesis [79].

It is important to note that although some mechanisms likely are ontogenically conserved, the molecular regulators of lineage potential and differentiation have mainly been studied in the context of adult hematopoiesis. In the adult, generation of CLPs, the common ancestor of B, T and NK cells [83] (**Figure 3**), from lymphomyeloid progenitors requires signaling through cKit and fms-like tyrosine kinase 3 (Flt3) via their respective ligands, SCF and Flt3-ligand (Flt3l) [84, 85]. The survival of CLPs, and thus their ability to produce mature lymphocytes, is additionally dependent on the TF Bcl11a in fetus as well as in adult [86, 87]. B cell development is governed by the TFs Ikaros (Ikzf1), Gfi1, Tcf3, Foxo1, Ebf1 and Pax5, which act in synergy to promote the generation of B cells while simultaneously suppressing myeloid programs in LMPPs and CLPs, and subsequently restricting T and NK cell potential in progenitors downstream of the CLP [82]. Intriguingly, the generation of B1 B cells during fetal life does not require PU.1 [88], but is strictly dependent on Lin28b-mediated expression of Arid3a, and loss of this TF blocks B1 B cell generation from fetal pro-B cells [66]. B cell progenitors, including CLPs, express the receptor IL-7Rα and B cell development is severely compromised in mice lacking its ligand, IL-7 [89]. Interestingly, while myeloid potential is nearly completely lost at the CLP stage of lymphopoiesis *in vivo*, myeloid programs have been shown to be partially reactivated during *ex vivo* culture of adult CLPs under lymphomyeloid differentiation conditions [90].

T and NK cells share a developmental origin with B cells [83], but their generation is governed by a distinct set of molecular cues. Like progenitors of B cells, NK cells develop mainly in the BM in the adult. Differentiation of CLPs towards NK cells occurs via the early innate lymphoid progenitor (EILP) and the NK progenitor (NKP), and is driven by TFs such as Nfil3, Id2, Ets1, Eomes and T-bet [91]. NK cell development is additionally mediated by extracellular stimuli, and shows a specific requirement for IL-15, in addition to other lymphopoiesis-promoting cytokines such as IL-7 and Flt3l, although these are not strictly required for mature NK cell generation following commitment to the NK lineage [92].

During fetal as well as adult life, T cell generation occurs primarily in the thymus, and activation of the Notch signaling pathway via interaction of CLP-derived early

thymic progenitors (ETPs) with the thymic microenvironment is indispensable for proper T cell development. Adult T cell generation is additionally heavily dependent on the cytokines SCF, Flt3l and IL-7, all of which are produced in the thymus. Differentiation of ETPs towards mature T cells is governed by the TFs Tcf1 and Gata3, and irreversible commitment to the T cell lineage, and concomitant loss of NK potential, is mediated by Bcl11b [93]. Notably, similarly to the differential requirement for PU.1 in B2 and B1 B cell development [88], Bcl11b is not strictly required for the generation of fetal-specific subsets of γδT cells [94].

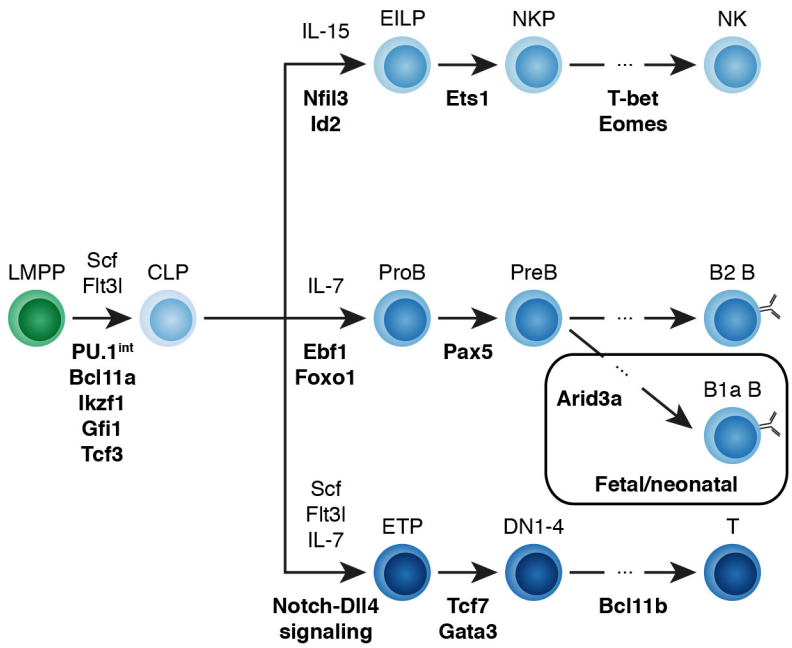

Figure 3. Examples of TFs and cytokines involved in lymphopoiesis. Commitment to the lymphoid lineage and subsequent production of mature B, T and NK cells is governed by a multitude of TFs and cytokines which act in synergy to promote differentiation towards one lineage while suppressing other lineage-associated GRNs.

Initiation and propagation of myeloid programs in MPPs and LMPPs is governed mainly by the TFs PU.1, CEBPα and ε, Gfi1 and Irf8 (**Figure 4**). Following PU.1-mediated commitment to the myeloid lineage, further myeloid differentiation towards GMPs is dependent on CEBPα, while downstream generation mature myeloid cells is driven by Gfi1, CEBPε (neutrophils) and Irf8 (monocytes and DCs) [81]. Irf8 is a highly important regulator of lineage choices between neutrophils, monocytes and DCs, and represents another example of a TF driving lineage commitment in a dose-dependent manner; lack of this TF promotes neutrophil

generation whereas low and high levels drive differentiation towards inflammatory Ly6C$^+$ monocytes and DCs, respectively [80]. Extracellularly, adult myelopoiesis is strongly stimulated by cytokines such as granulocyte-colony stimulating factor (G-CSF), macrophage (M)-CSF and IL-6 [95, 96].

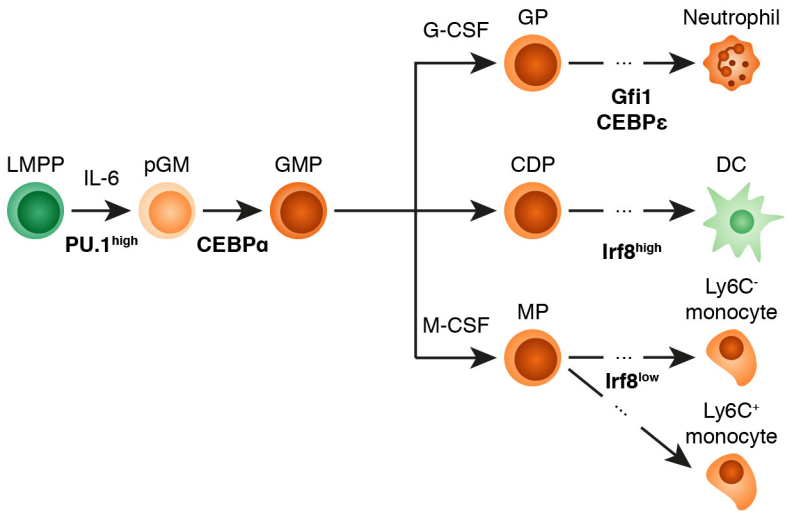

Figure 4. Examples of TFs and soluble factors involved in myelopoiesis. Following PU.1- and CEBPα-mediated commitment of a lymphomyeloid progenitor to the myeloid lineage and the subsequent production of GMPs, differentiation towards neutrophils, dendritic cells and monocytes occurs via the granulocyte progenitor (GP), the common DC progenitor (CDP) and the monocyte progenitor (MP), respectively. Different levels of Irf8 direct differentiation towards monocytes and DCs, whereas neutrophil production is driven mainly by the TFs CEBPε and Gfi1.

Although subject to some debate [12, 97], megakaryocytes and erythrocytes have traditionally been believed to share a common progenitor, the MEP [8] (**Figure 5**). In addition to MEPs, specific subsets of HSCs and MPPs which can act as a direct source of megakaryocytes upon inflammatory stress have been identified within the adult hematopoietic system [12, 98]. Megakaryocyte development and erythropoiesis are dependent on TPO and erythropoietin (EPO) signaling, respectively. Gata1 and Fog1 represent key transcription factors in driving MegE commitment in MPPs, while the subsequent generation of erythrocytes and megakaryocytes from MEPs is governed by c-Myb, Scl and Klf1, and Gata2 and Fli1, respectively [99].

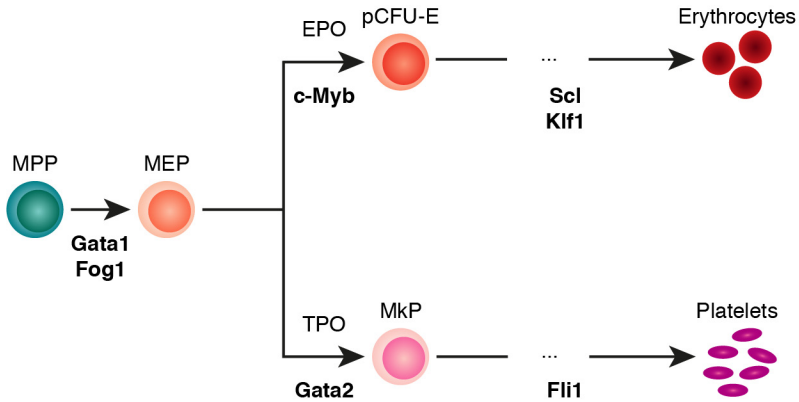

Figure 5. Examples of TFs and soluble factors governing erythro- and megakaryopoiesis. Differentiation MPPs towards MEPs is governed by the TFs Gata1 and Fog1. The TFs c-Myb, Scl and Klf1, and Gata2 and Fli2 drive differentiation of MEPs towards erythrocytes and platelets, respectively.

As mentioned previously, known differences exist in the dependency of fetal and adult HSPCs on TFs considered as fundamental components of GRNs regulating lymphomyeloid differentiation, as well as on cytokine signaling via, for example, SCF and its receptor cKit, which is dispensable for fetal, but not adult, B and T lymphopoiesis [85]. Further characterization of the molecular programs active in fetal HSPCs is necessary in order to fully understand ontogeny-specific factors governing mature cell output at different developmental stages.

Inflammation

Inflammation and inflammatory signaling play a profound role in the regulation of fetal as well as adult hematopoiesis [100]. Adult HSPCs express an array of receptors for inflammatory cytokines and can thus react directly upon inflammatory insult to facilitate production of the various mature cell subsets necessary to resolve the inflammation or infection [22]. Exposure of adult HSCs to Type I interferons (IFNs) drives the cells out of quiescence and skews differentiation, resulting in a massive production of myeloid cells and megakaryocytes [101]. The necessity for rapid megakaryocyte production during inflammation is made evident by the existence of megakaryocyte-biased HSCs and MPPs, which can bypass several intermediate progenitor stages to facilitate rapid production of platelets upon exposure to inflammatory stress [12, 98]. Whether such cell subsets additionally exist in the fetus has not been established.

Following reestablishment of homeostasis post-inflammation, adult HSCs return to quiescence. Importantly, while HSCs can tolerate even extended periods of inflammatory stress, chronic exposure to IFNs renders HSCs sensitive to any

additional proliferative pressure, such as that associated with transplantation, in which case failure to reestablish quiescence ultimately leads to cell death and HSC exhaustion [102]. Intriguingly, the proliferation-promoting effect of IFNs has been utilized in the treatment of leukemia, where administration of IFN is believed to drive dormant leukemic cells into cycle, thus sensitizing them to killing by chemotherapeutic agents [103].

The impact of inflammation on hematopoiesis has mainly been studied in an adult setting. However, differential expression of genes associated with inflammation is one of the most prominent features distinguishing fetal and adult HSPCs, where adult cells show significantly higher expression of multiple Type I IFN target genes [104, 105]. Additionally, stimulation with Type I IFNs has been shown to promote AGM HSPC generation and maturation [105, 106], and Type I IFN signaling was recently identified as one of few clear drivers of the transition from a fetal-like to and adult-like HSC state [107].

Reactive oxygen species

ROS are crucial regulators of cellular signaling in all tissues, including the hematopoietic system. In the cell, ROS levels are carefully balanced by ROS scavengers such as superoxide dismutase (SOD), thioredoxins and the glutathione (GSH) system. Failure to maintain this balance can lead to protein damage, increased mutational rate and cell death [108]. As previously mentioned, adult HSCs preferentially utilize anaerobic glycolysis for ATP generation, which, although considerably less efficient than OXPHOS, generates significantly less ROS [59]. This is critical for maintenance of adult HSC quiescence and function, as exposure to ROS induces proliferation in adult HSCs, which ultimately results in their exhaustion [109, 110]. Fetal HSCs do not require quiescence to maintain stemness; yet fetal HSCs, like adult, are adversely affected by accumulation of ROS. Increased ROS levels as a consequence of ineffective clearance of mitochondria or loss of mitochondrial SOD impairs the reconstitution capacity of fetal HSCs and is associated with erythroid defects [111, 112].

Post-transcriptional regulation in fetal and adult hematopoiesis

Due to technical limitations, comprehensive comparative studies of the molecular networks at play in fetal and adult HSPCs have historically almost exclusively been performed at the level of mRNA [104, 113, 114]. However, several bodies of work have shown that cellular processes such as metabolism, response to inflammation and even malignant transformation are regulated at the protein level in HSPCs [98, 115-118]. Furthermore, adult HSCs, which are hallmarked by a low protein synthesis rate [54], have recently been shown to have a significantly higher rate of transcription compared to more downstream HPCs [119]. This suggests that while mRNA-based analysis can provide an accurate picture of the cells' potential, it is an inadequate predictor of the actual cellular phenotype in quiescent HSCs. In line with

this, the correlation between protein and mRNA expression is particularly poor in HSCs, although that observed in MPPs is only slightly higher [120]. Maintaining a diverse transcriptome may facilitate lineage plasticity in stem cells and MPPs, allowing for rapid translation of the appropriate transcripts and subsequent lineage commitment and mature blood cell production in response to the need of the organism, as recently proposed by Mansell et al [119]. This theory is corroborated by reports showing that upon inflammation-induced emergency megakaryopoiesis, proteins associated with a megakaryocytic cell fate are upregulated in HSCs, while mRNA levels of the same genes are unchanged [98].

Leukemia

Leukemia (blood cancer) is characterized by an overproduction and accumulation of poorly-, or non-functioning, immature hematopoietic cells within hematopoietic tissues. Leukemic cells (blasts) outcompete healthy HSPCs and mature hematopoietic cells, ultimately leading to severely compromised blood and immune system function. In 2021, leukemia is estimated to result in over 20,000 death in the US alone [121]. Importantly, while leukemias are rare relative to other cancers in adults, they account for 25% of all cancers diagnosed in children, making leukemia the most common form of pediatric cancer [122].

Leukemias can be subdivided into different classes based on the kinetics of disease progression (acute or chronic) as well as on the hematopoietic lineage affected (e.g. myeloid, lymphoid or mixed lineage). As with most other cancers, the incidence rate of chronic myelogenous and chronic lymphocytic leukemia (CML and CLL, respectively) increases with age, and CML and CLL are rarely observed in younger patients. Similarly, acute myeloid leukemia (AML) has a peak incidence in adults over 60. Critically, this is not the case for acute lymphoblastic leukemia (ALL), which is rare in adults, but represents the most common form of leukemia in children [123].

Disease outcome, survival rate and treatment options for leukemia are heavily influenced by disease subtype as well as several additional factors, including age and mutational status. For example, while the overall 5-year survival rate for children with ALL is nearing 90%, only 25% of older ALL patients survive more than 5 years after disease onset [124]. Additionally, certain mutations are associated with a poor prognosis and response to treatment regardless of the age of the patient [125, 126].

Research conducted over several decades has established the existence of a population of self-renewing cancer stem cells in leukemia [127]. Like normal hematopoiesis, leukemia is believed to be organized in a hierarchical fashion, where leukemia stem cells (LSCs) situated at the apex of the “cancer hierarchy” are

responsible for maintaining a continuous output of blasts which make up the bulk of the tumor tissue (**Figure 6**) [128-130]. At least part of the LSC population is considered to reside in a quiescent state, making these cells difficult to eradicate with most chemotherapeutic agents, which preferentially target actively cycling cells [131, 132]. Critically, LSCs persisting following treatment frequently acquire additional mutations and drive relapse of an often times even more aggressive disease. Considerable efforts have been made to develop therapeutic strategies which target LSCs specifically, but to date, few such treatments have reached the clinic [127]. The development of targeted therapies has been hindered in part by the inability to accurately characterize the immunophenotype of LSCs, which may additionally vary between patients [133] or even between different LSC populations in the same patient [134]. As such, further exploration of the molecular features of LSCs and other populations of leukemia cells is urgently required in order to improve current treatment strategies and minimize the risk of relapse.

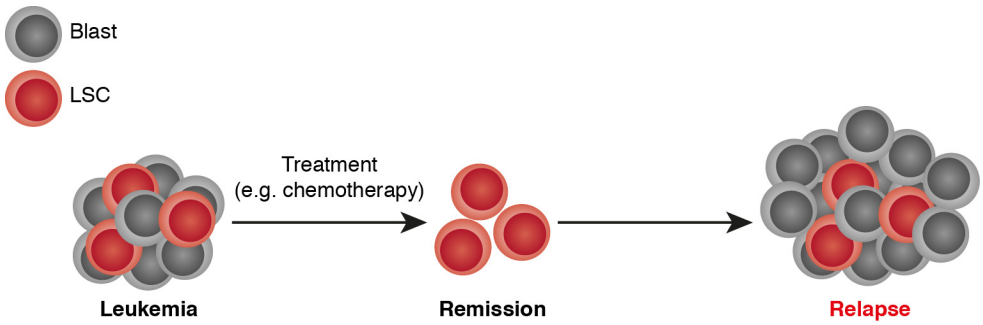

Figure 6. The leukemia stem cell hypothesis. Quiescent, self-renewing LSCs make up a minor fraction of the bulk tumor tissue. Conventional chemotherapeutic approaches effectively eradicate leukemic blasts but often fail to eliminate the non-cycling LSCs. LSCs persisting following treatment can give rise to new blasts, thus driving relapse.

Infant and childhood acute leukemia

Mutations and disease subtypes

Infant (<1 year of age) and childhood (<18 years of age) acute leukemias are often initiated already *in utero*, as has been made evident by twin studies and identification of cells harboring leukemic mutations in retrospective analyses of blood samples taken from newborns [135, 136]. Several different first-hit mutations acting as drivers of infant and childhood (c) ALL have been identified. The most prevalent mutation in infant ALL involves fusion of the mixed lineage leukemia (MLL) gene with one of its many partners, most commonly ALL-1 fused gene from

chromosome 4 (AF4), while the fusion of ETV6 and RUNX1 genes is the most common driver mutation in cALL [137]. The ETV6-RUNX1 translocation is present in approximately 1% of newborns, but requires additional mutational events for disease to develop. Thus, most children born with the translocation never develop overt leukemia [138]. In stark contrast, MLL-translocations are highly potent oncogenes that rarely require additional mutations to produce aggressive leukemias. MLL-rearranged (MLLr) ALL has a peak incidence in children below 2 years of age [139], indicating that leukemic transformation is complete or nearly complete even before birth.

Different genetic lesions preferentially give rise to different leukemia subtypes. For example, ETV6-RUNX1 is almost exclusively associated with B cell precursor leukemia, while MLL-rearrangements typically give rise to pro-B cell leukemia or AML when occurring in infants and children [140]. The fusion of the PML and RARA genes (PML-RARA), which can be found in approximately 20% of pediatric AML cases, is instead associated with a specific subtype of AML termed acute promyelocytic leukemia [141].

Hereditary and environmental risk factors

Few concrete predisposing factors have been identified in infant and childhood leukemia. Compared to the rest of the population, children with Down syndrome are at a 500-fold and 20-fold increased risk of developing acute megakaryoblastic leukemia (AMKL) and ALL, respectively [142, 143]. This has recently been proposed to be a consequence of an elevated mutational rate in fetal trisomy 21 HSPCs [57]. Hereditary mutations in lymphopoiesis-associated genes, such as Ikfz1 [144], modestly increase the risk of cALL [145]. Exposure to ionizing radiation is the only environmental factor that has been relatively consistently linked to an increased risk of pediatric leukemia, while studies investigating the association between childhood leukemia incidence rates and pre- or postnatal exposure to other possible mutagens, such as pesticides, have shown highly variable results [146]. For children born with the ETV6-RUNX1 translocation, a strong correlation has been observed between delayed exposure to infection in early postnatal life and the risk of acquiring secondary mutations which subsequently lead to the development of cALL [140]. However, in MLLr as well as in ETV6-RUNX1-driven leukemia, the environmental or hereditary factors promoting the acquisition of the initial driver mutation *in utero*, if any such factors exist, remain elusive.

Treatment and prognosis

Over the past 50 years, the overall survival rates for cALL have increased from a mere 10% to nearly 90% [124]. However, current treatment schemes involve intensive chemotherapy which is associated with severe, sometimes life-long, side effects [147]. Additionally, despite even the youngest leukemia patients receiving a relatively harsh treatment, the prognosis for infant leukemia remains dismal with an

overall survival rate below 50% [125, 126]. Alternative or complimentary therapeutic approaches include HSC transplantation (HSCT) and the recently approved chimeric antigen receptor (CAR)-T cell therapy. Critically, HSCT is not associated with better, but rather worse, outcomes in MLLr infant leukemia [125]. CAR-T cell therapy has shown promising results in cALL, but is as of yet not implemented on a large scale and, similarly to chemotherapy, is associated with considerable adverse systemic effects [148]. As such, there is a critical need for development of novel, targeted therapies which could eradicate leukemic cells while sparing other organs, thus improving treatment outcomes and alleviating some of the more severe side effects associated with anti-leukemia therapy.

MLL-rearranged acute leukemia

MLL fusion partners and leukemia subtypes in infant and adult MLLr leukemia

The fusion of the MLL gene with a partner gene produces a potent oncogene which can initiate and drive a highly aggressive acute leukemia with few or no cooperating mutations. Translocations involving the MLL gene are associated with myeloid as well as lymphoid leukemias, but the incidence rate for the two subtypes is remarkably different between young children and adults. MLLr ALL incidence rates peak before 2 years of age, followed by a sharp drop during later childhood and a modest increase in the elderly population. MLLr AML is rarely observed in infants and is instead more common in adults over 60 years of age [139]. MLL-translocations are present in as much as 80% of infant ALL, and in approximately 50% of the few infant AML cases that do occur [126, 149]. Perplexingly, *de novo* MLLr acute leukemias are relatively rare in adults, where MLL-translocations are instead more commonly associated with AML arising following anti-cancer treatment with a specific class of chemotherapeutic agents known as topoisomerase II inhibitors [150]. Even when accounting for secondary leukemias, MLL-rearrangements are found in only 9% of all adult acute leukemia cases [151]. Nevertheless, the presence of an MLL-translocation is a predictor of poor outcome in infant as well as in adult leukemia [125, 150].

Close to 80 fusion partners of the MLL gene have been identified to date [152]. Some of these are extremely rare, and only 5 of all known MLL-fusions account for 80% of MLLr leukemia cases (**Figure 7**). In infant as well as in adult MLLr leukemia, AF4 represents the most common fusion partner of MLL, followed by ALL1-fused gene from chromosome 9 (AF9). For other MLL fusion partners, however, distinct age-associated patterns have been observed. The fusion of MLL with the gene eleven-nineteen leukemia (ENL) is present in 18% of infant MLLr cases, but only in 8% of adult cases. This fusion protein (FP) is additionally associated with different disease phenotypes in infant and adult MLLr leukemia: the MLL-ENL fusion is found in 22% of infant, but only 12% of adult, ALL cases, and

is very rarely observed in infant MLLr AML. MLL-AF9 on the other hand, is frequently found in infant ALL as well as AML, but is almost exclusively associated with AML in adults [139]. The reason behind the different behavior of specific MLL-FPs in infant an adult leukemia remains a matter of open investigation.

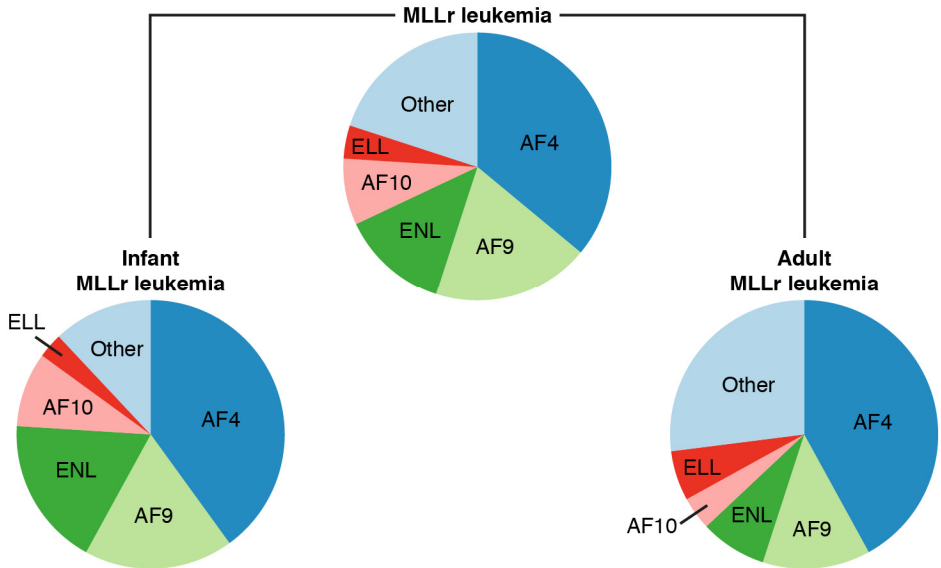

Figure 7. Occurrence of different MLL fusion partners in MLLr leukemia. AF4 is the most common MLL fusion partner in infant as well as in adult MLLr leukemia. The MLL-ENL and MLL-AF10 fusion oncogenes are more commonly observed in infant than in adult MLLr leukemia, whereas the opposite is true for MLL-ELL. Data from [139].

Molecular features of MLLr leukemia

The structure and function of the wild-type (WT) MLL protein have provided important insight into the molecular mechanisms governing MLL-FP-driven leukemogenesis (**Figure 8A**). The N-terminal part of the MLL contains, among other elements, three AT-hooks which enable direct binding of MLL to AT-rich DNA sequences, while the SET domain near its C-terminus confers the protein with methyltransferase activity [153]. These structural elements enable the MLL protein to act as a potent transcriptional regulator, and its targets include a multitude of genes indispensable for normal development, such as a group of evolutionarily conserved TFs known as the Hox genes. The breakpoint cluster region within the MLL gene is positioned in such a way that while the amino terminus of MLL is retained in MLL-rearrangements, the SET domain is invariably lost upon fusion of MLL with a partner gene (**Figure 8B**) [154]. Despite this, the MLL-FP retains its ability to regulate transcription, and the expression of MLL-FPs such as MLL-ENL

is associated with extensive alterations in histone methylation, which promotes transcription of genes that confer the cell with leukemic potential [155]. Other MLL-FPs have been reported to enforce aberrant gene expression by promoting transcriptional elongation. These features of the oncogenic FP have led to MLLr leukemia being described as an epigenetic disease [156].

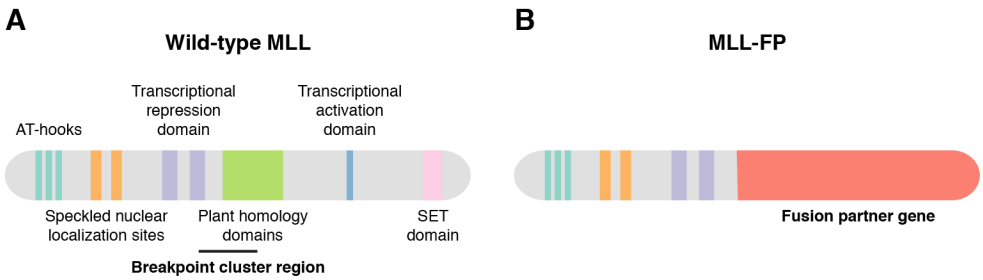

Figure 8. Structure of the wild-type and fusion MLL protein. A) The WT MLL protein is 3969 amino acids long and consists of several functional domains. The three AT-hook domains near the N-terminus are involved in DNA binding. The AT-hook domains are followed by two speckled nuclear localization sites, a transcriptional repression domain, plant homology domains, a transcriptional activation domain and, finally, a SET domain at the extreme C-terminus of the protein. B) The N-terminus of the WT MLL protein is retained in MLL-FPs, while the C-terminus containing the SET domain is lost and replaced by a fusion partner.

Compared to other leukemias, MLLr leukemia is hallmarked by a highly distinct gene expression profile [157]. By driving expression of genes such as the Hox genes and Meis1, MLL-fusions confer cells with stem cell-like properties [158], including acquisition of self-renewal capacity in otherwise short-lived lineage-committed HPCs which can then function as potent LSCs [159-162]. Upon expression of MLL-AF9 in GMPs, a large number of HSC-associated genes are re-activated [162]. More recent work has outlined two distinct sets of MLL-ENL target genes, which involve interaction of the FP with either chromatin modifier DOT1L or positive transcription elongation complex b (P-TEFb). The interaction of MLL-ENL with DOT1L and P-TEFb leads to enhanced expression of leukemia-associated TFs (e.g. HoxA cluster genes, Meis1 and Mecom) and genes involved in protein translation (e.g. Myc), respectively [163]. In line with the induced differentiation arrest in cells transformed by MLL-fusions [158], the expression of genes associated with lineage commitment are downregulated in MLL-FP-expressing LSCs [163].

Cell of origin in infant and adult MLLr leukemia

MLLr is associated with profound alterations in the composition of the HSPC pool, which impedes attempts at identifying the cell of leukemia origin once the disease

is established. As such, the majority of our current understanding about leukemia-initiating cells (LICs) in MLLr leukemia comes from mouse and xenograft models [164]. However, despite decades of research, no consensus has yet been reached about the exact identity of the LIC(s) in MLLr leukemia. Studies utilizing retroviral approaches to induce MLL-FP expression in HSPCs have indicated that HSCs can act as potent LICs in MLLr leukemia, and that HSCs expressing the fusion oncogene give rise to a more aggressive AML-like disease compared to more downstream hematopoietic progenitors [165, 166]. In stark contrast, more recent work utilizing a doxycycline (DOX)-inducible, transplantation-based murine model of MLLr leukemia has shown that while several subsets of lineage-biased/lineage-committed HPCs (including GMLPs, CLPs and pGMs) expressing MLL-ENL give rise to AML with close to 100% penetrance, HSCs fail to establish leukemia [159, 160]. These discrepant results are perhaps unsurprising considering the vastly different approaches used in the different studies. Retroviral models require *ex vivo* manipulation of cells and often produce gene expression levels far higher than those observed in physiological conditions [159], which may have a severe impact on cellular functionality and leukemia competence. Potential caveats with transplantation-based models have also been reported, including an accelerated leukemia progression and myeloid skewing as a consequence of transplantation-associated stress in the hematopoietic system [167].

Regardless, while a host of different HSPCs, ranging from HSCs to committed T cell progenitors, have been nominated as potential LICs in adult MLLr AML, less is known about the cell of origin in infant MLLr leukemia [164]. Age-specific cell characteristics have recently been shown to play a detrimental role in leukemias driven by the NUP98-HOXA9 [168] and the ETV6-RUNX1 oncogenes [169]. This has also been suggested for some MLLr leukemias, where faithful recapitulation of the distribution of leukemic phenotypes has proven particularly difficult, as most model systems have a strong bias towards AML [164]. However, MLLr lymphoid leukemia has been successfully produced from murine FL cells [170] and human cord blood cells [171-173]. Additionally, a recent study has shown that fetal and neonatal HSPCs are more susceptible to MLL-ENL-mediated transformation than their adult counterpart [167], providing a possible explanation for the higher prevalence of MLL-rearrangements in infant relative to adult acute leukemia. Other work has demonstrated a profound role of the developmental stage of the niche in determining disease phenotype, and that the neonatal microenvironment more efficiently drives a lymphoid-like leukemia compared to the ABM [174]. Collectively, these studies highlight the importance of the developmental stage from which the LIC is derived and in which the leukemia is propagated in determining disease phenotype and progression. Further exploration of the molecular makeup of fetal and adult HSPCs and their microenvironment is critical in order to elucidate the factors responsible for the cells' differential susceptibility to initiation of MLLr ALL and AML.

Tools for the study of hematopoiesis

Mice as a model system

Since the first definitive proof of the existence of HSCs was demonstrated in the mouse [175, 176], mice have remained one of the most widely used model organism in pre-clinical hematology research. In-bred laboratory mice are genetically homogeneous, thus allowing for assessment of HSPC function in a live organism with limited influence of biological variability. Mice have short gestation periods and reach sexual maturity relatively early in life, which enables rapid breeding of large quantities of animals and renders them an excellent model for studying the impact of genetic manipulation in mammalian systems. In hematology research, mice play an especially important role in the field of developmental hematopoiesis, as the dynamics of prenatal hematopoiesis are highly similar between mouse and man [177] and access to human fetal material is both limited and subject to considerable ethical debate. Murine models are additionally invaluable for the study of initiation events in malignancies where humans remain largely asymptomatic until later disease stages, as well as for diseases with a rapid progression and low survival rate in humans, such as acute leukemia [164].

While studies conducted in mice have undoubtably laid the foundation for our current knowledge and understanding of hematopoiesis, murine models are not without limitations. A general issue with animal models relates to the lack of immune stimuli in the controlled laboratory environment, which poorly mimics the relatively high level of pathogen exposure most humans are subjected to in daily life. Furthermore, mouse and human HSPCs exhibit differences in cell cycle kinetics [178], immunophenotype [179, 180] and telomere length, among other characteristics with strong implications in cellular function [181]. It is therefore unsurprising that several human malignancies, including leukemia, are difficult to faithfully recapitulate in murine disease models [164]. The development of a variety of humanized mouse models [182-184] has expanded the possibilities for investigating the dynamics of hematopoiesis and hematological malignancies derived directly from primary human cells by xenotransplantation. Some human malignancies remain difficult to model even when using primary patient material xenografted into immunocompromised mice [185]. However, the number of human diseases which can be accurately recapitulated in mice is increasing rapidly as more advanced humanized mouse models are being generated [182].

In summary, while mice may be regarded as an imperfect surrogate for modeling human development and disease in many aspects, the numerous benefits of murine models render them an indispensable part of hematology research. Recent progress in the characterization of human HSPCs [186] will further aid in raising awareness of similarities as well as differences between the mouse and human hematopoietic

system, which will further facilitate translation of findings in animal models to the human setting.

Mass spectrometry-based quantitative proteomics

An introduction to mass spectrometry-based proteomics

Mass spectrometry (MS)-based proteomics represents the gold standard technology for global characterization of cellular proteomes. In addition to providing a means for protein identification, both relative and absolute protein quantification can be achieved using a range of different methodologies, making MS an extremely powerful tool for interrogating and comparing different cell types or cellular states at the level of the main functional units of the cells - the proteins.

In the most widely used MS-based proteomics techniques – termed "bottom-up proteomics" - protein identity is inferred from peptide sequences generated by enzymatic digestion (most commonly using trypsin) of the proteome [187]. In its simplest form, the initial steps of a typical bottom-up cellular proteomics workflow involve extracting proteins by cell lysis, followed by protein denaturation to increase accessibility of the peptide sequence for subsequent proteolytic digestion (**Figure 9**). The digested peptides are usually introduced into the MS instrument after some form of pre-fractionation to reduce the complexity of the peptide mixture. In addition, the peptide mixtures are most commonly separated by hydrophobicity-based separation using reverse-phase (RP) liquid chromatography (LC) directly coupled to the MS instrument, a method referred to as LC-MS [187].

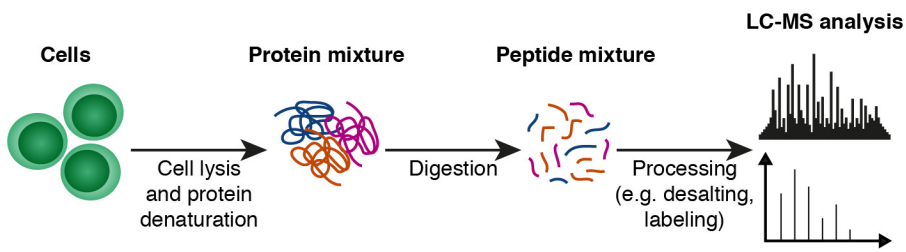

Figure 9. General workflow for cellular MS-based proteomics. Cell samples are lysed and treated with denaturing agents to extract proteins and increase peptide sequence accessibility. Protein mixtures are enzymatically digested to peptides and processed further for e.g. salt removal, pre-fractionation and labeling prior to LC-MS analysis.

Peptides eluting from the LC are ionized and introduced into the MS instrument, where they undergo one (MS^2) or several (MS^n) fragmentation steps, allowing for subsequent peptide identification and extraction of quantitative information. Global

MS analyses can be performed in either data-dependent or -independent acquisition (DDA and DIA, respectively) mode. In the former, only the most intense peptide ions are selected for subsequent fragmentation, and the number of peptides selected from the first-level MS scan is restricted by setting a limit for either the number of peptides (Top-N method) directly, or by limiting the time allowed for the scans (Top-Speed method). In contrast, DIA approaches do not rely on peptide ion intensity for selection of ions for fragmentation. Instead, fragmentation is performed on all ions that are present within a given mass over charge (m/z) range, often separated into multiple m/z-windows [187]. As such, DIA represents a significantly less biased approach for protein identification and quantification relative to DDA approaches [188]. However, both methods are associated with their own set of advantages and caveats, which will be discussed in more detail below.

Quantification strategies in MS-based proteomics

MS-based protein quantification can be achieved by an array of different methods which are associated with more or less manipulation of cells, proteins or peptides during sample preparation. In label-free quantification (LFQ), proteins are quantified mainly based on the intensity of the peptide ions [189]. LFQ has the advantage of not requiring introduction of any labeling reagents during proteomic sample preparation, and can be implemented in DDA as well as DIA approaches [188]. However, a significant disadvantage of LFQ approaches in both DDA and DIA is the need to keep samples separate during all steps of the proteomic workflow [190], which renders this quantification method prone to technical variability arising from inconsistencies in sample handling or in the performance of conventional LC-MS instrumentation.

To diminish the influence of sample-to-sample variation, a number of labeling methods which enable combination of several samples at different stages of the sample preparation workflow have been developed. Metabolic labeling by stable isotope labeling of amino acids in culture (SILAC) generates samples which can be distinguished already at the cell level, allowing for multiplexing at a very early stage of the proteomic sample preparation workflow [191]. However, the utility of this protocol is limited to cell types which can be maintained in culture, which, until very recently [192], did not include primary HSPCs [193].

Chemical labeling approaches do not require pre-culture of cells, and are thus suitable for a broader range of sample types. Such approaches include dimethyl labeling [194] and labeling with isobaric tags [195, 196]. The former method utilizes introduction of different isotopomers of formaldehyde and cyanoborohydride to the peptide mixture, which results in the generation of peptide ions with a difference in mass, allowing for up to three samples to be combined and analyzed together. Peptides stemming from different samples can be distinguished and quantified relative to one another in the first, full MS, scan (**Figure 10**) [194]. Dimethyl labeling is an attractive quantification approach, in large part due to the accessibility

and inexpensiveness of all required reagents. This method does, however, come with certain drawbacks, with two key issues being the low level of multiplexing achievable and the, compared to other approaches, larger risk of missing quantitative values as a result of unequal peptide ion sampling from the different assayed conditions, which is inherent to MS^1-based quantification methods in DDA approaches.

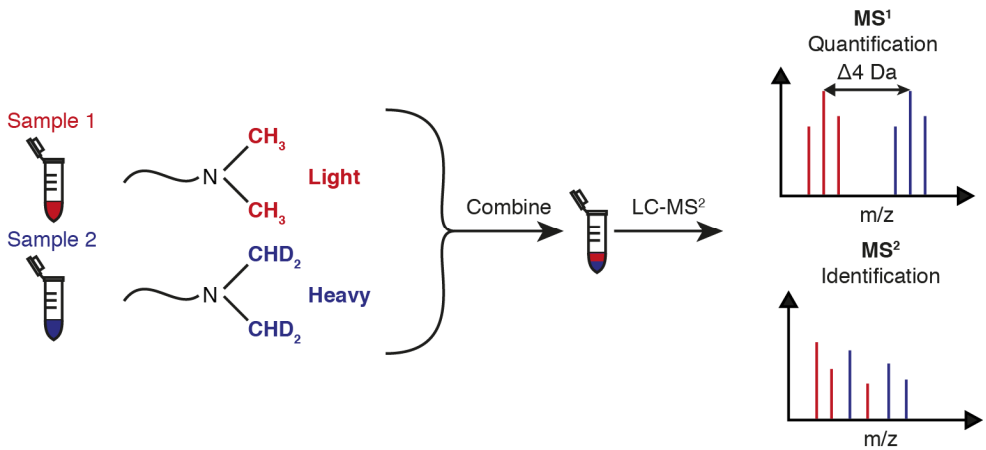

Figure 10. Principles of dimethyl labeling for multiplexed quantitative proteomics. Samples are differentially labeled with "light" and "heavy" reagents. Samples are then combined and processed together. Peptides stemming from the "light"- and "heavy"-labeled samples are separated by a 4 Da mass difference in the full MS scan, allowing for the different samples to be distinguished.

Both of these issues are addressed elegantly in isobaric tag-based quantification methods. Two such methods, isobaric tags for relative and absolute quantitation (iTRAQ) and tandem mass tag (TMT) labeling, have been commercialized by Sciex and Thermo Fisher Scientific, respectively [195-198]. While initially only allowing for multiplexing of 4-6 samples, the most recent isobaric labeling reagents (TMTpro; Thermo Fisher Scientific) enable combined analysis of up to 16 different samples in one MS experiment [199]. Because these labels are isobaric, they are indistinguishable from one another in the full MS scan. Every peptide ion selected for fragmentation will thus most likely include labels from all samples, which greatly increases the chance of obtaining quantitative information for all interrogated conditions in the multiplex. The mass reporter part of the TMT label can be cleaved off by higher-energy collision-induced dissociation (HCD), and relative quantification can subsequently be performed by comparing the intensity of the different reporter ions in tandem MS (**Figure 11**) [196].

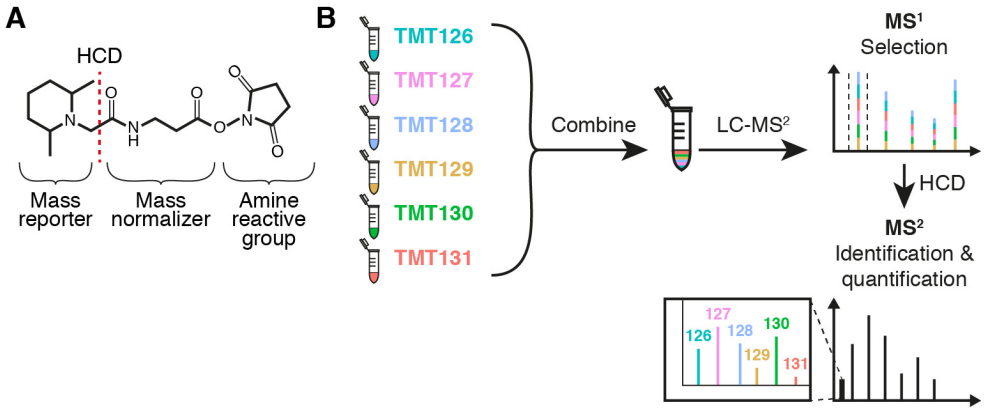

Figure 11. Principles of TMT labeling for multiplexed quantitative proteomics. A) Each TMT label is made up of a mass reporter, a mass normalizer and an amine reactive group. The amine reactive group binds to the N-terminus and lysine residues in the peptide. The mass normalizer compensates for weight differences in the mass reporter. The mass reporter can be cleaved of using HCD, enabling relative quantification of peptides in up to 16 samples in one MS experiment. B) Example workflow for a TMT6plex experiment. Labeled samples are combined and subjected to LC-MS^2 analysis. TMT labels are indistinguishable from one another in the first MS scan, greatly increasing the chance of generating quantitative values for all samples in the multiplex. HCD-mediated peptide ion fragmentation results in the reporter ions being cleaved off and relative quantification to be performed by assessing reporter ion intensities.

The caveats associated with isobaric labeling approaches relate directly to the chemical composition of the labels. Because the reporter ions are separated by a disappearingly small mass difference (less than 0.01 Da in the case of TMTpro [199]), methods utilizing isobaric labeling require high-resolution MS instrumentation to resolve the reporter ions. In addition, co-fragmentation of peptides with very similar mass can lead to an underestimation of differentially expressed peptides. The quantitative accuracy can be improved considerably by implementing synchronous precursor selection (SPS)-MS^3 methodology, in which a collision-induced dissociation (CID)-based fragmentation step is performed following the initial full scan, and fragment ions generated by CID are subsequently selected for HCD-based MS^3 analysis, allowing for substantially higher resolution and lower occurrence of co-fragmentation events [200]. Additionally, recent developments in gas phase fractionation technology have substantially improved quantitative accuracy in MS^2-based approaches [201], which have the advantage of requiring less expensive instrumentation compared to MS^3-based methods and are thus more widely available. Importantly, while the multiplexing capacity and quantitative accuracy of isobaric tag-based methods is unparalleled by any other quantification strategy, the demand for high resolution renders quantitative MS-

methods utilizing isobaric labeling slower than approaches with acquisition at lower resolution, which results in a significant decrease in the number of peptides quantified per unit of time.

Moving towards proteomics at the single HSPC level

Unlike mRNA, the protein content of a cell can not be amplified in the test tube. Thus, comprehensive proteomic characterization of rare cell types, such as primary HSPCs which are challenging to expand in vitro [193], has historically been difficult. MS instrumentation has long possessed sufficient sensitivity for peptide detection at or close to the single-cell level. However, in order to resolve complex protein mixtures with a dynamic range spanning at least seven orders of magnitude in the case of a mammalian cellular proteome [202], considerable pre-processing of samples is required prior to introduction into the MS instrument, and such pre-processing often results in substantial sample loss. Several sample preparation protocols designed specifically for low-input samples have been developed to combat these issues, including nanodroplet processing in one pot for trace samples (nanoPOTS) [203] , single-pot, solid-phase-enhanced sample preparation (SP3) [204] and in-StageTip (iST)-based methods [205]. SP3- and iST-based sample preparation protocols have been used to identify over 3000 proteins from a starting material of as little as 1 µg of cell lysate (corresponding to approximately 20,000 HSPCs) with high quantitative reproducibility [206]. Proteome coverage can additionally be increased by performing pre-fractionation prior to MS analysis, which reduces the sample complexity and as such enables identification of low-abundant peptide species which would otherwise be masked by high-abundant components in an unfractionated peptide mixture. For this purpose, iST-based high pH (HpH)-RP pre-fractionation has proven particularly well-suited for starting materials of 10-100 µg, allowing for identification of almost 5000 proteins even in the lower part of this range [207].

Isobaric labelling approaches which enable multiplexing prior to MS analysis have proved highly beneficial for low-input proteomics. Apart from reducing technical variability, isobaric labelling provides a means of signal amplification in MS^1 and thus facilitates subsequent quantification and comparison across the assayed conditions. Isobaric labels have a central part in the recently developed single-cell proteomics by mass spectrometry (SCoPE-MS) technology, where protein quantification in single cells is made possible by including a larger, less refined sample in the multiplex (a so-called carrier proteome), which facilitates detection of ions in MS^1 and thus increases identification rate in the single cell samples [208, 209]. This technique has been applied to resolve macrophage heterogeneity, where over 3000 proteins across approximately 1500 single cells were successfully quantified using SCoPE-MS [209]. Further development of single-cell proteomics will however be required in order to increase the peptide identification confidence to the levels currently achievable with bulk proteomics.

Only a handful of MS-based studies detailing the proteomic composition of HSPCs have been performed to date [98, 115, 120, 210, 211]. The earlier of these studies utilized "classic" sample preparation methods to gain impressive coverage of the adult HSPC proteome from a starting material of 400,000-1,000,000 fluorescence activated cell sorting (FACS)-purified cells [98, 115, 210]. In more recent work, deep proteomic coverage (>4,000 identified proteins) has been achieved in as little as 50,000 mouse and 25,000 human HSPCs by implementing iST-based sample preparation and advanced label-free MS approaches [120, 211]. As the field of low input/single-cell proteomics continues to advance, it is highly plausible that the first MS-based single-cell proteomic characterization of the hematopoietic system is only a few years away.

Flow cytometry and FACS

Flow cytometric technologies build upon the light-scattering ability of particles/cells and the differential excitation and emission properties of natural and synthetic fluorophores. In addition to cell size and granularity, modern multi-laser flow cytometers and FACS instruments can analyze up to 20 parameters detectable by fluorophore-conjugated antibodies or fluorescent dyes [212].

The relative ease at which hematopoietic tissues can be obtained and homogenized into single-cell suspensions has enabled detailed interrogation and coupling of the surface immunophenotypes and function of mouse HSPCs by flow cytometry and FACS (**Table 1**). Since the late 1980's, it has been known that all HSC activity in mice is contained within a population of cells which express cKit and stem cell antigen-1 (Sca-1) but lack surface expression of mature lineage (Lin; e.g. Gr-1 [myeloid], B220 [B cells], Ter119 [erythroid]) markers (LSK cells) [213, 214]. Subsequent work have resulted in the discovery of several additional HSC cell surface markers [215, 216], and adult long-term (LT)-HSCs defined immunophenotypically as LSK $CD150^+$ $CD48^-$ $EPCR^+$ can now be separated from non-repopulating HPCs at a purity of >60% by FACS [217]. A similar immunophenotype can be used to purify FL HSCs [216]. However, fetal HSCs additionally express CD11b/Mac-1 [35] and low to intermediate levels of Flt3 [43, 218], whereas expression of these two markers is associated with myeloid cells and MPPs, respectively, within the adult hematopoietic system [8, 9]. Flt3 in combination with CD48 and CD150 can additionally be used to separate non-LT-HSCs within the LSK population into short-term (ST)-HSCs (LSK $CD150^-$ $CD48^-$ $Flt3^-$), LMPPs (LSK $CD150^-$ $CD48^+$ $Flt3^{high}$) and other more or less lineage-biased MPP subsets [9].

MegE- and myeloid-restricted HPCs are contained within the Lin^- $Sca\text{-}1^-$ $cKit^+$ (LS^-K) population of the FL and BM [219, 220]. Based on the expression of CD150, CD41, CD16/32 and Endoglin, LS^-K myeloerythroid HPCs in the adult can be further sub-fractionated into MkPs (LS^-K $CD41^+$ $CD150^+$), GMPs (LS^-K $CD41^{low}$

CD150$^-$ CD16/32$^+$) and pGMs (LS$^-$K CD41^{low} CD150$^-$ CD16/32^{low} Endoglin$^-$), as well as other more mature MegE precursors [8]. The immunophenotype and function of the corresponding HPC populations in the fetus are less well-defined.

Lymphoid-restricted CLPs can be found within a cell population immunophenotypically defined as Lin$^-$ Sca-1^{low} cKit^{low} (LS^{low}K^{low}) Flt3^{high} IL-7Rα^+ [7, 8, 42]. Adult CLPs can be further subdivided into multipotent Ly6D$^-$ and largely B cell-restricted Ly6D$^+$ cells [221], whereas the existence of a similar branch point in fetal lymphopoiesis has not been established.

It's important to note that while the immunophenotypes described above mark HSPCs in the most commonly used strains of laboratory mice, surface marker expression is known to differ between mouse strains [222], and is additionally subject to change upon disruption of homeostasis [103]. As such, care must be taken when aiming to quantify HSPC populations based on immunophenotype alone, in particular in perturbed experimental settings.

Table 1. Cell surface markers for different populations of HSPCs in fetus and adult.

Cell type	Fetal	Adult
LT-HSC	LSK CD150$^+$ CD48$^-$ Flt3^{low} Mac-1$^+$ EPCR$^+$	LSK CD150$^+$ CD48$^-$ EPCR$^+$ Flt3$^-$
ST-HSC	LSK CD150$^-$ CD48$^-$ Flt3^{int}	LSK CD150$^-$ CD48$^-$ Flt3$^-$
LMPP	LSK CD150- CD48+ Flt3^{high}	LSK CD150$^-$ CD48+ Flt3^{high}
MkP	?	LS$^-$K CD41$^+$ CD150$^+$
pGM	?	LS$^-$K CD41^{low} CD150$^-$ CD16/32^{low} Endoglin$^-$
GMP	?	LS$^-$K CD41^{low} CD150$^-$ CD16/32$^+$
CLP	LS^{low}K^{low} Flt3^{high} IL-7Rα^+	LS^{low}K^{low} Flt3^{high} IL-7Rα^+ Ly6D$^{+/-}$

In vitro assays

In vitro assays provide a means of assessing HSPC function while circumventing many of the ethical issues associated with *in vivo* experiments such as transplantation and administration of drugs directly to laboratory animals. Over the

past decades, several model systems which allow for interrogation of HSPC lineage potential *ex vivo* have been established. The OP9 and OP9DL1 stromal cell lines, developed in the mid 1990's and early 2000's, respectively, support efficient generation of lymphomyeloid progeny from HSPCs over the course of several days to weeks [223, 224], thus allowing for discrimination of bipotent lymphomyeloid and unipotent lymphoid- or myeloid-restricted HSPC populations. The OP9 system has, however, been shown to strongly promote myelopoiesis even in progenitor populations which *in vivo* only give rise to lymphoid cells [90]. As such, *in vitro* differentiation assays performed in co-culture with stromal cell lines should perhaps be regarded as a measure of HSPC potential, but not necessarily of their steady-state function. HSPC differentiation potential can additionally be assessed in liquid culture or in colony-forming cell (CFC) assays performed in semi-solid, defined media such as methylcellulose [222], which unlike the OP9 co-cultures are not associated with an artificial amplification of myelopoiesis [90]. The limited mobility of cells in semi-solid media additionally provides a means of assessing HSPC colony-forming capacity at a per-cell basis. However, in contrast to OP9/OP9DL1 co-cultures, most methylcellulose-based CFC cultures do not support lymphopoiesis [222]. Thus, a combination of different assays is necessary in order to establish the full *in vitro* potential of HSPCs.

In vivo assays

Transplantation of cells into irradiated recipient animals is considered the gold standard for assessing HSPC function, and is to date one of very few assays which can formally demonstrate HSC activity. Beyond providing a means of interrogating the ability of HSCs to serially reconstitute multilineage hematopoiesis in live animals [225], transplantation assays can be used to assess the fitness of a cell following treatment or genetic manipulation if performed in a competitive manner [226], rendering it a powerful tool for investigating HSPC function.

Transplantation assays typically require myeloablative pre-treatment of the recipient animal with lethal or sublethal doses of irradiation. While such pre-treatment is necessary to liberate niche space for the transplanted cells, it additionally results in a high proliferative pressure being exerted on the transferred HSPCs, which alters cell cycle kinetics as well as differentiation output of the cells [167, 227]. As such, it is unlikely that transplantation assays reflect physiological behavior of HSPCs. A multitude of different lineage tracing mouse models which allow for characterization of native hematopoiesis have been developed; however, conclusions regarding the steady-state behavior of HSPCs in such models appear to strongly depend on the approach used to track the cells [227-230].

Regardless of the developmental stage of the assayed cell type, the absolute majority of *in vivo* studies of HSPC function have been carried out in adult recipients. As several studies have shown that the behavior of HSPCs is heavily influenced by the

developmental stage of the niche, both in normal hematopoiesis [231] and in leukemia [174], assessing the function of fetal- and neonatal-derived cells within an adult microenvironment is suboptimal. However, transplantation into neonatal mice remains challenging, not least because of technical difficulties with injections into very small veins and because of the vulnerability of newborn animals to radiation-associated adverse effects [232]. Intrahepatic transplantation has been shown to result in high chimerism and survival in neonatal recipients [232] and thus represents an attractive alternative to traditional transfer of HSPCs via intravenous injection. Broader application of this transplantation method might aid in overcoming the technical barrier towards routine assessment of HSPC function in a developmentally appropriate niche.

Ethical considerations

Research involving animals is a topic of abundant debate. Naturally (and thankfully, one might add), most people feel a strong aversion towards even the thought of hurting animals in any way. Yet, it is important to consider that the majority of past as well as more recent scientific advances in the field of pre-clinical and clinical research would not have been possible without the use of laboratory animals. As such, animal research is oft regarded as a "necessary evil", without which scientific progress would be severely halted.

Animal research is subject to strict national and international legislation, which only allows for animal experiments where the benefits to human health and society outweigh the risks to animal welfare. All proposed research involving animals must further adhere to the principles of "The Three R:s"– replacement, reduction, and refinement [233]. The first R – replacement – states that whenever possible, experiments conducted in animals should be replaced by alternative models, such as *in vitro* assays utilizing cell lines or primary human material. Indeed, while animal models may never be fully replaceable, their use will likely diminish significantly as induced pluripotent stem cell (iPSC)-based [234] disease models become more advanced and thus gain applicability for more human malignancies. In cases where *in vitro* models are deemed insufficient and animal experiments are required, experiments must be designed in such a way that the least possible number of animals are used (reduction) and that measures aimed at reducing the discomfort of the animal during the course of the experiment are taken (refinement). Improving laboratory animal welfare is a research topic in itself, and novel refinement strategies are continuously being developed and implemented [235].

Sex and gender perspectives

Both female and male sex hormones (estrogen and testosterone, respectively) have documented effects on the blood and immune system in normal development and in disease [236-238]. Examples of this include a higher occurrence of autoimmune disorders in females compared to males [237], whereas the opposite is true for cALL, which is observed more frequently in male than in female children [238]. Importantly, HSCs express sex hormone receptors in mice [239] and in humans [240], respond to stimulation with sex hormones [241], and exhibit sex-specific differences in self-renewal and proliferation kinetics [238]. Collectively, these studies highlight that sex distribution among test subjects merits careful consideration even at the level of laboratory HSPC research.

Aims of the thesis

The overarching aim of my graduate studies has been to characterize the differences in the molecular makeup and functionality of fetal and adult HSPCs and to understand how ontogeny-specific molecular cues drive differential outcome in normal hematopoiesis and in leukemia. The specific aims of this thesis were:

- To elucidate differences in proteomic makeup and redox homeostasis between fetal and adult HSPCs (Papers I & II)
- To characterize the ontogenic shifts in the proteome and functionality of lineage-biased HPCs (Paper III)
- To characterize the ontogeny-specific intra- and extracellular factors driving MLLr leukemia initiation and progression (Paper IV)

Summary of included papers

Papers I & II

Several efforts have been made to characterize the molecular underpinnings of the ontogenic changes in the hematopoietic system on the level of mRNA [104, 113, 114]. However, the proteomic makeup of fetal and adult HSPCs and its relation to the functionality of these cells has remained largely unexplored. To overcome this knowledge gap, we utilized an MS-based quantitative proteomics approach to perform the first ever in-depth characterization of the cellular proteome of FACS-purified E14.5 FL and ABM LSK HSPCs (*Paper I*) and subsequently characterized the redoxomes of the same cell populations (*Paper II*).

Our proteome analysis in *Paper I* yielded in the identification of nearly 7000 proteins, out of which we found 454 to be differentially expressed between fetal and adult HSPCs. Importantly, 26 proteins with a four-fold expression difference on the protein level had not previously been described to have differential mRNA expression between fetal and adult HSCs, highlighting the need for a shift towards proteomic-centric approaches in the field. Our data showed that whereas the proteomic signature of fetal HSPCs is mostly enriched for processes related to cell proliferation, adult HSPCs are defined by a larger set of proteins which are involved in a broader variety of biological processes, such as immune response and glucose metabolism.

We uncovered discrepancies between fetal and adult HSPCs in protein complex stoichiometry as well as numerous important cellular processes and pathways, with proteins involved in antimicrobial defense, defense against ROS and in Type I IFN signaling displaying particularly dramatic expression differences between the two cell types. Intriguingly, our proteomics data as well as subsequent functional analyses pointed towards fetal HSPCs being generally less well-equipped with immune response proteins and proteins involved in antioxidant defense via S-glutathionylation, possibly indicating a higher sensitivity to ROS-induced protein damage compared to the adult cells. To explore this idea further, we undertook an MS-based redox proteomics approach to characterize reversible cysteine modifications in the fetal and the adult HSPCs (*Paper II*). In line with the lower expression of proteins involved in antioxidant defense in fetal relative to adult HSPCs, we found a generally higher level of cysteine oxidation in the fetal cells, as well as a higher number of cysteine peptides significantly higher oxidized in fetal

relative to adult cells than vice versa (174 versus 31 peptides). This points to a particularly important regulatory role for redox signaling in fetal hematopoiesis. Fetal-enriched redox sensitive proteins were associated with biological functions relating mainly to metabolism and translation initiation, both of which processes are subject to considerable ontogenic remodeling in HSPCs [24].

Our final investigations in *Paper I* uncovered that the NSP Elane as well as the inflammatory cytokine IFNα have differential roles in fetal and adult HSPC functionality. Importantly, we showed that despite residing in an IFNα-low environment and expressing low levels of proteins involved in Type I IFN signaling relative to adult HSPCs, fetal HSPCs can respond to IFNα and do so in a dose-dependent manner; low levels of exposure promote expansion of fetal lymphomyeloid progenitors *in vivo*, whereas higher levels impair the repopulation capacity of fetal HSPCs. We further showed that HSPCs acquire adult-like expression levels of the IFNα receptor (IFNaR) and MHC Class I less than a week after birth, indicating that enhancement of inflammatory response pathways is an early event in the shift from a fetal-like to an adult-like state of hematopoiesis.

Collectively, the studies presented in *Paper I* and *Paper II* represent a significant advancement in the understanding of the molecular landscape of fetal and adult HSPCs, and provides important insight into what factors may be responsible for the functional differences between fetal and adult hematopoiesis.

Paper III

The molecular composition and function of lineage-biased HPCs situated at the apex of lymphomyeloid bifurcation has mainly been studied in the adult system, and until very recently [120], exclusively at the transcript level [6, 8, 9]. The lack of insight into molecular programs governing differentiation in fetal compared to adult HPCs represents a significant hurdle towards understanding differential lineage choices at various stages of ontogeny, both in normal hematopoiesis and in leukemia.

To address these issues, in *Paper III*, we set out to perform a comprehensive and comparative functional and proteomic characterization of fetal and adult LMPPs, CLPs and GMPs. Our initial *in vitro* differentiation assays of the fetal and adult cells showed that previously established adult immunophenotypic definitions of LMPPs, CLPs and GMPs [7-9, 43] mark cell populations with lymphomyeloid, lymphoid-biased and myeloid-restricted lineage potential, respectively, also within the fetal hematopoietic system. We further uncovered that adult LMPPs, CLPs as well as GMPs possess a stronger myeloid potential compared to their fetal counterparts.

The rarity of LMPPs, CLPs and GMPs within FL and ABM severely limited the number of cells which could be collected for MS-based proteome analysis within a reasonable time frame. We therefore invested considerable effort into optimizing a proteomic workflow where we implemented iST-based sample preparation and pre-fractionation together with isobaric labeling to be able to reduce the required input cell number five-fold compared to *Paper I*. Using this approach, we were able to identify and quantify 4,189 proteins from a starting material of 100,000 fetal and adult LMPPs, CLPs and GMPs. We found that protein expression accurately separated the six cell types on ontogenic stage as well as on lineage potential. Strikingly, our data indicated that generic fetal-enriched features such as those identified in *Paper I* and in other previous work [104], including a high expression of proteins involved in cell cycle- and translation-related processes, are prevalent in lymphoid-competent (LMPPs and CLPs) but largely absent from myeloid-restricted (GMPs) progenitors. Features traditionally attributed to adult hematopoiesis however, such as a high expression of immune response- and redox-associated genes (*Paper I* and [104]), were conserved across lymphoid and myeloid adult progenitors. The unique protein signature of fetal GMPs was enriched for inflammatory and wound healing processes and showed a strong association to adult MkPs, which together with the lower capacity of the fetal cells to produce mature myeloid cells indicates that fetal GMPs have an effector cell-like rather than a progenitor-like phenotype. In line with our *in vitro* differentiation data, we additionally found myeloid protein signatures to be suppressed in fetal relative to adult lineage-biased HPCs.

Surprisingly, we found that one of the main proteomic features distinguishing fetal and adult LMPPs was a significantly higher expression of several member or the

myosin, tropomyosin and troponin family of proteins in the adult cells. Although the role of these proteins in HSPCs is relatively poorly characterized, myosins have been implicated in inflammation-induced emergency hematopoiesis [98] and in leukemia [242]. By carrying out experiments in which myosin phosphorylation and thus myosin activity was decreased via inhibition of either Rho kinase (ROCK) or myosin light chain (MLC) kinase, we revealed that fetal LMPPs show a greater sensitivity to disruption of myosin activity compared to adult LMPPs.

Interestingly, we found the myelopoiesis master regulator and TF Irf8 to be differentially expressed between fetal and adult GMPs. In line with this TFs previously described prominent role in driving monocytic differentiation [81], we observed a diminished capacity of fetal relative to adult GMPs to generate fully mature Ly6C$^+$ monocytes. The defect in monopoiesis could be partially rescued by enhancing Irf8 expression in the fetal cells, thus confirming its role in mediating a previously unknown ontogenic shift in the monocyte differentiation capacity of myeloid-restricted HPCs.

Taken together, our work in *Paper III* has illuminated a number of previously unknown functional and molecular differences between fetal and adult lineage-biases HPCs as well as provided novel insight into the molecular cues governing ontogenic shifts in cellular fate at the protein level.

Paper IV

A growing body of work suggests that ontogeny-specific intra- and extracellular cues play a prominent role in determining disease phenotype and outcome in MLLr leukemia [165, 167, 174]. However, little is known about the molecular events governing the earliest steps of leukemic transformation, in particular in fetal-derived leukemia. In *Paper IV*, we aimed to delineate the contribution of ontogenic factors in the cell-intrinsic and -extrinsic milieu to the development and progression of different subtypes of MLLr leukemia.

To this end, we utilized an inducible mouse model of MLL-ENL-driven leukemia (iMLL-ENL mice) [159] to perform a proteomic and functional characterization of fetal- and adult-origin MLLr leukemia. We focused our investigation on leukemia initiated in LMPPs, as these progenitors have previously been shown to be among the most potent LICs within the adult progenitor cell pool upon induced expression of MLL-ENL [159] and show marked ontogeny-specific differences in differentiation capacity and molecular makeup (*Paper III*).

In vitro, differentiation was partially blocked in fetal as well as adult MLL-ENL-expressing cells and an accumulation of cells expressing stem cell marker cKit could be observed over time, suggesting that MLL-ENL confers LMPPs with LSC-like properties regardless of the cells' developmental origin. Intriguingly, fetal LMPPs harboring MLL-ENL expression additionally produced a population of $cKit^+$ $Flt3^+$ cells, a phenotype reminiscent of early lymphoid progenitors. Such cells were largely absent among the progeny of adult MLL-ENL-expressing cells, which instead intermittently produced high numbers of myeloid cells. Thus, the intrinsically programmed difference in lineage potential that we have previously observed in WT fetal and adult LMPPs (*Paper III*) appears to be retained upon leukemic transformation *ex vivo*.

A strong contribution of the developmental stage of the niche to outcome in both normal hematopoiesis and in leukemia has been reported [174, 231]. We therefore next sought to determine how different extracellular environments may influence disease phenotype and progression in fetal- and adult-origin MLLr leukemias. To this end, we transplanted fetal and adult LMPPs derived from iMLL-ENL mice into neonatal and adult recipients. We found that only fetal cells could successfully sustain multilineage hematopoiesis in neonatal recipients long-term, although none of the recipient mice transplanted as neonates developed leukemia during the course of the experiment. Adult recipients developed AML with equal latency regardless of the donor cell source, confirming that both fetal and adult LMPPs can give rise to leukemia *in vivo* upon induced expression of MLL-ENL.

To investigate the molecular events governing the earliest steps of leukemic transformation, we applied the proteomic workflow developed in *Paper III* to fetal and adult WT and iMLL-ENL LMPPs in which expression of the fusion oncogene

had been induced *ex vivo*. From a starting material of 40,000 cells per sample and a TMT 16-plex approach, we were able to quantify close to 3,000 proteins across all assayed conditions. We found that protein expression most clearly separated the cell types based on ontogenic stage, but we additionally observed strong expression differences between healthy and pre-leukemic cells in both fetus and adult. The main ontogenically conserved molecular features associated with MLL-ENL-mediated leukemic transformation were a suppression of proteins associated with differentiation and carbohydrate metabolism, indicating that a block in differentiation and metabolic rewiring represent early features of malignant transformation in fetal- as well as adult-derived MLLr leukemia. Intriguingly, relative to WT cells, adult MLL-ENL expressing cells showed an upregulation of proteins associated with an inflammatory, myeloid-like phenotype, a signature which was suppressed in fetal pre-leukemic relative to WT cells.

In summary, our work in *Paper IV* showed that MLL-ENL-mediated leukemogenesis is governed by a complex interplay of intra- and extracellular factors which act in synergy to promote development of different leukemia subtypes. Importantly, a better understanding of the ontogenically shared and distinct features of MLLr leukemogenesis will aid in the development of novel therapies which can selectively target leukemia cell vulnerabilities in infant and adult MLLr leukemia.

General discussion and future perspectives

The work presented in this thesis represents a step towards understanding ontogeny-specific molecular mechanisms governing the outcome of normal and malignant hematopoiesis beyond the level of the transcriptome. This is especially important in light of recent evidence suggesting that the correlation between mRNA and protein expression is poor in normal adult and ageing HSPCs [120], which further highlights a role of post-transcriptional regulation in the temporal variance of hematopoiesis. Undoubtedly, such regulation is additionally highly prevalent during the complex shift from a fetal-like to and adult-like state of hematopoiesis as well as during leukemia development, as suggested by our findings in *Paper I, Paper II* and *Paper IV,* and other previous studies [116].

Transcriptomic and epigenetic characterization at the single-cell level has provided evidence for a considerable heterogeneity among HSPC populations previously perceived as relatively pure [11]. Similar trends have been observed upon single-cell proteome analysis of different monocyte and macrophage populations [209]. It is therefore important to consider that although the transition from a fetal-like to an adult-like state of hematopoiesis is often described as a "switch", this process may occur in a gradual and uncoordinated manner, meaning that at any given time of perinatal development the hematopoietic system likely consists of a mix of cells with a fetal-like, adult-like or intermediate phenotype, as recently suggested [107]. In lieu of evidence at the single- cell level, features referred to as ontogeny-specific/enriched should therefore be regarded as a description of the phenotype of the majority of cells at a particular stage of ontogeny. Nevertheless, it is plausible that as in the recently described "punctuated continuum" model of hematopoiesis [11], some defined cellular states do exist during the transition from a fetal-like to an adult-like HSPC identity, and such states represent an important target for investigation in order to understand functional ontogenic variation in normal hematopoiesis and in leukemia.

The cell-intrinsic and -extrinsic events that trigger the transition from a fetal to an adult HSC state remain elusive. Very recent work utilizing single-cell RNA-sequencing to characterize fetal, neonatal and adult HSCs identified a prenatal spike in Type I IFN signaling as the only coordinated event in the ontogenic switch in HSC state [107]. This is in line with previous work showing that stimulation with

Type I IFNs drives maturation of AGM HSCs [105], as well as our findings regarding a strong differential expression of proteins involved in Type I IFN signaling between fetal and adult HSPCs and the rapid acquisition of adult-like expression levels of IFNaR and MHC Class I soon after birth (*Paper I*). In the study by Li et al, increased levels of IFNα and IFNβ were observed locally in the FL [107], suggesting that at least the initial events of the transition from a fetal to an adult HSC state occurs independently of the relocation of HSPCs from the FL to the BM. Fetal HSCs additionally maintain certain fetal-specific characteristics, such as a high proliferative rate and B1a B cell potential, even when transplanted into an adult environment [50, 56], perhaps suggesting that the fetal-to-adult switch in HSC state is predominately intrinsically programmed. Considering this, it is surprising that a recent study showed that adult HSPCs expressing the MLL-AF9 fusion oncogene give rise to a myeloid and lymphoid-like leukemia in adult and neonatal mice, respectively, leading the authors to propose that the age of the microenvironment is a stronger determinant of leukemia phenotype than the developmental origin of the LIC in MLLr leukemia [174]. Our findings in *Paper III* and *Paper IV* rather suggest that at least some subsets of fetal HPCs are intrinsically biased towards production of lymphoid cells in normal development and in leukemia, but that extracellular factors play a role in influencing the *in vivo* behavior of the cells upon leukemic transformation. In line with the report by Rowe et al, fetal as well as adult MLL-ENL-expressing LMPPs exclusively gave rise to AML when transplanted into adult recipients in our study. A neonatal microenvironment did however not appear to promote lymphoid fate in adult-derived MLL-ENL expressing cells, and adult cells largely failed to engraft neonatal recipients. Notably, while fetal cells harboring MLL-ENL expression showed higher engraftment potential in neonatal mice compared to adult cells, they too failed to give rise to leukemia in neonatal recipients. It is possible that augmenting the experimental conditions by increasing the radiation dose and/or changing the transplantation route may alter the outcome of these experiments by facilitating engraftment in the neonatal mice.

While a broad consensus exists regarding the *in utero* origin of infant MLLr leukemia, the developmental time window during which leukemogenic transformation occurs has not been established. An embryonic lymphomyeloid progenitor existing prior to the emergence of definitive HSCs was recently identified as a target cell for leukemia initiated by the ETV6-RUNX1 oncogene [169]. While ETV6-RUNX1 is almost exclusively associated with cALL [137], MLL-rearrangements are present in infant as well as adult acute leukemia [139], suggesting that the target cell type for the oncogenic hit is an HPC derived after the establishment of definitive hematopoiesis rather than a progenitor which is exclusive to a particular ontogenic stage. Nevertheless, MLL-rearrangements are considerably more common in infant than in adult leukemia [126, 149]. In light of this, it is tempting to speculate that the anabolic nature of fetal hematopoiesis in

combination with a high mutational rate [57] and poorly developed immune and antioxidant defense mechanisms (*Paper I*, *Paper II* and *Paper III*) renders fetal HSPCs more vulnerable to leukemic transformation compared to their adult counterpart. Indeed, proteins involved in immune response and inflammatory processes are further downregulated in fetal LMPPs upon leukemia initiation mediated by the MLL-ENL fusion oncogene (*Paper IV*). Despite their high penetrance, MLL-rearrangements within the hematopoietic system are not embryonically lethal, suggesting either that the translocation occurs late in gestation, and/or that fetal HSPCs harbor some degree of resistance towards complete transformation. In line with the latter hypothesis, a recent study found that Lin28b, the expression of which decreases dramatically during early postnatal life [50], serves as a barrier against leukemic transformation [167]. It is not unlikely that the sterile intrauterine environment in combination with maternal-derived protective mechanisms [37] are additional factors in the prenatal resistance to leukemic transformation. Further characterization of the molecular mechanisms involved in fetal-derived MLLr leukemia initiation and disease progression during infancy will thus illuminate how to best enhance leukemia resistance and exploit age-specific leukemia vulnerabilities, and aid in the development of novel preventative and treatment strategies for infant MLLr leukemia.

Populärvetenskaplig sammanfattning

Varje sekund skapas över 2 miljoner nya blodceller i vår kropp. De allra flesta (>99%) är röda blodkroppar som transporterar syre till våra organ, medan resterande består av blodplättar och immunceller som tillhör det medfödda eller det adaptiva immunförsvaret. Att vi kan ha en konstant tillverkning av blodceller är tack vare en liten population celler i vår benmärg som kallas blodstamceller och som ger upphov till alla olika blodceller i kroppen genom en process som kallas differentiering. När en blodstamcell differentierar gör den det i flera olika steg, där varje ny cell som bildas är mer specialiserad än den förra tills en fullmogen blod- eller immuncell slutligen bildas. Cellerna som befinner sig i ett mellanläge mellan blodstamcell och mogen cell kallas för blodprogenitorer.

Blodstamcellerna bildas tidigt under fostertiden i embryots artärer, och färdas därefter till levern som är det huvudsakliga blodbildande organet fram till kort före födseln, när blodstamcellerna flyttar sig till sin slutgiltiga destination – benmärgen. Utvecklingen av blodets immunceller skiljer sig avsevärt åt hos foster och vuxna. Den fetala blodutvecklingen är en aktiv process, där cellerna delar sig snabbt och ökar i antal för att kunna tillgodose det växande fostrets behov och bygga upp grunden till blodsystemet. Dessutom bildas vissa typer av immunceller enbart under fostertiden. I den vuxna individen däremot är det viktiga med blodutvecklingen att upprätthålla balans i blodsystemet. Många vuxna blodceller är därför under normala förhållanden i ett vilande stadium där de inte genomgår celldelning. Det är dock inte bara under normal blodutveckling som foster och vuxnas blodutveckling ser olika ut. Det finns stora skillnader i vilken typ av blodcancer (leukemi) som oftast drabbar barn och vuxna, och hur känsliga cellerna är för uppkomsten av cancer. När det gäller akut leukemi, som är den mest aggressiva typen av leukemi, drabbas barn oftast av typen akut lymfatisk leukemi (ALL), medan vuxna oftare drabbas av en annan typ som kallas akut myeloisk leukemi (AML). Tyvärr har man ännu inte kunnat identifiera det molekylära maskineriet som ligger till grund för cellernas olika egenskaper, vilket hindrar utvecklingen av nya typer av behandling mot leukemi.

I alla artiklar som inkluderas i den här avhandlingen har vi förutom att undersöka fetala och vuxna blodcellers funktion studerat hur proteomet – som är den samlade bilden av alla proteiner inom en cell – skiljer sig mellan fetala och vuxna blodceller. Fördelen med det tillvägagångssättet över ”traditionella” metoder, där förlagan till proteinet (så kallat messenger RNA, eller mRNA) studeras, kommer av att det är

proteinerna som utgör cellens maskineri och att studera dem därför ger en mer korrekt bild av vad som pågår i cellen.

I artikel I kartlade vi hur proteomet skiljer sig mellan fetala och vuxna blodstamceller och tidiga blodprogenitorer. Vi detekterade och kvantifierade ett stort antal proteiner och upptäckte att fetala celler är dåligt utrustade med proteiner som deltar i immunförsvaret och i cellens försvar mot fria radikaler, som kan leda till att proteiner oxideras och ändrar eller tappar funktion. I artikel II kunde vi sedan visa att proteiner i fetala blodstamceller är mer oxiderade än motsvarande proteiner i vuxna celler, vilket kan göra de fetala blodcellerna känsligare för skador på viktiga proteiner och för uppkomst av mutationer.

I artikel III fokuserade vi på fetala och vuxna blodprogenitorer som redan är programmerade att bara bilda celler som ingår i det medfödda eller adaptiva immunförsvaret, eftersom dessa celler tidigare har visat sig vara mottagliga för mutationer som kan leda till leukemi. Vi utvecklade en metod som lät oss detektera och kvantifiera många proteiner från få celler och kunde med hjälp av proteindatan och funktionella studier bland annat visa att fetala blodprogenitorceller är sämre på att göra så kallade myeloiska celler än motsvarande vuxna blodceller. Till viss del berodde de fetala cellernas lägre myeloiska förmåga på att de saknar det viktiga proteinet Irf8 som krävs för att producera mogna celler av en myeloisk celltyp som kallas monocyter och som ingår i det medfödda immunförsvaret.

En mutation där genen MLL slagits ihop med genen ENL (MLL-ENL) ger upphov till väldigt aggressiv ALL hos spädbarn och AML hos vuxna. Mutationen förekommer oftare i spädbarnsleukemi än i leukemi hos vuxna och tros uppkomma under den fetala utvecklingen. I artikel IV undersökte vi hur olika inre och yttre faktorer påverkar leukemiutveckling när cancergenen MLL-ENL uttrycks i fosters eller vuxnas blodceller. När vi odlade cellerna utanför kroppen så gav fosterceller med MLL-ENL upphov till cancerceller som påminde om ALL medan de vuxna cellerna inte gjorde det, vilket tyder på att fosters och vuxnas blodceller har en inre programmerad partiskhet mot utveckling av lymfatisk respektive myeloisk leukemi. När fosters och vuxnas MLL-ENL-celler transplanterades till vuxna möss gav de däremot enbart upphov till AML, vilket innebär att yttre faktorer i cellernas omgivning samarbetar med cellernas inre molekylära program för att styra leukemiutveckling. Genom att undersöka proteomet hos friska och leukemiska blodceller från foster och vuxna kunde vi kartlägga flera processer som sker vid leukemiutveckling oavsett ålder, samt cellulära processer som är väldigt specifika för leukemi som utvecklas från fosters respektive vuxnas blodceller.

Sammantaget har vi i de fyra arbetena som ingår i avhandlingen kartlagt de proteomiska och funktionella skillnaderna i foster och vuxnas blodutveckling under normala förhållanden och under initiering och utveckling av olika blodcancertyper. Vi har förväntan att vårt arbete ska leda till nya strategier för upptäckt, förhindrande och behandling av blodcancer hos barn och vuxna.

Краткое резюме научной работы

Каждую секунду в нашем теле создается более 2 миллионов новых клеток крови. Подавляющее большинство (>99%) - это красные кровяные тельца, которые транспортируют кислород к нашим органам, а оставшиеся состоят из тромбоцитов и иммунных клеток, принадлежащих к врожденной или адаптивной иммунной системе. То, что мы можем иметь постоянное производство клеток крови, связано с небольшой популяцией клеток в нашем костном мозге, называемой стволовыми клетками крови, которые, посредством процесса, называемого дифференцировкой, дают начало всем различным клеткам крови в организме. Дифференциация стволовых клеток крови происходит в нескольких различных стадиях, где каждая новая формирующаяся клетка является более специализированной, чем предыдущая, до тех пор, пока, наконец, не сформируется полностью зрелая кровь или иммунная клетка. Клетки, которые находятся в промежуточном положении между стволовыми клетками крови и зрелыми клетками, называются предшественниками крови.

Стволовые клетки крови формируются на ранних этапах развития плода в артериях эмбриона, а затем перемещаются в печень, которая является основным кроветворным органом. Незадолго до рождения стволовые клетки крови перемещаются к своему конечному месту назначения - костному мозгу. Развитие иммунных клеток крови у плодов и взрослых значительно различается. Развитие крови плода - это активный процесс, при котором клетки быстро делятся и увеличиваются в количестве, чтобы удовлетворить потребности растущего плода и заложить основу для системы крови. Кроме того, определенные типы иммунных клеток образуются только во внутриутробном периоде. С другой стороны, у взрослого человека при развитии крови важно поддерживать баланс в кровеносной системе. Поэтому многие взрослые клетки крови в нормальных условиях находятся в стадии покоя, когда они не подвергаются клеточному делению. Однако не только при нормальном развитии крови формирование крови плода и взрослого человека выглядит по-разному. Существуют также большие различия в типе рака крови (лейкемии), который чаще всего поражает детей и взрослых, и в том, насколько чувствительны клетки к возникновению рака. В случае острого лейкоза, который является наиболее агрессивным типом лейкемии, дети чаще всего страдают от типа острого лимфолейкоза (ОЛЛ), в то время как взрослые

чаще страдают другим типом, называемым острым миелоидным лейкозом (ОМЛ). К сожалению, пока не удалось идентифицировать молекулярный механизм, лежащий в основе различных свойств клеток, что препятствует разработке новых видов лечения лейкемии.

Во всех наших статьях, включенных в эту диссертацию, помимо изучения функции эмбриональных и взрослых клеток крови, мы изучали, то как протеом - который представляет собой общую картину всех белков в клетке - различается между клетками крови плода и взрослого. Преимущество этого подхода перед «традиционными» методами, в которых изучается модель белка, заключается в том, что именно белки составляют механизмы клетки и поэтому их изучение дает более точную картину происходящего в клетке.

В Статье I мы составили аналитическую карту, чем протеом различается между эмбриональными и взрослыми стволовыми клетками крови и ранними предшественниками крови. Мы обнаружили и количественно оценили большое количество белков и обнаружили, что клетки плода плохо оснащены белками, которые участвуют в иммунной системе и защите клетки от свободных радикалов, что может привести к окислению белков и изменению или потере функции.

В Статье II мы смогли показать, что белки в стволовых клетках крови плода более окислены, чем соответствующие белки во взрослых клетках, что может делать клетки крови плода более чувствительными к повреждению важных белков и привести к возникновению мутаций.

В Статье III мы сосредоточились на предшественниках крови плода и взрослого человека, которые уже запрограммированы на формирование только клеток, которые являются частью врожденной или адаптивной иммунной системы, поскольку ранее было показано, что эти клетки подвержены мутациям, которые могут привести к лейкемии. Мы разработали метод, который не только позволил нам обнаруживать и количественно определять многие белки из нескольких клеток, но и помог, с помощью данных о белках и функциональных исследований, среди прочего показать, что клетки-предшественники крови плода хуже вырабатывают так называемые миелоидные клетки, чем соответствующие взрослые клетки крови. В некоторой степени более низкая миелоидная способность клеток плода была связана с отсутствием важного белка Irf8, необходимого для производства зрелых клеток типа миелоидных клеток, называемых моноцитами, которые являются частью врожденной иммунной системы. Мутацию, в которой ген МЛЛ сцеплен с геном ЕНЛ (МЛЛ-ЕНЛ), вызывает очень агрессивный ОЛЛ у младенцев и ОМЛ у взрослых. Мутация чаще встречается при лейкозах младенцев, чем при лейкозах взрослых, и считается, что она возникает во время внутриутробного развития плода.

В Статье IV мы исследовали, как различные внутренние и внешние факторы влияют на развитие лейкемии, когда раковый ген МЛЛ-ЕНЛ экспрессируется в клетках крови плода или взрослого человека. Когда мы культивировали клетки вне тела, плодные клетки с МЛЛ-ЕНЛ дали начало раковым клеткам, которые напоминали ОЛЛ, в то время как взрослые клетки - нет, что указывает на то, что эмбриональные и взрослые клетки крови имеют внутреннюю запрограммированную предвзятость в сторону развития лимфатических и миелоидных клеток лейкемии. Напротив, когда эмбриональные и взрослые клетки МЛЛ-ЕНЛ были трансплантированы взрослым мышам, они продуцировали только ОМЛ, что означает, что внешние факторы в клеточной среде взаимодействуют с внутренними молекулярными программами клеток для контроля развития лейкемии. Изучая протеом здоровых и лейкозных клеток крови плодов и взрослых, мы смогли отобразить несколько процессов, которые происходят при развитии лейкемии независимо от возраста, а также клеточные процессы, которые очень специфичны для лейкемии, которая развивается из клеток крови плода и взрослого человека.

Всего в четырех работах, включенных в диссертацию, мы регистрировали и анализировали протеомные и функциональные различия в развитии крови плода и взрослого человека в нормальных условиях, а также время инициации и развития различных типов рака крови. Мы ожидаем, что наша работа приведет к развитию новых стратегий обнаружения, профилактики и лечения рака крови у детей и взрослых.

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